DIFFERENTIAL GENE EXPRESSION OF *Piscirickettsia salmonis* STRAINS IN ADAPTIVE RESPONSE TO DIFFERENT CULTURE SYSTEMS: A STRATEGY TO IDENTIFY POSSIBLE INTRACELLULAR SURVIVAL MECHANISMS AND PATHOGENICITY

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DOCTORAL THESIS

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DIFFERENTIAL GENE EXPRESSION OF *Piscirickettsia salmonis* STRAINS IN ADAPTIVE RESPONSE TO DIFFERENT CULTURE SYSTEMS: A STRATEGY TO IDENTIFY POSSIBLE INTRACELLULAR SURVIVAL MECHANISMS AND PATHOGENICITY

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by

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LIST OF ABBREVIATIONS

2D-DIGE	Two-Dimensional difference gel electrophoresis
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
AcOH	Acetic acid
AcOK	Potassium acetate
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATCC	American Type Culture Collection
BCG	Bacillus Calmette-Guérin
bp	base pairs
BSA	Albumin from bovine serum
CHAPS	3[(3-cholamidopropyl) dimethylammonio] propane sulfonate
CHSE214	Chinook salmon embryo cell line
COGs	Clusters of Orthologous Groups of proteins
Ct	Threshold cycle
DAB	3,3 diaminobenzidin
DEPC	Diethylpyrocarbonate
Dot/Icm	Defect in organelle trafficking/intracellular multiplication
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
EtOH	Ethanol
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FCS	Fetal Calf Serum
FCV	*F. tularensis*-containing vacuole
FMN	Flavin mononucleotide

FPI	Francisella Pathogenicity island
Gt	Generation time
HCl	Hydrochloric acid
IAM	Iodoacetamide
IEF	Isoelectric focusing
IPG:	IEF in immobilized pH gradients
IFAT	Immunofluorescence antibody test
L-Cys	L- cysteine
LCVs	*Legionella*-containing vacuoles
LPS	Lipopolysaccharide
M-MLV	Moloney Murine Leukemia Virus
MOI	Multiplicity of infection
MOPS	Morpholino propane sulfonic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NADPH	Nicotinamide adenine dinucleotide phosphate
NO•	Nitric oxide
OD_{600nm}	Optical density measured at 600nm wavelength
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCVs	Pathogen containing vacuoles
PMSF	Phenyl methyl sulfonyl fluoride
RLOs	Rickettsia-like organisms
RNI	Nitrogen intermediates
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SHK1	Atlantic salmon head kidney cell line
SOD	Superoxide dismutase
SPI-1	Salmonella pathogenicity island 1
SPI-2	Salmonella pathogenicity island 2
SRS	Salmonid rickettsial septicemia, piscirickettsiosis

$TCID_{50}$	Median tissue culture infective dose
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TNT	Tris-HCl-NaCl-Tween 20
Tris	Tris(hydroxymethyl)aminomethane
TS	Tris-sucrose
TSA	Trypticase soy agar
TSB	Tryptic soy broth
T3SS	Type III secretion system
T4SS	Type IV secretion system
T6SS	Type VI secretion system

RESUMEN

P. salmonis es una bacteria gram-negativa, intracelular facultativa, agente causal del Síndrome Riketsial Salmonídeo (SRS), que en los últimos treinta años ha causado grandes pérdidas en la industria chilena del salmón. Se describió por primera vez en salmón coho en el sur de Chile, en 1989 por Bravo y Campos y en un primer momento se calificó como intracelular obligada y hace ya unos años se logró cultivar en medios artificiales sólidos, libres de células. Sin embargo, debido a la naturaleza intracelular de esta bacteria los programas de control basados en antibióticos han tenido pobres resultados, lo que hace necesario mejorar la cantidad y calidad de la información del patógeno.

En la presente tesis se estableció el objetivo general de analizar patrones de expresión global de la bacteria a nivel de transcriptoma y proteoma para identificar genes y proteínas relevantes que pueden estar involucrados en patogenicidad y virulencia. Para ello, se emprendió la búsqueda de genes involucrados en: i) proceso de infección de la línea celular; ii) supervivencia intracelular; iii) y genes relacionados a la adaptabilidad de esta bacteria al crecimiento en medio libre de células. Esta estrategia también incluye análisis comparativos de proteoma y transcriptoma de una cepa virulenta (IBM040) con la cepa de referencia (LF89), al igual que análisis proteómico comparativo (2D-DIGE) de esta última cepa, sometida a condiciones de vida intracelular con respecto a crecimiento en medio libre de células.

Para el cumplimiento de estos objetivos se desarrolló un medio líquido para el cultivo óptimo de este patógeno, el cual permitió además demostrar que la bacteria no pierde su capacidad de infección en línea celular posterior a múltiples pasajes en medio líquido.

Los resultados obtenidos en el análisis comparativo entre las cepas IBM040 y LF89, mostraron expresión diferencial de diversos genes, entre los cuales destacan componentes del sistema de secreción tipo IV y factores asociados a virulencia como PurL, AcrAB y AhpC. Por su parte, el análisis proteómico comparativo de la cepa LF89, en condiciones de vida intracelular respecto a crecimiento en medio libre de células, mostró la expresión diferencial de reguladores globales de la virulencia como PNPase y CsrA. Ninguno de los genes coreespondientes a estas proteinas ha sido analizado hasta ahora en esta bacteria, lo cual constituye un hallazgo importante de esta tesis. Por tanto, se constituye en un aporte relevante que contribuirá a dilucidar el efecto que tienen las diferencias de expresión génica en la supervivencia intracelular y patogenicidad de *P. salmonis*.

ABSTRACT

Piscirickettsia salmonis is a gram-negative, facultative intracellular bacterium and is the causative agent salmonid rickettsial septicemia (SRS), which in the last thirty years has caused great losses in the Chilean salmon industry. The disease was described for the first time in 1989 affecting to coho salmon cultured in Chile, initially *P. salmonis* was described as an obligated intracellular pathogen, able to grow only in the host cell cytoplasm, and only a couple of years ago it was demonstrated its ability to grow in solid cell-free media. Due to the intracellular nature of this bacterium, antibiotic- based control programs have been ineffective, which makes necessary an improvement in the quantity and quality of the information about this pathogen.

The overall objective of this thesis was to analyze global expression patterns of the bacterium at the transcriptome and proteome level to identify relevant genes and proteins that may be involved in pathogenicity and virulence. To do this, we undertook the search for genes involved in: i) the infection process of the cell line ii) intracellular survival, iii) genes related to the adaptability of this pathogen to growth in cell-free medium. This strategy also includes comparative proteome and transcriptome analysis of a virulent strain (IBM040) with the reference strain (LF89) as well as comparative proteomic analysis (2D-DIGE) of the latter strain, under intracellular living conditions with respect to growth in cell-free medium.

To fulfill these objectives it was necessary to develop a liquid medium for the optimal culture of this pathogen, which also allows us to show that the bacteria do not lose their ability to infect the cell line after multiple subcultures in liquid medium.

The results obtained in the comparative analysis of LF89 and IBM040 strains showed differential expression of several genes, including components of

the type IV secretion system and factors associated with virulence as PurI, AcrAB and AhpC. Meanwhile, proteomic analysis of strain LF-89 under intracellular conditions compared to growth in cell-free medium showed differential expression of global virulence regulators PNPase and CsrA. None of these genes has been described so far for this bacterium, which is an important finding of this thesis. Therefore, this work constitutes a significant contribution to gain insight in mechanism of intracellular survival and pathogenicity revealing underlying differential gene expression of *P. salmonis*.

1. INTRODUCTION

Aquaculture is the fastest growing food-production sector in the world, providing a significant supplement and substitute for wild aquatic organisms. However, disease is a primary constraint to the growth of many aquaculture species and is now responsible for severely impeding both economic and socio-economic development in many countries of the world (Subasinghe *et al*., 2001). So aquaculture requires continued research, scientific and technical developments and innovation (Toranzo *et al*., 2005).

The marine fish culture is dominated by Atlantic salmon (*Salmo salar*) led by Norway, followed by Chile, United Kingdom, Canada and Ireland (Toranzo *et al*., 2005). In Chile, salmon industry began in the early 1980 achieving three decades of exceptional growth. This allowed Chile to position itself as one of the leading global suppliers of salmon, in its various forms of presentation. From almost negligible levels of production and export in the early 1980s, twenty years later production runs along the 700 thousand tons (Katz *et al*., 2011).

The appearance and development of fish diseases in the salmon farms is the result of interaction among pathogen, host and environment. Therefore, multidisciplinary studies involving the characteristics of potential pathogenic microorganisms for fish, as well as aspects of the biology of fish hosts will allow a better understanding of the environmental factors affecting such cultures (Toranzo *et al*., 2005).

Some of the most threatening bacterial diseases occurring in fish cultured in marine waters worldwide are vibriosis, “winter ulcer”, photobacteriosis, furunculosis, flexibacteriosis, “winter disease”, streptococcosis, lactococcosis, bacterial kidney disease (BKD), mycobacteriosis and piscirickettsiosis (SRS) (Toranzo *et al.,* 2005).

In Chile *Piscirickettsia salmonis* pathogen, causing the disease

piscirickettsiosis (SRS), is responsible for 56.37% of diagnoses reported by laboratories to Chilean National Service of Fisheries and Aquaculture (Sernapesca) during January-September period of the year 2012, this shows the prevalence of this pathogen in national salmon farming (http://www.salmonxpert.cl).

1.1. PISCIRICKETTSIOSIS (SRS)

Piscirickettsiosis is a septicemic condition of salmonids, also called salmonid rickettsial septicemia (SRS) (Fryer and Lannan, 1996; Almendras and Fuentealba, 1997; Smith *et al*., 1999; OIE, 2003) the causal agent being *Piscirickettsia salmonis* (Fryer *et al*., 1992). The disease was described for the first time in 1989 affecting coho salmon cultured in Chile. The first signs of this disease began 6-12 weeks after fish were transferred from fresh water to sea water and cumulative mortality ranged between 30% and 90% (Bravo and Campos, 1989; Cvitanich *et al*., 1991).

Natural outbreaks of piscirickettsiosis typically occurred a few weeks after smolts are transferred to the sea (Fryer *et al*., 1990; Cvitanich *et al*., 1991). However, the disease has also been observed in fresh water facilities (Bravo, 1994; Gaggero *et al*., 1995). Although horizontal transmission is one of the main routes of infection, in certain cases, existence of vertical transmission of *P. salmonis* has been demonstrated (Larenas *et al*., 2003).

From 1992, both the disease and the pathogen have been reported in a wide geographical distribution, including at coasts of Ireland (Rodger and Drinan, 1993), Norway (Olsen *et al*., 1997), Scotland (Birrell *et al*., 2003), and both the west (Brocklebank *et al*., 1992) and east coasts of Canada (Jones *et al*., 1998; Cusack *et al*., 2002), its pathogenesis in fish farms in Chile seems to be more severe than its pathogenesis elsewhere (Marshall *et al.,* 1998).

Currently in Chile four main species are cultivated for export: Atlantic

salmon (*Salmo salar*), Coho salmon (*Oncorhynchu. kisutch*) and rainbow trout (*Oncorhynchus mykiss*) and to a lesser extent, Chinook salmon (*Oncorhynchus tschawytscha*) (DirectorioAqua.com, online reference) All these species are susceptible to infection by *P. salmonis* (Fryer and Hendrick, 2003).

Salmonids have not been the only target fish of Rickettsia-like organisms (RLOs), and several reports have been published describing rickettsial infections as responsible agent of epizootic outbreaks in nonsalmonid fresh water and marine fish such as species of tilapia in Taiwan, imported blue-eyed plecostomus (*Panaque suttoni)* in USA (Khoo *et al*., 1995; Chen *et al*., 2000), European seabass (*Dicentrarchus labrax*), (McCarthy *et al*., 2005), which is an important farming species in the northern hemisphere (Marshall *et al*., 2007, FAO Fisheries and Aquaculture Department, online reference), and has shown that it is able to infect a species of great economic value, as white seabass (*Atractoscion nobilis*) naturally living between the coasts of southern California in USA and northern Baja California in Mexico (Arkush *et al*., 2005; GMSAIN online reference).

In the majority of cases, no comparison between these Rickettsia-like organisms and *P. salmonis* isolates have been conducted, but immunohistochemistry studies demonstrated antigenic similarities between RLOs from *European seabass* and *P. salmonis* (Steiropoulos *et al*., 2002).

Reported clinical signs of fish affected by piscirickettsiosis are lethargy, anorexia, darkening of the skin, pale gills, inflamed kidneys, extended spleen and mottled liver, as well as a hematocrit below average (Bravo and Campos, 1989; Fryer *et al*., 1990). We can also observe severe necrosis and inflammation in the liver, spleen, intestine, kidney hematopoietic tissue, and mild injury in cardiac, pancreatic and ovarian tissue (Cvitanich *et al*., 1991). The main macroscopic lesions characteristic is the presence of white to yellow subcapsular nodules, measuring up to 2cm in diameter, scattered throughout the liver (Almendras and Fuentealba, 1997; Lannan *et al*., 1999) and specific histological changes in the liver include vasculitis characterized by hypertrophy of endothelial cells,

endothelial cell vacuolation, presence of leukocytes within the blood vessel wall and fibrin thrombi into the lumen of affected blood vessels. However, often the fish die without any visible clinical signs (Almendras *et al*., 2000).

1.2. THE BACTERIUM *Piscirickettsia salmonis*

It is a gram-negative bacterium, non-mobile, not capsulated, pleomorphic but generally cocoid with a diameter ranging between 0.2 and 1.5 microns (Bravo and Campos, 1989; Fryer *et al*., 1990; Rojas *et al*., 2009).

Initially *P. salmonis* was described as an obligated intracellular pathogen, able to grow only in host cell cytoplasm, where it reproduces by binary fission within cytoplasmic vacuoles (Fryer *et al*., 1990; Garcés *et al.*, 1991; Fryer and Lannan, 1996; Almendras *et al*., 1997; McCarthy *et al*., 2008). This pathogen is grown in numerous cell lines derived from fish, commonly employed in virology as CHSE 214 (Chinook Salmon Embryo), SHK1 (Atlantic Salmon Head Kidney), EPC (Epithelioma Papulosum Cyprini), BB (Brown Bullhead Catfish) and others where its growth is controlled, causing the gradual appearance of cytopathic effect (Fryer *et al*., 1990; Almendras *et al*., 1997; Almendras and Fuentealba, 1997). Recently it was shown that *P. salmonis* is able to survive and replicate in macrophages isolated from rainbow trout head kidney and successfully infected cell lines with monocyte and macrophage characteristics as RTS11 (McCarthy *et al*., 2008; Rojas *et al*., 2009). Besides in fish-derived cell lines, *P. salmonis* has been grown successfully in the insect cell line Sf21 reaching better titers, as when propagated in cell line CHSE-214 (Birkbeck *et al*., 2004).

Recently it has been demonstrated that *P. salmonis* is able to grow in cell-free media, containing high levels of cysteine, which changes its classification to facultative intracellular (Mikalsen *et al*., 2008; Mauel *et al*., 2008). These character to be a potentially extracellular bacteria had been previously suggested in a study of semi-purified preparations of the bacterium, where there was greater survival of bacteria in salt water, while the bacteria did not survive in fresh water suspensions

(Lannan and Fryer, 1994).

The bacteria's name was attributed on the basis of certain similarities with rickettsial species, such as cytoplasmic inclusions replication in host cells, and cell wall undulating., However, subsequent analysis of the reference strain LF-89, places *P. salmonis* within the Class III, *gamma proteobacteria*, to which the genera *Coxiella, Legionella* and *Francisella belong*, with which it shares phenotypic characteristics such as intracellular replication, and requirement of high concentrations of L-cysteine in artificial growth media, but not the *alpha* subdivision to which genus Rickettsia belongs (Fryer *et al*., 1992; Fryer and Hendrick, 2003).

The classification given in Bergey's Manual of Systematic Bacteriology, is based on phylogenetic analysis of 16S rRNA gene sequences where the *phylum proteobacteria* groups all bacteria whose 16S rRNA gene sequences are related to members of the order Pseudomonadales.

P. salmonis is the only described species of the genus *Piscirickettsia,* which belongs to the family II: *Piscirickettsiaceae,* and order V*: Thiotrichales*, Class III: *gamaproteobacteria*.

The Family *Piscirickettsiaceae* is formed by gram-negative, aerobic, coccoid, rod- or spiral-shaped bacteria, occasionally pleomorphic. *"Piscirickettsiaceae"* includes six genera, with 16S rRNA similarities as the major unifying factor, but share a limited number of phenotypic characteristics. The remaining five genera of the family are composed of rod-, comma-, or spiral-shaped bacteria. They are all motile by means of a single polar flagellum. All can be cultured on artificial media. None is pathogenic or replicates within cells of its hosts. Optimum temperatures are higher than those of *Piscirickettsia* and all were isolated from aquatic environments. (Garrity, G. M., 2005).

It is important to note that there are reports about a small and infective

variant of *P. salmonis*, which has been recovered from cultures of both cell line CHSE-214 infected with *P. salmonis*, as well as from naturally infected fish (Rojas *et al*., 2008). *In vitro* this small variant was enriched after longer time post infection. Atomic force microscopy showed that its size is less than 0.2µm and could be also specifically recognized by standard antibodies against *P. salmonis*. This small variant is transcriptionally active and infectious, showing a delayed appearance of cytopathic effect, but in time this effect is fully recovered, as well as its size (Rojas *et al*., 2008).

Reference strain is called LF89 and was first isolated in southern Chile in 1989. However, to date have been isolated and characterized further variants, including a strain that showed high virulency. The reference strain LF89 is currently attenuated in virulence due to the large number of years maintained in cell lines without any contact with its natural host. The virulent strain IBM040, which in field trials has shown to be highly efficient in infection of salmon, achieve high levels of mortality. IBM040 was isolated recently by SGS Chile Ltda., and facilitated to our laboratory. Both LF89 as well as IBM040 were isolated from kidney of *O. kisutch* affected by SRS, from fish farms located in the X Region de los Lagos, Chile.

Despite the above mentioned background, little is known about the infection strategy of *P. salmonis*, primarily due to experimental limitations, up to now imposed by the intracellular nature of this bacteria (Rojas *et al*., 2009).

At the beginning of descriptions of SRS the existence of *P. salmonis* in cytoplasmic vacuoles in hepatocytes and macrophages associated with liver, kidney, spleen and peripheral blood was shown (McCarthy *et al.,* 2008). *In vitro* it was shown that *P. salmonis* is capable of infecting many cell lines derived from fish (Rojas *et al.,* 2009), so that the ability of these bacteria to infect macrophages and monocytes alike, successfully proposed a new model for studying and evaluating host pathogen interactions formerly unknown to *P. salmonis* (Rojas *et al*., 2009).

1.3. MICROBICIDAL ACTIVITY OF THE PHAGOSOME

Generally professional phagocytic cells have a large and sophisticated microbicide system, which is able to ingest and destroy invading microorganisms. During phagocytosis the microorganism is trapped with a vacuole extracellular fluid, or phagosome derived from the plasma membrane, where its content is harmless, which is followed by a dramatic conversion to acquire the microbicidal and degradative features associated with innate immunity. This conversion, known as phagosomal maturation, is accomplished through a strictly choreographed sequence of fusion and fission events that involve defined compartments of the endocytic pathway (Flannagan *et al*., 2009).

One component of the microbicidal response is the acidification of the phagosomal lumen, which is brought about by V-ATPases delivered via fusion with membranes of the endocytic pathway (Figure 1). Acidification of the phagosomal lumen favors the lytic activity of a variety of degradative enzymes and also promotes generation of hydrogen peroxide. In the case of phagosomes, an acidic luminal pH is not only essential for optimal activity of a variety of microbicidal agents but also appears to be required to coordinate the fusion of membranes that promote maturation, culminating in formation of phagolysosomes (Huynh and Grinstein, 2007).

Professional phagocytes destroy pathogens in part through reactive oxygen species (ROS) generated directly or indirectly by NADPH oxidase. The active oxidase transfers electrons from cytosolic NADPH to molecular oxygen, releasing O_2^- into the phagosomal lumen. Within the phagosome, O_2^- can dismutate to H_2O_2, which can in turn react with O_2^- to generate hydroxyl radicals and singlet oxygen. Collectively, these highly reactive, toxic ROS effectively kill intraphagosomal microorganisms (Figure 1).

Similarly to ROS, nitric oxide (NO•) and derived reactive nitrogen species

(RNS) are important antimicrobial effectors. The activity of the inducible nitric oxide synthase, or NOS_2, is regulated at the transcriptional level. RNS production requires *de novo* synthesis of the protein in response to proinflammatory agonists (Figure 1). This synthase functions as a dimer: one subunit transfers electrons from NADPH to FAD, then to FMN (flavin mononucleotide) and to the haem iron of the adjacent subunit, to produce NO• and citrulline from L-arginine and oxygen. Unlike superoxide, NO• is synthesized on the cytoplasmic side of phagosomes, but has the ability to diffuse across membranes to reach intraphagosomal targets. In the luminal environment, where it encounters ROS, NO• can undergo either spontaneous or catalytic conversion to a range of RNS, ROS and RNS synergize to produce highly toxic effects on intraphagosomal microorganisms. They interact with numerous microbial targets, such as thiols, metal centres, protein tyrosine residues, nucleic acids and lipids. As a result, proteins are inactivated and lipids are converted by oxidative damage. In addition, microbial DNA can undergo irreparable damage. Together, these reactions can impair bacterial metabolism and ultimately inhibit replication (Flannagan *et al*., 2009; Diacovich and Gorvel, 2010).

There is a set of antimicrobial proteins and peptides that antagonizes bacterial growth. They can be grossly subdivided into those that prevent growth and those that compromise the integrity of the microorganism. Growth prevention can be accomplished by limiting the availability of essential nutrients inside the phagosome. To this end, phagocytes secrete scavengers into the lumen or insert transporters into the phagosomal membrane (Flannagan *et al*., 2009). One such scavenger is lactoferrin, a glycoprotein contained in neutrophil granules that is released into the phagosome lumen, where it sequesters iron that is required by some bacteria.

More direct mechanisms deployed by phagosomes to disrupt the integrity of pathogens involve defensins, cathelicidins, lysozymes, and assorted lipases and proteases (Figure 1). Defensins, which are small, disulphide-bridged polypeptides of ≈10kDa, bind to negatively charged molecules on the microbial surface. They

subsequently induce membrane permeabilization of gram-positive and gram-negative bacteria by forming multimeric ion-permeable channels. Phagosomes are also equipped with an assortment of endopeptidases, exopeptidases and hydrolases that degrade various microbial components. Not all proteases are acquired simultaneously by the maturing phagosome, implying that they are delivered by distinct organelles. Cathepsin H is predominant in early phagosomes, whereas cathepsin S is typically present in late phagosomes. Hydrolases that target carbohydrates (for example, α-hexosaminidase, β-glucuronidase and lysozyme) and lipids (for example, phospholipase A2) are also delivered to the phagosomes (Flannagan *et al*., 2009).

All these studies seem to indicate that the fusion of pathogen containing vacuoles (PCVs) with lysosomes results in bacterial killing by lytic enzymes and acidic pH. Therefore, to successfully establish a replicative niche, almost all intracellular pathogens inhibit or delay lysosome fusion (Diacovich and Gorvel, 2010).

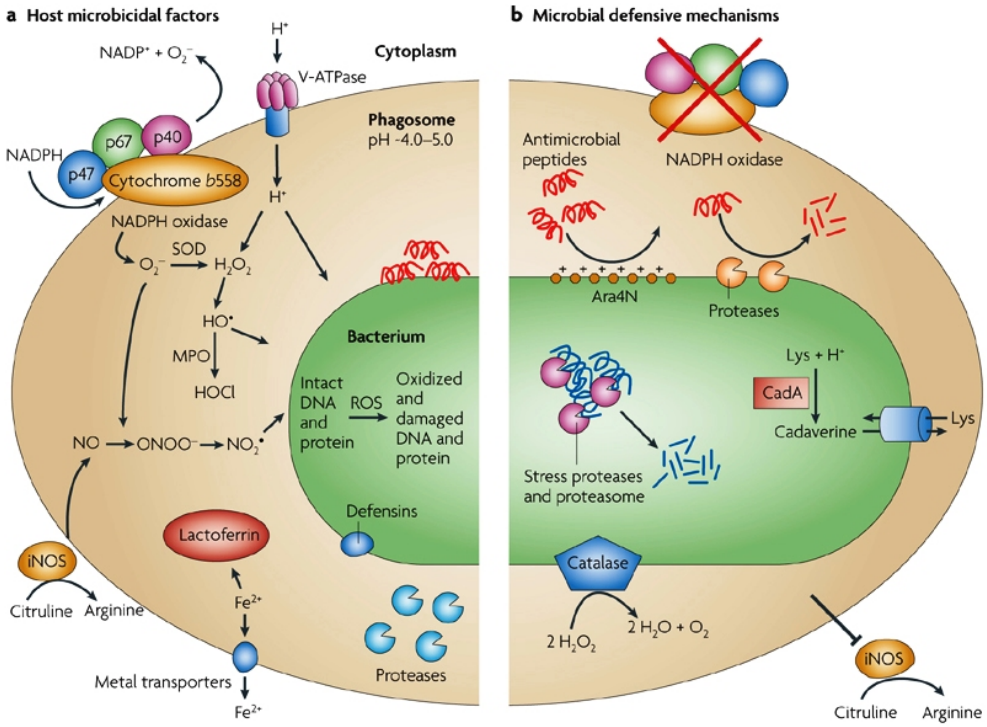

Figure 1: The microbicidal arsenal of phagocytes versus the defensive mechanisms of the microorganism. (a) The host microbicidal mechanisms include the NOX2, NADPH oxidase, inducible NO synthase (iNOS), iron scavengers and exporters, such as lactoferrin and natural resistance-associated macrophage protein 1 (NRAMP1), plus antimicrobial peptides and proteins that permeabilize and degrade the bacteria. **(b)** Bacterial defensive mechanisms include modification of their surface to resist or break down antimicrobial peptides, and expression of enzymes, such as catalase, that convert reactive species to less harmful compounds or prevent recruitment of the protein complexes that synthesize reactive nitrogen species (RNS) or reactive oxygen species (ROS). SOD, superoxide dismutase (Flannagan *et al*., 2009).

1.4. INTRACELLULAR SURVIVAL STRATEGIES

Intracellular bacterial pathogens have the remarkable ability to manipulate host cell processes in order to establish a replicative niche within the host cell. In response, the host can initiate immune defenses that lead to the eventual restriction and clearance of intracellular infection (Shin, 2012).

The bacteria that is internalized by the host cells, once inside the cell follows alternative pathways of intracellular trafficking to establish a replication niche. Some bacteria reside in specialized vesicles that are termed vacuoles or phagosomes and can manipulate host cell molecular motors to induce vacuolar and vesicular movement along microtubules. Vacuolar bacteria can also interact with different host cell compartments of the endocytic pathway (such as endosomes and lysosomes) and can replicate in host cell compartments such as the endoplasmic reticulum (ER), (Figure 2) strategy used by bacteria as *Brucella* spp. and *Legionella* spp. both encode specialized T4SSs that can transfer effectors from the bacterial cytosol into the host cytoplasm. To successfully establish a replicative niche, almost all intracellular pathogens inhibit or delay lysosome fusion (Diacovich and Gorvel, 2010).

S. typhimurium and *M. tuberculosis* are bacteria that despite inducing the production of nitric oxide synthase (NOS_2) at the site of infection, have developed mechanisms to detoxify ROS and reactive nitrogen intermediates (RNI). For example in *Salmonella* spp. periplasmic superoxide dismutases (SodCI and SodCII) detoxify ROS (De Groote *et al.*, 1997) and in *M. tuberculosis* several gene products have been associated with the detoxification of ROS and RNS, KatG is a catalase peroxidase that protects *M. tuberculosis* from killing by hydrogen peroxide and alkylhydroperoxide reductase AhpC is capable of catalyzing breakdown of peroxynitrite. *M. tuberculosis* also produces two superoxide dismutase (SOD) proteins, SodA and SodC, achieving elimination of toxic effects of O_2^- (Piddington *et al*., 2001).

Other bacteria escape from vacuoles to remain in the cytosol and then promote actin-mediated motility. This strategy is used by bacteria such as *Burkholderia pseudomallei*, *L. monocytogenes, Rickettsia rickettsii*, *Mycobacterium marinum* and *Shigella flexneri* (Figure 2).

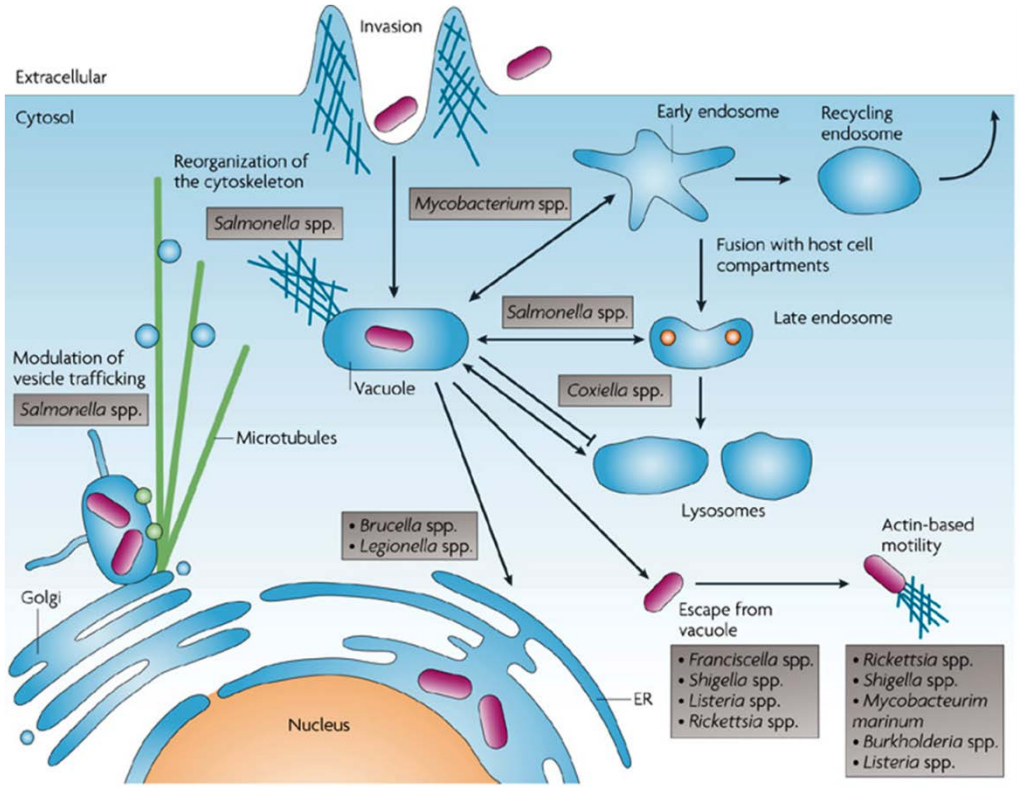

Figure 2: Intracellular pathogens manipulate host membrane trafficking to resist innate immunity and promote survival and replication. Intracellular pathogens follow alternative pathways of intracellular trafficking to establish a replication niche. Some bacteria reside in specialized vesicles and can manipulate host cell molecular motors to induce vacuolar and vesicular movement along microtubules. Vacuolar bacteria can also interact with different host cell compartments of the endocytic pathway and can replicate in host cell compartments such as the endoplasmic reticulum (ER). Other bacteria escape from vacuoles to remain into the cytosol and then promote actin-mediated motility (Diacovich and Gorvel 2010).

In the case of *L. monocytogenes*, the cholesterol-dependent cytolysin listeriolysin O (LLO) creates pores in the phagosomal membrane as early as 5min after infection (Beauregard *et al*., 1997). The effect of LLO is restricted to the

phagosome, as it needs to be activated by acidification and/or by the host enzyme GILT (Gamma-interferon Inducible Lysosomal Thiolreductase) that is found inside the phagosome (Singh *et al.,* 2008). Secretion of LLO inhibits maturation of phagosomes due to a loss of luminal H^+ and Ca_2^+, which are thought to be required for fusion with endosomes and/or lysosome (Shaughnessy *et al.,* 2006). Listeria also expresses two membrane-active phospholipase C enzymes, phosphoinositol-specific phospholipase C (PI-PlC; encoded by plcA) and broad-range phospholipase C (PC-PlC; encoded by plcB). Together with LLO, PI-PlC and PC-PlC cause breakdown of the membrane of *L. monocytogenes*-containing phagosome and thereby enable the bacteria to escape and take up residence in the cytosol, where bacterial replication occurs (Shaughnessy *et al.,* 2006). Bacteria in the cytosol are mobile, usurping the host's actin cytoskeletal machinery, where in the surface protein ActA induces actin assembly by recruiting the host cell Arp2/3 complex, G actin family members VASP protein, this process substantially contributes to the spread of *L. monocytogenes* during infection (Figure 3 A) (Lambrechts *et al*., 2008).

One strategy used by *Mycobacterium tuberculosis* is to arrest formation of the phagolysosome. Vacuoles containing *M. tuberculosis* are characterized by the presence of Rab5a, but recruitment of Rab5a effectors, such as EEA1 (early endosome antigen) and hVPS34, is impaired and as a result, PI3P does not accumulate (Vergne *et al*., 2005). The mycobacterial phosphoinositide lipoarabinomanan is a component of the cell wall that is shed from live bacteria and becomes distributed through the endocytic network (Beatty *et al.,* 2000), preventing increase in cytosolic Ca^{2+} that normally accompanies the phagocytosis and that is thought to be required to activate the kinase hVPS34 through calmodulin (Vergne *et al.,* 2003). This bacterium also affects calcium flux by inhibiting cytosolic sphingosine kinase, which converts sphingosine to sphingosine-1-phosphate, which promotes efflux of Ca^{2+} from the lumen of the ER (Malik *et al.,* 2003). *M. tuberculosis* also produces SAPM phosphatase that specifically hydrolyzes PI3P and the combined strategy effectively eliminates PI3P

from early phagosomes and prevents transition to late phagolysosomal stages (Figure 3 B) (Flannagan *et al*., 2009).

1.5. *Gammaproteobacteria* INTRACELLULAR SURVIVAL MECHANISMS

The pathogenic microorganisms*, Coxiella, Legionella* and *Francisella*, belonging to the Class III, *gammaproteobacteria*, share phenotypic characteristics with *P. salmonis* such as intracellular replication, and require high concentrations of L-cysteine in artificial growth media (Fryer *et al*., 1992; Fryer and Hendrick, 2003). These bacteria might have similar mechanisms affecting intracellular traffic and creating a niche suitable for microorganism replication.

1.5.1. *Francisella tularensis*

Francisella tularensis is one of the most infectious and pathogenic bacteria known. It is the etiological agent of the debilitating febrile illness tularemia. The bacterium is a gram-negative, capsulated, facultative intracellular pathogen and is one of the members of the genus *Francisella* of the *Gammaproteobacteria* class (Pechous *et al*., 2009; Jones *et al*., 2012).

F. tularensis can infect macrophages of humans, mice, rats, rabbits, and guinea pigs, and it is generally believed that macrophages are the major reservoir of *Francisella in vivo*. In the past few years several research groups have begun to define the niche occupied by this organism inside host cells and to delineate how bacterial growth and survival are modulated by specific virulence factors (McLendon *et al*., 2006).

Positively charged antimicrobial peptides, capable of disrupting the negatively charged bacterial membrane, are present extracellularly on mucosal surfaces as well as within macrophages and neutrophils. The literature to date has described at least two different mechanisms by which *Francisella* can either resist or evade the action of host antimicrobials: (i) by altering the charge of its surface

and thus being able to use electrostatic interactions to repel cationic antimicrobial peptides and (ii) by encoding a number of efflux systems that are necessary for resistance to antimicrobials as well as for virulence *in vivo*. *Francisella*, like many other bacteria, including *Neisseria*, *Pseudomonas*, and *Salmonella*, encodes at least one known multidrug efflux pump that allows resistance to a number of different antimicrobials and detergents, the AcrAB/TolC efflux pump (Jones *et al*., 2012).

Francisella enters cells through the process of phagocytosis. It has been reported that *F. tularensis* may utilize an unusual mechanism involving the formation of spacious asymmetric pseudopod loops. This process involves actin rearrangement through phosphatidylinositol 3-kinase signaling and is strongly dependent on the presence of complement factor C3 and complement receptor CR3 (Clemens *et al*., 2005). *Francisella* may also enter cells via the mannose receptor; type I and II class A scavenge receptors, and the Fc receptor (Pechous *et al*., 2009). Following internalization into host cells, *F. tularensis* is able to alter normal bactericidal processes.

ROS are produced by the NADPH oxidase, a membrane-bound multicomponent enzyme system that converts molecular oxygen into toxic superoxide anions. Upon phagocytosis of a microbe, the cytosolic subunits traffic to the phagosome and assemble with membrane subunits to create the active NADPH oxidase that then produces ROS. *Francisella* species use several approaches to inhibit ROS, including blocking initial assembly of NADPH oxidase components at the phagosomal membrane (McCaffrey *et al*., 2006), blocking ROS production in complexes that have assembled, and detoxifying ROS that are generated. Although *Francisella* significantly suppresses activation of the NADPH oxidase, low levels of ROS are produced in the phagosome during infection. Like many pathogens, *Francisella* can directly detoxify ROS using proteins, including catalase and superoxide dismutases, limiting its exposure to superoxide or other reactive oxygen by-products (Jones *et al*., 2012).

The organism initially resides in a membrane-bound compartment that acquires limited amounts of early endosomal and late endosomal-lysosomal markers. The *F. tularensis*-containing vacuole (FCV) fails to acquire the acid hydrolase cathepsin D and does not fuse with lysosomes (Clemens *et al*., 2004). In addition, *F. tularensis* alters host cell trafficking by escaping from the phagosome and entering the host cell cytosol, where it undergoes extensive replication (Pechous *et al*., 2009).

Phagosomal escape requires viable *F. tularensis* and occurs via an unknown mechanism that involves degradation of the surrounding lipid bilayers. At roughly 12h postinfection, *Francisella* begins to replicate in high numbers within the host cell cytosol, eventually leading to cell death, egress of *Francisella*, and presumably infection of nearby cells. Escape of *F. tularensis* from the phagosome and replication within the host cell cytosol is dependent on genes present in the *Francisella* pathogenicity island (FPI) (Pechous *et al*., 2009), The FPI encodes a putative type VI secretion system (T6SS) that is essential for *Francisella* replication and pathogenesis (Nano and Schmerk, 2007; Jones *et al*., 2012). *Francisella* has also been shown to reside in vesicles similar to autolysosomes at 24h postinfection, prior to host cell death, indicating that *Francisella* may reenter the endocytic pathway through host cell autophagy (Checroun *et al*., 2006).

1.5.2. *Legionella pneumophila*

Legionella pneumophila is a gram-negative bacterium, found ubiquitously in aquatic environments, growing in biofilms or in side of protozoa (Flannagan *et al*., 2009).

The lifestyle of a facultative intracellular bacterium is characterized by exposure to the environment both intracellular and extracellular, so *L. pneumophila* must adapt to these changes, adopting a biphasic life cycle that is characterized by the transition from the replicative phase to transmission or virulent phase. In this transition different patterns of gene expression in the stationary growth phase are reflected, preferentially expressed virulence gene for

the transmission and infection of host cells, which are repressed in the replicative phase, during which components of aerobic metabolism are upregulated. This global change in gene expression is governed by a complex regulatory network, including alternative sigma factors such as RpoS (σ^{38}) and FliA (σ^{28}), like RNA binding proteins such as Hfq and CsrA (RsmA). The latter is an overall transmission repressor and activator essential in intracellular replication (Tiaden *et al*., 2008).

In humans, after inhalation of *L. pneumophila*, is internalized by alveolar macrophages and epithelial cells, replicate in intracellular vacuoles, which is possible since this bacterium is able to redirect the phagosome maturation to create a unique intracellular niche, suitable for replication (Newton *et al*., 2006; Flannagan *et al*., 2009). The evasion of the endocytic pathway, occurs in two evolutionarily distant hosts both protozoan and human cells and is mediated by the type IV secretion system (T4SS), Dot/Icm, which is encoded by 26 genes dot/icm (Al-Khodor *et al.,* 2008). This secretion system is a system that translocates effector proteins of bacteria to the host cell cytoplasm, some of which interfere with the traffic on the host cell modulating GTPase activity or phosphoinositide metabolism (Tiaden *et al*., 2008).

The major outer membrane protein MOMP, on the surface of the bacteria effectively fixes complement promoting phagocytosis by macrophages via complement receptors, which leads to the formation of *Legionella*-containing vacuoles (LCVs) (Payne *et al.,* 1987; Bellinger and Horwitz, 1990).

LCV subsequently recruit Rab1 GTPase and Sec22b of the host cell to establish a replicative vacuole, surrounded by the ER (Newton *et al*., 2006). Secretory vesicles derived from the ER, covered with SarI-COPII merge with LCV in a process that requires the Rab1GTPase, which together with another GTPase, ARF1 regulates vesicular transport between the ER and the Golgi complex (Figure 3 C). The extent and timing of recruitment of Rab1 to the LCV, is tightly regulated by system effector proteins Dot/Icm (Flannagan *et al*., 2009).

In sum *L. pneumophila* replicates within large acidic vacuoles with some features of the lysosome, although it is unclear if the acidification is necessary for optimal replication. It is clear that the merger with ER vacuoles is crucial to deliver the time to develop resistance to vacuolar environment. Interaction between ER membranes derived with LCV has been associated with autophagy the host cell, and it was suggested that *L. pneumophila* can delay the formation of autophagelysosome, allowing greater survival (Flannagan *et al*., 2009). Appointed effectors are only a fraction of those required for successful bacterial replication; there are over 300 different effectors of the T4SS likely to have been involved in this process (Segal, 2013).

1.5.3. *Coxiella burnetii*

Coxiella burnetii is the causative agent of Q fever, a highly infectious gram-negative, intracellular pathogen (Flannagan *et al*., 2009; Ghio *et al*., 2009). As recently published *Coxiella burnetii* growth in host cell-free medium in a microaerophilic atmosphere could facilitate the molecular characterization of the microorganism to develop protective measures against Q fever (Omsland *et al*., 2009). *Coxiella burnetii* has a biphasic developmental cycle that consists of a variant infectious small cell variant (*Coxiella* phase 1) and a large cell variant (*Coxiella* phase 2) that replicates intracellularly, this bacterium resides in a lysosome-like acidified compartment, in which it replicates in the presence of various antimicrobial factors. Phagocytosis of *Coxiella* Phase 1 occurs after engagement by the bacterium of leukocyte response to integrin αVβ3, which activates a cell signaling cascade that induces localized actin polymerization, promoting internalization and *Coxiella* phagosome formation. By contrast, internalization of a virulent *Coxiella* phase 2 is through engagement αVβ3, CR3 or through hydrophobic binding mediated by the bacterial lipopolysaccharide, which does not produce the same signaling cascade (Flannagan *et al*., 2009).

After internalization, the phagosome interacts with *Coxiella* classical endocytic pathway. Simultaneously, *C. burnetti* begins to alter the phagosome maturation, conferring properties autophagosome to the vacuole. Specifically LC3 protein, characteristic of autophagosomes, is recruited to the phagosome, delaying their fusion with lysosomes, giving the bacteria time to start the transition to replication-competent, large cell variant (Figure 3 D) (Flannagan *et al*., 2009).

Within 48 hours after infection *C. burnetii* resides in a large compartment, which contains several lysosomal proteins, including the V-ATPase. This acidic compartment (pH about 4.8), is called replicative *Coxiella* vacuole, which is a requirement for replication of *C. burnetii* (Flannagan *et al*., 2009). Physiological studies reveal that it behaves similarly to an acidophilus microorganism which requires low pH for certain metabolic activities. Most of the factors that allow intracellular survival remain to be identified, but a familiar example is the DNA repair system, SOS, which protects the pathogen chromosomal damage from exposure to ROS (Flannagan *et al*., 2009).

Coxiella regulates multiple events during infection via effector proteins that are delivered to the cytoplasm by the T4SS Dot/Icm. Currently the genetic manipulation of this organism is difficult, so that the system Dot/Icm of *Coxiella* has been identified using bioinformatics and *Legionella pneumophila* as a model of T4SS (Voth and Heinzen, 2009).

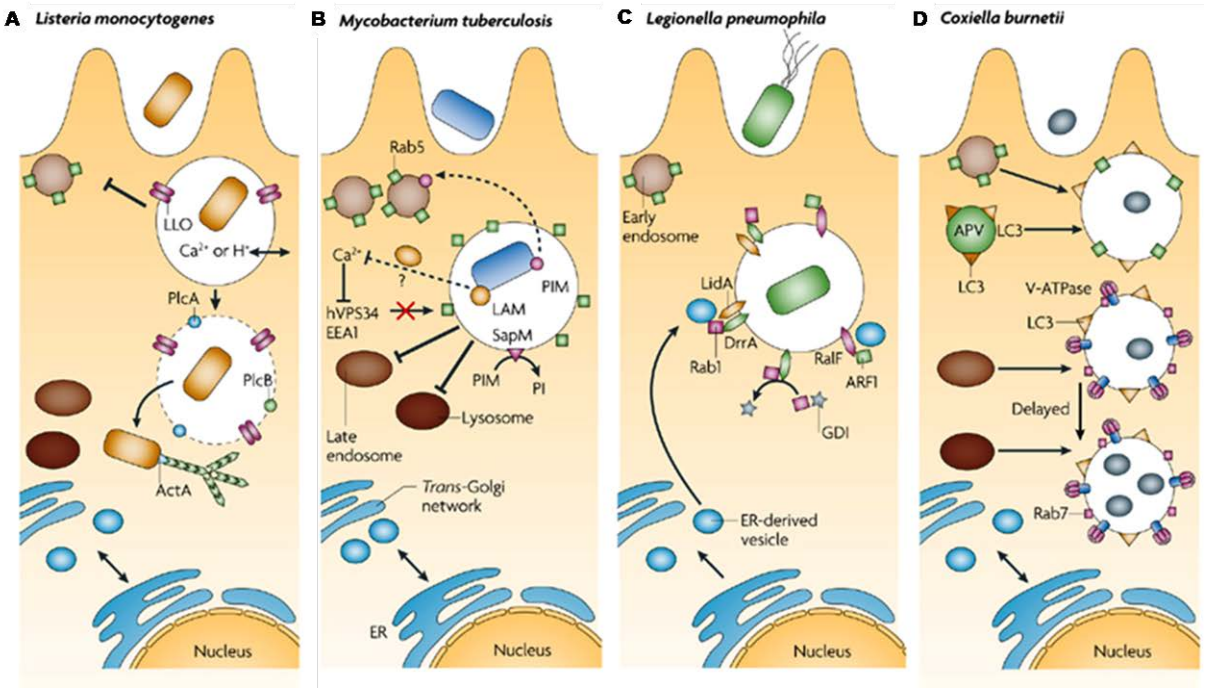

Figure 3: Strategies used by professional intracellular bacterial pathogens to modulate phagosome maturation. (A) *L. monocytogenes*, evades phagolysosomal fusion after internalization by escaping the phagosome through secretion of LLO and two phospholipases, PlcA and PlcB. Once in the cytoplasm, bacterium replicates and becomes motile by using actin 'comet tails' generated by the effector ActA. **(B)** After internalization, *M. tuberculosis* uses an array of effector molecules to arrest phagosome maturation at an early stage. **(C)** *L. pneumophila*, impairs fusion of the LCVs with endolysosomal compartments, and instead promotes fusion with ER-derived membranes. **(D)** Phagosomes containing *C. burnetii,* undergo delayed maturation as they fuse with autophagocytic vesicles bearing LC3. The delay enables *C. burnetii* to acquire features that allow it to replicate in a membrane-bound compartment that resembles phagolysosomes (Flannagan *et al*., 2009).

1.6. HYPOTHESIS AND OBJETIVES

Although it has been confirmed that *P. salmonis* is a bacteria affecting not only salmonideas species which causes great economic loss in production, it is most important to expand knowledge about *P. salmonis*, and facilitate the cultivation of the bacteria in a culture medium free of cells, despite the fact that some progress has been made recently in this area little is known about their infection strategy.

Considering the above background, the following hypothesis is proposed:

Hypothesis:

1. Replication and growth of *P. salmonis* in cell-free culture media and cell culture generates a differential gene expression.
2. Under identical culture conditions, there is differential gene expression between the reference strain LF89 and the pathogenic strain IBM040.

To prove this hypothesis the following general and specific objectives have been established:

General objective:

Analyze the expression patterns of the bacterial pathogen *P. salmonis* at transcriptome and proteome level, aimed at identifying genes and proteins probably involved in pathogenicity.

Specific objectives:

1. Optimize the system under study

1.1. Design a liquid culture medium for optimal growth of *P. salmonis.*
1.2. Identify the optimal conditions of infection in cell line SHK1, evaluated by immunocytochemistry.
1.3. Purify the bacteria from an infected cell line, using isopycnic centrifugation.

2. Analyse proteome in two-dimensional gels for differences in protein expression pattern of LF89 strain cultivated in cell-free medium versus grown in SHK1cell line.

2.1. Evaluate the differential expression of proteins in *P. salmonis*, grown in a cell-free medium or in SHK-1 cell line, through the technique DIGE.
2.2. Sequence proteins of pre-selected spots, identify, analyze and classify them according to functionality.
2.3. Validate the differential expression of pre-selected genes by qRT-PCR

3. Analyse proteomes of two strains of *P. salmonis*, LF89 (reference strain) and IBM040 (virulent strain), through two-dimensional gels, comparing the differences in their expression pattern.
3.1. Evaluate the differential expression of proteins between the two strains of *P. salmonis*, grown in a cell-free medium through 2D gels.
3.2. Sequence proteins of pre-selected spots, identify, analyze and classify them according to functionality.
3.3. Validate the differential expression of pre-selected genes by qRT-PCR

4. Analyze transcriptome and analize differentially expressed genes likely involved in virulence and pathogenicity of the strain IBM040 (pathogenic) versus the reference strain LF89 in liquid culture medium.
4.1 Identify gene sequences that are potentially involved in phatogenicity, through transcriptome sequencing of strains LF89 and IBM40.
4.2. Validate the differential expression of pre-selected genes by qRT-PCR.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Equipment and Instruments

- Class II Biological Safety cabinet: Nuaire NU425-400E.
- Centrifuges: Sigma 2-16 PL, Sigma 1-14 Microfuge, Boeco C-28A. 35, High Speed Refrigerated centriguges Hitachi Himac CR22-GII, Preparative Ultracentrifuge Hitachi Himac CP20-WX.
- Electrophoresis systems: Biorad Mini-Protean III, First-Dimension Isoelectric Focusing (IEF) System Biorad Protean IEF Cell, Serva HPE™ Tower System.
- Electroblotting system: Labnet Enduro modular Vertical gel system.
- Freezer -20ºC: Cónsul.
- Freezer -80ºC: Ult-Freezer 560 liters.
- Horizontal gel box electrophoresis: Labnet Enduro.
- Incubator: Zhicheng ZSD 1270.
- Incubator Shaker: Zhcheng, ZHWY-200B.
- Microware oven: Somela Faney WT1700.
- Microscope: LW-Scientific I4 Series, Olympus CKX41, Inverted Microscope.
- Micropipettes: Labnet, Biopette™ A Pipettes.
- Molecular Imager VersaDoc™ MP 4000 System.
- Power supplies: Biorad Power PacTM Universal Power Supply, Enduro E0303 model 300V power supply.
- Potter-Elvehjem Tissue Grinder 5mL: Weathon Science.
- Platform Shaker: Heidolph polymax 1040.
- Precision Balance: Sartorius TE4101.
- pH meter: Benchtop precision pH meter WTW InoLab pH720.
- Real-Time PCR System: Stratagene MX 3000P.

- Refrigerator: Fenza.
- Spectrophotometer: Thermo Scientific Evolution 60, GE Healthcare NanoVue.
- Thermoblock: Labnet, AccuBlock™ Digital Dry Baths.
- Thermal Cycler: Labnet, MultiGene™ OptiMax Thermal Cycler, Eppendorf, Mastercycler personal.
- Ultrasonic processor Cole Parmer, CPX130PB.
- UV transilluminator: Syngene, INGENIUS.
- Vortex mixer: Brarnstead International, MAXIMIX II VORTEX MIXER.
- Water Bath: N-Biotec, NB-301.

2.1.2. Chemical reagents

Table 1: Chemical reagents grouped by manufacturer.

Merck	Winkler	Promega
– Chloroform – Ethanol – EDTA – Glycine – Isopropanol – perhydrol – SDS – Sodium hydroxide – Potassium Chloride – Triplex III – Trypticase soy broth (TSB)	– TEMED – Bovine Serum Albumin – Sodium chloride – Acrylamide: Bisacrylamide – Bradford Reagent – Tween-20 – Triton X-100 – β-Mercaptoethanol – Potassium phosphate monobasic – Sucrose – Ammonium persulfate	– Random Primer – GoTaq Flexi DNA polymerase – M-MLV Reverse Transcriptase – Nucleotides, set dNTPs – Recombinant RNAsin ribonuclease inhibitor – RQ1 RNAse-Free DNAse – GoTaq qPCR Master Mix – SV Total RNA Isolation System, Promega (USA) – Wizard® Genomic DNA Purification Kit

Invitrogen	**Becton Dickinson**	**Ambion**
– Syber-Safe – Trypsin 0.05% – Fetal Bovine Serum	– Gram Staining – Trypticase soy agar (TSA)	– Kit RiboPure-Bacteria – Kit MICROBExpress – DEPC water
Sigma	**UsBiological**	**Hy Clone**
– DAB – Glucose – PMSF – Optiprep – Protease Inhibitor Cocktail For Bacterial Cells	– Tris base – L-cysteine – boric Acid – Urea – DTT	– Leibovitz L-15 – Trypan Blue
		Bios Chile
		– Indirect SRS-FluoroTest
Serva	– **Calbiochem**	**Fermentas**
– IPG Blue Strips 24cm – Serdolit MB-1 analytical grade – SERVALYT™ Carrier Ampholytes	– Iodoacetamide – CHAPS	– GeneRuler DNA Ladder Mix – PageRuler Plus Prestained Protein
	Gelcompany	**Thermo Scientific**
	– 2DGel Flatbed NF Large 12.5% – LavaPurple Staining	– Buffer Ripa
DyeAgnostics	**Rockland**	**Microtek International Inc**
– Refraction-2D labeling kit with G-Dye100, G-Dye200 and G-Dye300	– Anti-SHEEP IgG (H&L) (RABBIT) Antibody Peroxidase Conjugated	– Anti-SRS IgG (SHEEP) Antibody

2.1.3. Solutions

- **Buffer SB 1X:** 10mM sodium hydroxide, pH adjusted to 8.5 with boric acid.
- **Buffer MOPS:** 20mM MOPS pH 7.0, 2mM sodium acetate, 1mM EDTA pH 8.0.
- **Buffer Tris-Sucrose**: 10mM Tris–HCl pH 7.2, 0.25 mM Sucrose.
- **Buffer lysis total protein extraction:** 0.02% Triton X100; 1X Protease Inhibitor Cocktail; 0.1mM PMSF, in RIPA buffer 1X.
- **Formaldehyde loading dye:** 1mM EDTA pH 8.0, 50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol.
- **PBS:** 136.89mM NaCl, 2.68mM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4 pH 7.4.
- **Immunocytochemistry:**
  - Fixing solution: 4% paraformaldehyde in PBS.
  - Blocking solution: 1% BSA, 5% nonfat milk, 0,3% Triton x-100 in PBS.
  - TNT wash solution: 0.1M Tris–HCl pH 7.5, 0.15M, NaCl, 0.05%.Tween 20.
  - Development solution: 0.25mg/mL DAB, 0.0225% perhidrol, in PBS.
- **Polyacrylamide gel electrophoresis:**
  - Loading buffer SDS-PAGE: 62.5mM Tris-HCl, pH 6.8; 20% glicerol, 2% SDS, 5% β-mercaptoetanol 0, 002% bromophenol Blue.
  - Running buffer SDS-PAGE: 25mM Tris, 192mM glycine, 0.1% SDS; pH 8.3.
  - Stacking gel buffer SDS-PAGE 4x: 0.5M Tris-HCl pH 6.8, 0.4% SDS.
  - Resolving gel buffer SDS-PAGE 4x: 1.5M Tris-HCl pH 8.8, 0.4% SDS.
- **Coomassie Staining:**
  - Fixing solution: 50% methanol and 12% glacial acetic acid.
  - Coomassie blue staining solution for polyacrylamide gels: 0.3% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid.
  - Destaining solution: 30% methanol and 7% glacial acetic acid.
- **Siver Staining Compatible with Mass-Spectrometry:**
  - Fixing solution 40% ethanol, 10% acetic acid.

- Washing: 30% ethanol.
- Sensitizing solution: 0.02% (w/v) sodium thiosulfate ($Na_2S_2O_3$).
- Staining: 0.1% (w/v) silver nitrate (AgNO3).
- Developing solution: 3% (w/v) sodium carbonate (Na_2CO_3), 0.05% formaldehyde.
- Stop solution: 0.05M EDTA.

- **2D Spots preparation for sequencing:**
  - Solution A: 50mM NH_4HCO_3.
  - Solution B: 25mM NH_4HCO_3, 50% acetonitrile.
  - Trypsin digestion solution: 6.25ng/µl trypsin in solution A.
  - Extraction solution: 5% formic acid.

2.1.4. Software and on line tools

- Microsoft Office Excel 2007.
- NormFinder.
- Mascot Server, Mascot Deamon.
- Progenesis SameSpot.
- CLC Genomics wokbench 6.
- R-3.0.1, RStudio.
- http://www.ncbi.nlm.nih.gov/ *National Center for Biotechnology Information* (NCBI).

2.1.5. Biological material

- *Piscirickettsia salmonis* reference strain, LF-89 (ATCC VB 1361).
- *Piscirickettsia salmonis* field strain, IBM040.
- Cell line SHK-1, arising from Atlantic salmon head kidney .

2.2. METHODS

2.2.1. Culture biological material

2.2.1.1. Cell culture

Cell line SHK-1 from head kidney of *S. salar*, which exhibits properties of macrophages (Dannevig *et al*., 1997), was grown in culture flasks of 75 and 150cm^2, with culture medium Leibovitz's L-15 and 10% fetal bovine serum (FBS). The incubation temperature used for growth was 20°C.

Confluent cell layers were subcultured weekly, and the cells used in this study were between passages 90 and 120.

2.2.1.2. Bacterial culture in cell line

The Cell line SHK1 was used at 90% confluence and remained with L-15 medium and 2% FBS, to be infected with *P. salmonis* using multiplicity of infection (MOI) between 10 and 200 depending on the purpose of the test to be performed. Cultivation time after infection was 6-10 days until 90% cytopathic effect.

2.2.1.3. Bacterial culture in solid medium

Bacterial culture of LF-89 and IBM040 in cell-free medium, was performed on supplemented tryptic soy agar (TSA).

This medium was prepared according to the following description: 40g/L TSA, whith 15g/L NaCl was sterilized by autoclaving, 121°C for 15min, and then was supplemented aseptically with glucose, L-Cysteine, Fetal bovine serum (FBS)

and hemoglobin, at final concentrations of 5g/L, 0.1g/L, 5%, 2% respectively. The medium pH was adjusted to 7.4 with 5M NaOH and plates were incubated at 17°C for 7 to 20 days.

2.2.1.4. Bacterial culture in liquid medium

Bacterial culture of the strain LF89 and IBM040 in cell-free liquid medium, was performed on tryptic soy broth (TSB) supplemented. This medium was prepared according to the following description: 30g/L TSB, whith 3g/L NaCl was sterilized by autoclaving and then supplemented aseptically with, 0.05% L-Cysteine, 2.5% FBS and 10g/l $FeCl_3$. The medium pH was adjusted to 7.0 with 5M NaOH and the culture was incubated at 17°C with constant shaking at 60rpm for 3 to 10 days.

2.2.2. Proteomic analysis of *P. salmonis*

The analysis of proteins expressed by *P. salmonis* was made from the microorganism propagated in liquid medium and in the cell line SHK-1, for which purification technique of this bacterium from the infected cell line was standardized.

2.2.2.1. Purification of *P. salmonis* propagated in cell line SHK-1

Cell line SHK1 at 90% confluence were maintained in L-15 medium with 1% FBS, 48h before the infection with *P. salmonis*. Infection was done at MOI of 100. 24h post-infection cells were washed once with PBS and the culture medium was changed. The fourth day after infection, culture medium was removed and 3 washes were performed with 10mL of PBS in order to remove bacteria present in the culture medium and work with those present within the cells.

Cells were incubated with 0.05% trypsin for 3-7min, and then trypsin was inactivated by adding equal volume of 10% FBS in L-15 medium. The cells were

transferred to a centrifuge tube, sedimented at 4000xg for 10min at 4°C, the pellet was resuspended in 1ml of TS buffer, cells were lysed by dounce homogenizer, to allow release of the intracellular bacteria.

This suspension was subjected to isopycnic centrifugation according to the methodology described by Enríquez *et al*., 2003, making some modifications: OptiPrep density gradient (iodixanol) 22-24% was prepared, the previously obtained suspension was carefully layered on top and centrifuged at 25000xg at 4°C for 3h. Then the band formed in the upper third of the gradient containing the bacteria was recovered. The sample was diluted tenfold in tris-sucrose buffer for washing and then concentrated by centrifugation at 13000xg for 15 minutes at 4°C.

Bacteria obtained from this procedure were used for extraction of total protein.

2.2.2.2. Indirect Immunofluorescence

Indirect Immunofluorescence was carried out with Indirect SRS-FluoroTest Kit, according to the procedure recommended by provider (BiosChile). SRS-FluoroTest Kit is based on the use of a mixture of highly specific monoclonal antibodies against *P. salmonis* and an anti-mouse IgG conjugated with FITC is used for visualization. This assay is highly sensitive, specific and reproducible to detect *Piscirickettsia salmonis*

2.2.2.3. Immunocytochemistry

SHK1cells infected with *P. salmonis* were fixed in 4% paraformaldehyde in PBS for 10min, washed twice with PBS and incubated in 3% hydrogen peroxide solution in methanol for 10min., Subsequently cells were washed three times in PBS and incubated for 30min in blocking solution. A commercial polyclonal antibody Anti SRS Microteck was used at a dilution of 1:250 in blocking solution, as recommended by the supplier, and incubated for 12 to 16h at room

temperature. Three washes were performed in TNT wash solution and samples were incubated with peroxidase-conjugated secondary antibody at 1:1000 dilution, for 1h at room temperature. This was followed by three washes in TNT wash solution and incubation for 15min in Development solution. All washes were performed for 5min with constant shaking and cells smears were stained with hematoxylin-eosin as contrast (modified according to Figueroa *et al*., 1994).

2.2.2.4. Total protein extraction of *P. salmonis*

Total protein were extracted from *P. salmonis* by pelleting bacteria obtained from cultivation in liquid medium or alternatively those obtained from purification of intracellular bacteria through isopycnic centrifugation.

The pellet was washed in sterile PBS and centrifuged at 4000xg, for 10min. The bacterial pellet was resuspended in lysis buffer and lysis was completed by short pulses generated by a sonicator at minimum amplitude. After centrifugation at 4000xg for 10min, the supernatant was recovered constituting total protein extract of *P. salmonis.*

The samples were kept at 4°C throughout the procedure and total proteins were stored in aliquots at -20°C until use.

2.2.2.5. Protein quantification

The Bradford method (Bradford, 1976) was used for proteins quantification. Calibration curve from 5 to 30µg of protein per ml reagent, BSA in distilled water was used as standard. The reading was done after 10min in a spectrophotometer at a wavelength of 595nm. To determine the concentration of protein samples, aliquots were used for each one following the procedure described above and the resulting values were interpolated in the calibration curve. All readings were performed against their respective blanc.

2.2.2.6. Total protein electrophoresis in one dimension (SDS-PAGE)

Analysis of proteins extracted from microbial cell culture was carried out by electrophoretic separation on polyacrylamide gels in denaturing conditions (Laelmmli, 1970). The resolving gel was prepared at a concentration of 12% containing 1x resolving gel buffer, 0.04% ammonium persulfate and 0.04% TEMED. The stacking gel was prepared at a final concentration of 3.8% polyacrylamide containing 1x stacking buffer, 0.01% amoniopersulfate, 0.04% TEMED. 10µg of total protein in sample buffer was used. For electrophoretic fractionation Running Buffer SDS-PAGE was used.

Electrophoretic fractionation conditions were: 30min at 80V, followed by 1.5h at 120V. Gels were fixed for 1h in fixing solution. Staining was performed with Coomassie blue staining solution for a minimum of 3h, with constant stirring. The visualization of proteins was achieved incubating the gel in destaining solution and changing several times solution until background was fully washed-out.

2.2.2.7. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The identification of a spot on the gel corresponding to a single protein was achieved using 2D-PAGE technique which allows to assess the relative abundance of each spot on two related samples, followed by isolation and sequencing of a spot of interest.

2D-PAGE consists of two basic stages: In the first stage first dimension electrophoresis is performed, also called isoelectrofocusing where proteins are separated according to their isoelectric point. A commercial system, SERVA IPG *Blue*Strip 4-7/24cm, was used according to supplier's instructions. In the second stage the proteins were separated based on their size, this stage was performed with the commercial 2DGel NF Flatbed large 12.5% of Gelcompany, following the supplier's instructions.

After the SDS-PAGE, the gels was stained with Lava purple for scanning images and then with silver staining to achieved direct visualization of spots. Scanned images were analyzed with the Progenesis SomeSpot sofware.

2.2.2.8. Two-Dimensional difference gel electrophoresis (2D-DIGE)

The technique 2D DIGE relies on pre-electrophoretic labeling of samples with one of three spectrally distinct fluorescent dyes: G-dye100, G-dye200, G-dye300. The samples were all electrophoresed in one gel and viewed individually by scanning the gel at different wavelengths, thus circumventing problems with spot matching between gels.

Labeling of samples was performed according to supplier's instruction, Dyeagnostic.

The experimental design of small Refraction-2D™ experiments (≤ 12 samples) require technical replicates of the 2D gels. Since in general, fluorescence dyes differ slightly in their binding preference to each protein, dye-swaps were included in the experiment. An internal standard was also included, which was created by pooling equal amounts of protein from each sample to a final amount of 50μg protein per 2D gel and labeled with G-Dye100.

2D-PAGE was conducted as was described previously in Section 2.2.2.7

2.2.2.9. Lava purple staining

This staining war performed, following the supplier's instructions, Gelcompany.

2.2.2.10. Silver staining

Silver staining was performed according to Blum *et al*. (1987) with slight modifications. Gels were fixed for 1h with gentle agitation (40% ethanol, 10%

acetic acid), followed by three washes for 20min each, the first two were in 30% ethanol and the next in deionized water.

The gels were incubated for 1min in a solution of sensitization then rinsed three times in deionized water for 20s each.

Staining was performed for 20min at 4°C, with the staining solution previously cooled to 4°C, followed by three rinses in water for 20s each. Incubation in developing solution was held for 5-10min, until the spot was clearly visualized, the reaction was stopped by incubating 10min in 0.05M EDTA.

2.2.2.11. Sample preparing for nano LC-MS-MS

Spots preselected to be sequenced were excised from the gel and treated individually. Consecutive washes were performed 10min each, first in solution A and then in solution B, repeating the procedure four times. Subsequently, gel slice was dried in a vacuum centrifuge for 40min at 30°C. 6µl of trypsin digestion solution, were added to each gel piece and incubated a 37°C overnight for in gel-digestion. The obtained peptides were eluted with 25µl of 5% formic acid and subjected to nano-liquid chromatography-MS/MS analysis.

2.2.3. Transcriptome analyses of *P. salmonis*

2.2.3.1. RNA isolation from bacteria grown on supplemented TSB

Bacterial cultures in exponential phase between 0.2-0.6 OD_{600nm} were sedimented at 4000xg for 10min at 17°C. The pellet was washed in sterile PBS, then total RNA extraction from the bacterial pellet using the RiboPure-Bacteria Kit (Ambion) was performed according to the manufacturer's instructions. RNA was quantified by spectrophotometry at 260nm using equipment NanoVue (General Electric), following the relation 1 AU = 40µg/mL.

2.2.3.2. RNA isolation from bacteria grown on cell line SHK1

The extraction of total RNA from *P. salmonis* grown SHK1 cell line was made from $1x10^6$ cells infected with a multiplicity of infection (MOI) of 50, according to the conditions described in section 2.2.1.2.

The fourth day after infection, culture medium was removed and 3 washes with 10mL of PBS were performed in order to remove bacteria present in the culture medium and worked with those present within the cells. The culture was incubated with 0.05% trypsin for 5-7min, and then trypsin was inactivated by adding equal volume of 10% FBS in L-15 medium. The cells were transferred to centrifuge tube where was sedimented at 4000xg for 10min at 4°C.

Total RNA extraction was performed with the Promega kit, following the manufacturer's instructions and RNA was quantified as described in the previous section. The bacterial RNA was-enriched, removing most of the eukaryotic mRNA from the cell line SHK1, MICROBEnrich Kit (Ambion) was used for this procedure according to manufacturer's instructions.

2.2.3.3. Denaturing agarose gel electrophoresis

This procedure was modified from Current Protocols in Molecular Biology, Section 4.9.

RNA samples were prepared in sterile tubes by mixing 1.2µg of total RNA 2.5µl 10X MOPS running buffer, 4.5µL 12.3M formaldehyde, 12.5µL formamide. It was incubated at 55°C for 15 minutes, in order to denature the RNA and then placed on ice where 1µl of 1mg/ml ethidium bromide (EtBr) was added, and 5µl of RNA loading buffer.

Total RNA was fractionated under denaturing conditions on 1% agarose gels which were prepared by melting 0.4g agarose in 28.8ml DEPC water at 70°C,

slightly cooled down to room temperature and 4ml of 10X MOPS running buffer and 7.2ml 12.3M formaldehyde were added, to obtain a final concentration of 1X and 2.2M, respectively.

Electrophoretic fractionation was performed in 1X MOPS running buffer, the gels were pre-run for 5 minutes 5V/cm, performing fractionation at the same voltage.

Once finished the electrophoretic run, the gels were exposed to U.V. light on a transilluminator to visualize the RNAs.

2.2.3.4. Sequencing transcriptome of LF89 and IBM040

A sample of each strain was used for sequencing of the transcriptome, total RNA was extracted as previously described in section 2.2.3.1, and was sequenced by the Center of Excellence for Fluorescent Bioanalytics KFB, by using the following kits:

- Ovation® Prokaryotic RNA-Seq System of NuGEN for Library Generation.
- KAPA Library Quant Kits from KAPABIOSYSTEMS for Library Quantification.
- TruSeq SR Cluster Kit v3 for Cluster Generation, from Illumina.
- TruSeq SBS Kit v3–HS how Sequencing Kit, from Illumina.

The data obtained were analyzed with the software CLC Genomics Workbench 6.0.

2.2.3.5. Primer design

Primers were designed for genomic sequences homologous to LF89, using program primer3, a program with free on line access.

Table 2: List of primers for conventional PCR and real-time PCR (qPCR).

Name	Forward primer Sequence (5'->3')	Reverse primer Sequence (5'->3')	Product length (bp)
16S	AGAGTTTGATCCTGGCTCAG	ACGGATACCTTGTTACGAGTT	1505
Ps-Detect	CATGGAGATGAGCCCGCGTTG	GTCACACCTGCGAAACCACTT	469
ATPsyn	TATGCGGGTTGCGCGATGGG	TGCCGCACGCTCAAGTAGGC	189
GroEl	ACACGCGCCGCAGTTGAAGA	ACAGCCGCTTCGCTACCTGC	185
DNApol B	TGGCCGCTCATGAGTTTCCTGT	TTCGATGCCCATCCGTCGCC	198
SucCoA	AACGTATGGGCCATGCCGGTG	TACGCACACCCGCCGCTTC	90
NDK	GCCCTGTCATGGTGCAAGTGCT	ATCGTGCCCGCTACTGCCTCA	100
Kat	AATTGTCCGCATGCCACCCGT	TGAACGCCCAGAGGGTGCTG	105
PNPase	TAGACCGGCCATTGCGTCCG	CCAACACGTGCCGCTCCCAT	181
Clpx	AACGGACGAGATGAGAGCAGCA	GGGGTAGGTAACTGACTGGGCT	191
ClpP	GGTATTCAGGGCCAGGCGACA	GCGGTAATACCCCGCTCAGCAA	209
CsrA	CACTGTGACTGTCCTCGGTATTCG	ACTGCCTGGGAAAGGGACACGA	187
Ggt	GTGGCAAGTCAGGAAGCTCT	CGCCTAAATTACCAGCACGC	133
IcmL	ACTCTGCGGTGATTAGCTGG	CTGCTCCCAGCCATATGAAGT	121
IcmP	GAGTCGGCCAGGAAAAGTGG	CAGCCAATGCGATCTTACCG	144
VgrE	AGTGATCAGGCAGGAAGTGC	AAGACTCTCGGCTTGTGGTC	79
DotA	TCAGATTGGTCATGGGGTGC	ATCGCCGCAGTAAATAGCGA	200
AcrB	AAAAAGCCGTTGCCAGTGTC	GCAATAACGGGTTGATCGGC	184
AcrA	AGGCACCAATATCGTCAGCC	CACGGCTTGCAAAGGTATCG	131
VasQ	TGAGTGATGCATCTGTTGCG	GCGCGCTGATCTTCTTTACC	139
VasA	AAAGTGTGATGGGGCGTGAT	TGGCTGTGGGCGAATAAACT	164
DotB	AGGCATTAGTGCCGACGATT	TCAGCCGTCGAACGATACAG	100
RecA	TGTGCTAGGGAACCAAACGCGA	GGCGGACATTTTCCTTACCTTGACC	212
PykA	GCCGGCAACAGAAACGCCTGA	CGCGCGGTGCATATGGTCTT	109
RpoS 1s	ACCGAGCAACCTTGATGCGACA	CACCCTGCTGAGAGCGCCTAGA	107
RpoD 1s	GCTCCGACAAGCAACATCGGC	GCGCTCTGCCGCTTCCTCAA	168
ProC 1s	CGATGCCAAACACACCGGCT	CTAAGCCCACCGCACGCATC	117
GyrA	ACACGCTTAGTGGTCACGCCG	GCGCGGCAAGCCGTTTAGG	135
DnaB	TTTTGCCCGAAGGGCGCAGT	TCGACTCAGGGCCGGTCTGC	105
Rho	TACCCGGGCGCGTAAACAAGATG	CCAAAGCGGCGAATTTGACTGGGA	192
RecF	TCAGGTTGGCGCCAGATGTTG	CTGGCGTTTCATGCCCTGTTCAC	120
GlyA	CGCGTACCATTGCAGATTTCGACC	GCTTCTAGCACACGCGGACTCG	124

Name	**Forward primer Sequence (5'->3')**	**Reverse primer Sequence (5'->3')**	**Product length (bp)**
Ffh	ACGCCGCATTGCTGCAGGTT	CCGGTTCCCGGCGGCATCAT	159
GlnA	CGTTAGAAGCGGTGTTAGCCACA	AACGGATCAAGCACCGCCGTC	235
AtpA	TTTGACGCGACTTCAGAATCACA	ATGGACCTAACACCACCGCA	181
PhbB	ATCGGTGTTTACATCGCACG	TTGAGCTTCTTGCTCCGCTA	120
NusG	AGTGCGTAATGTGCCTCGTG	TCTCGCCATCTTTCAAGCGAT	113
RpoZ	AATTAGCGTATGGCTCGGAGG	TTCGGCAATTTCACGTAAGGC	80
AhpC	TGCTCTTGATAAGCGTGTGGA	GTACCACGGAAAGCCACTCC	219

2.2.3.6. DNA isolation from bacteria grown on supplemented TSB

Bacterial cultures in exponential phase between 0.2-0.6 OD_{600nm} were sedimented at 4000xg for 10min at 17°C. The pellet was washed in sterile PBS, then total DNA extraction from the bacterial pellet was performed using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. DNA was quantified by spectrophotometry at 260nm using equipment NanoVue (General Electric), following the relation 1 AU = 50µg/mL.

2.2.3.7. First strand cDNA

The synthesis of first strand cDNA was performed by reverse transcription (RT-PCR) using M-MLV Reverse Transcriptase and Random Primer both Promega, according to the supplier's instructions.

2.2.3.8. Polymerase Chain Reaction PCR

To identify optimal annealing temperature for each pair of primers used in qPCR assays (Table 1), gradient PCR varying the annealing temperature of 50-65°C were performed. The PCR reaction was performed using the following temperature cycles: Initial denaturation 95°C during 5min, follow of 35 cycles of denaturation 95°C for 30s, annealing 50-65°C for 30s, extension 72°C for 20s, and Final extension of 10min at 72°C.

The PCR reaction was performed in a total volume of 25uL containing 0.5U de GoTaq flexi DNA polymerase, 1x Green GoTaq Flexi Buffer, 3mM $MgCl_2$, 0.2mM mix dNTPs, 0.5µM of each primer, 100ng genomic DNA LF89, in DEPC water.

2.2.3.9. Electrophoretic fractionation on agarose gels

The gels were prepared by melting 1.5% agarose in buffer 1X SB (Brody and Kern, 2004), then 0.5µl Syber Safe 10,000X (Invitrogen) were added, and the solution poured into a mold. Samples of the PCR product and molecular size marker (Fermentas) were loaded into the agarose gel and fractionated at 100V in 1X SB buffer.

After electrophoresis, gels were exposed to U.V. light on a transilluminator and a digital image was captured.

2.2.3.10. qPCR assay

Evaluation of the expression levels of genes preselected was performed using real-time kit qPCR GoTaq Master Mix (Promega) according to manufacturer's instructions and qPCR reaction was performed in a real-time thermocycler Stratagen MX300P.

At least four samples for each condition of the study were used, which was performed in experimental triplicate. The variation of relative expression was calculated according to the formula $2^{-(\Delta\Delta CT)}$. For statistical analysis of the data the R-3.0.1 software was used.

For each analysis the best gene and the best pair of genes that presented less variation for the group of samples was determined, using the program NormFinder, these genes were used as normalizer. Results were plotted using Microsoft Office Excel 2007.

3. RESULTS

The results presented below were divided into four main sections, which are derived from the specific objectives.

3.1. PART 1: OPTIMIZATION OF THE SYSTEM UNDER STUDY

3.1.1. Designing a liquid culture medium for optimal growth of *P. salmonis*

Bacteria employ multiple mechanisms to control gene expression, but depend not only on specific regulatory mechanisms, but also on bacterial growth conditions, because important global parameters such as the abundance of RNA polymerases and ribosomes are all growth-rate dependent, for this reason it was necessary to work with bacteria that are in the same growth phase to generate a liquid culture medium suitable for growth of this bacterium.

Previous reports showed the ability of *P. salmonis* to grow in culture media containing blood from sheep or fish and a high content of cysteine, (Mikalsen *et al*., 2008; Mauel *et al*., 2008) whereby the first approach to obtain a liquid culture medium for this pathogen was to find a replacement for blood or hemoglobin used in solid media, routinely utilized in our laboratory, because they are not appropriate supplements in liquid culture media.

Initially TSB (Tryptic soy broth) was used as base culture medium, because it has similar composition to the base medium TSA (Trypticase soy agar), used for solid culture of *P. salmonis*, and was supplemented with equal amounts of the reagents used in the solid medium of this pathogen, except for the commonly used blood or hemoglobin.

Considering that iron is an essential nutrient for bacteria and is considered

essential for survival of intracellular pathogens such as *Mycobacterium tuberculosis*, *Salmonella typhimurium*, *Legionella pneumophila* and *Francisella tularensis* among others, and that iron is a major component in the haem group of hemoglobin, three iron salts were tested to 10mg/L to replace blood used regularly. The concentration of iron salts were chosen based on the described for defined medium, Adapted to Prit, 1975 for the bacterium *Klebsiella aerogens*.

SHK1 cell culture supernatant infected with *P. salmonis* was initially used with 90-95% of visible cytopathic effect, the supernatant was centrifuged at 300xg to remove cellular debris and the concentration of bacteria present was estimated according to scale of McFarland. 22,500,000 bacteria per ml of culture medium was seeded and incubated at 50rpm at 17°C. On the ninth day growth was observed and measured OD_{600nm} obtaining a value of 0.2. These cultures were used as initial inoculums to generate growth curves, measuring the increase in OD_{600nm}.

The result was that the reference strain LF89 was able to grow in the three media tested, reaching OD_{600nm} of 1.3-1.5 (Figure 4 A-C).

Once achieved the main objectives of development of a new liquid medium for *P. salmonis* culturing, the next step was to produce a solid medium, with the same formulation of the liquid medium, except that 1.5% agar-agar was added and twice the concentration of $FeCl_3$ and FBS. Bacterial growth was observed at day 4 and the appearance of colonies visualized only after 9 days of incubation at 17°C (Figure 4 D).

To check the purity of the culture liquid and the increase of the optical density which corresponds to an increase in the number of microorganism, samples were taken at different times and Gram staining and IFAT was performed, which showed characteristic coccoid bacteria that retain properties staining and size chords as previously described. An increase in the number of microorganisms in the different phases of microbial growth (Figure 5 A) was also observed.

The final confirmation of the identity of the bacteria was obtained by performing nested PCR. In a first round of PCR with primers for eubacterial 16S an amplification product of 1505bp was obtained and a second round of amplification with primers specific for *P. salmonis*, an amplification product of 467bp was obtained (Figure 5 B).

In order to optimize the culture medium a variation in the concentration of the fetal bovine serum, L-cysteine and sodium chloride was tested, achieving a significant improvement in maximum OD_{600nm} reached. In each growth curve concentration of only one of the compounds was varied, to assess their effect on the behavior of the growth curve (Figure 6).

Decreased fetal bovine serum at 2.5% showed no significant change in the behavior of the growth curve, however calculating the generation time of bacteria, indicated a value of 13.3h to medium supplemented with 5% FBS and 12.3h to medium supplemented with 2.5% FBS, indicating that reducing the FBS concentration a slight improvement in the rate of replication can be achieved. (Figure 6 A). By eliminating the FBS in the composition of the culture medium, bacterial growth was achieved, but the lag phase was very long therefore it was decided to use 2.5% FBS in the following culture media.

By reducing the concentration of 1g/l L-cysteine used initially to 0.5g/l an increase in the maximum OD_{600nm} with values close to 5 was observed (Figure 6 B). A similar effect was observed by decreasing the sodium chloride concentration of 1.5% initially used to 0.8% (Figure 6 B). Choosing the concentration of sodium chloride was based on the concentration of this salt in L15 culture medium, which is used to grow SHK1 cell line, used to propagate the pathogen.

Both the reference strain as IBM040 strain were subcultured in the culture medium optimized and growth curves were constructed, starting from which the generation time of each strain was calculated, obtaining as a result a time of

generation to a third subculture 12.4h for LF89 and 8.3h for IBM040 (Figure 7 A, C). Interestingly on comparing growth curves carried out in a third subculture with those made in a fifth subculture (Figure 7 A, B) a decrease in generation time of 4.2h was shown, indicating that *P. salmonis* had adapted to the culture media used.

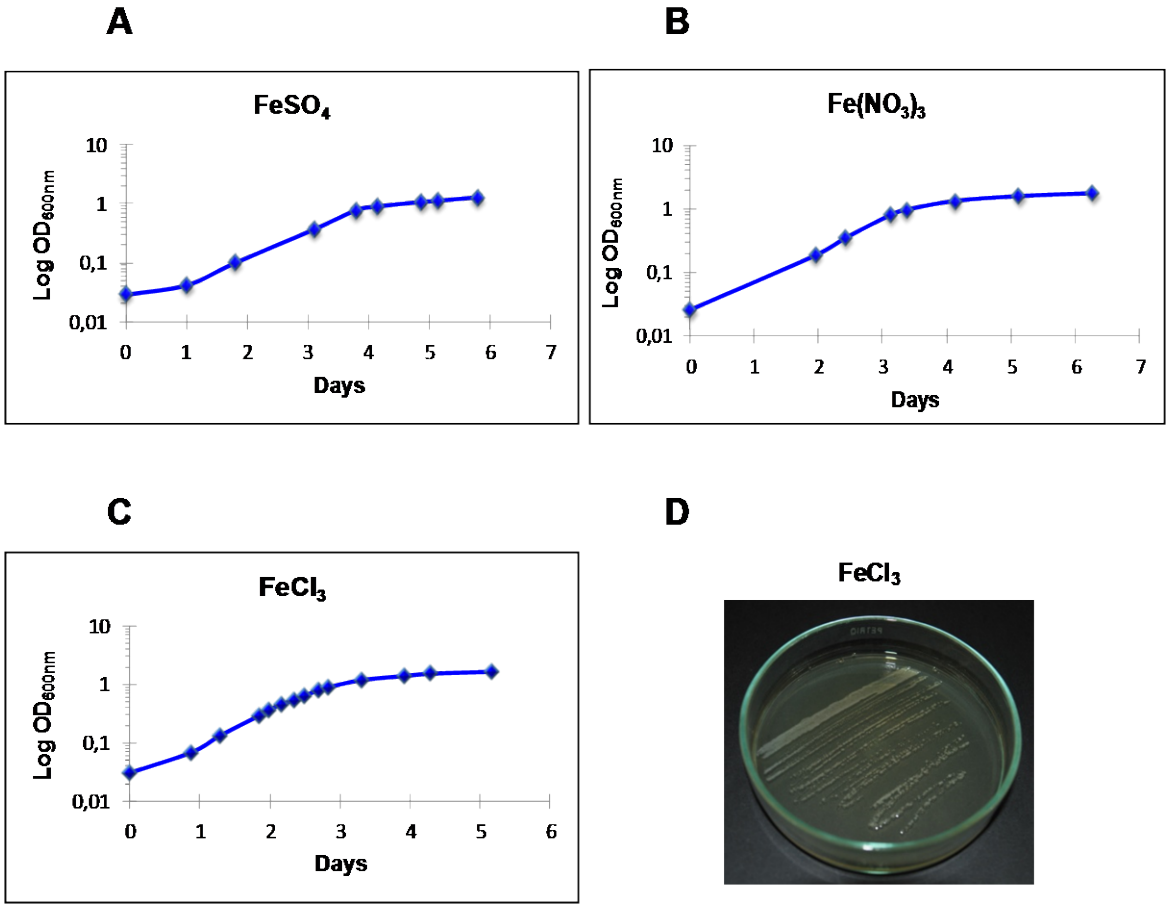

Figure 4: Growth in liquid and solid medium strain LF89. (A-C) Growth curves in liquid medium supplemented with different iron salts. **(D)** Growth in agar plate at day 12 of incubation at 17ºC.

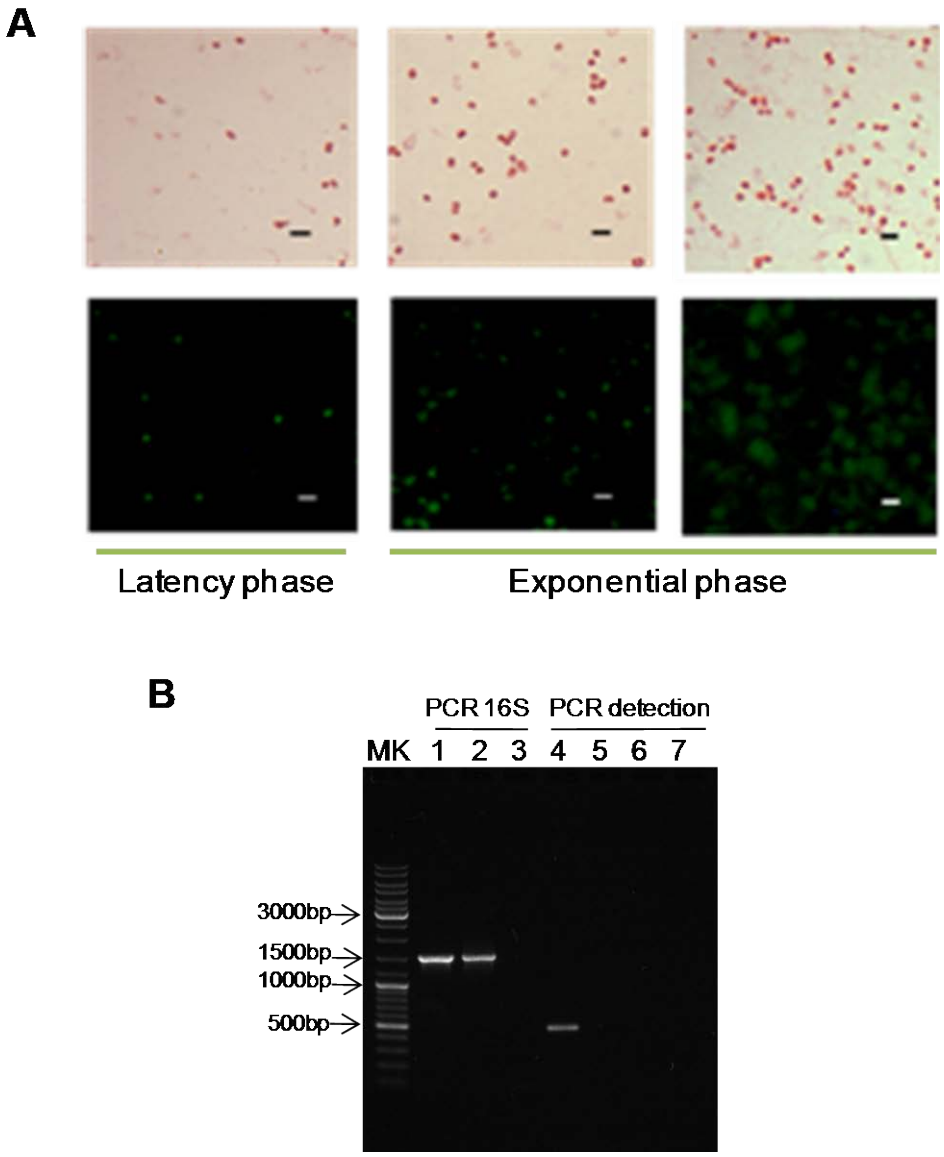

Figure 5: Verification of purity and identity of cultures. (A) Verification of the increased in number of bacteria in liquid medium. Gram staining and indirect immunofluorescence in samples taken from the lag and exponential phase. Bar 2µm. **(B)** Molecular verification of the identity of cultures. PCR analysis on day 5 of growth. MK gene ruler ladder mix (Fermentas). Lines 1 and 4: LF89, Lines 2 and 5: *E. coli*, lines 3, 6, 7: negative control nested PCR, first and second round.

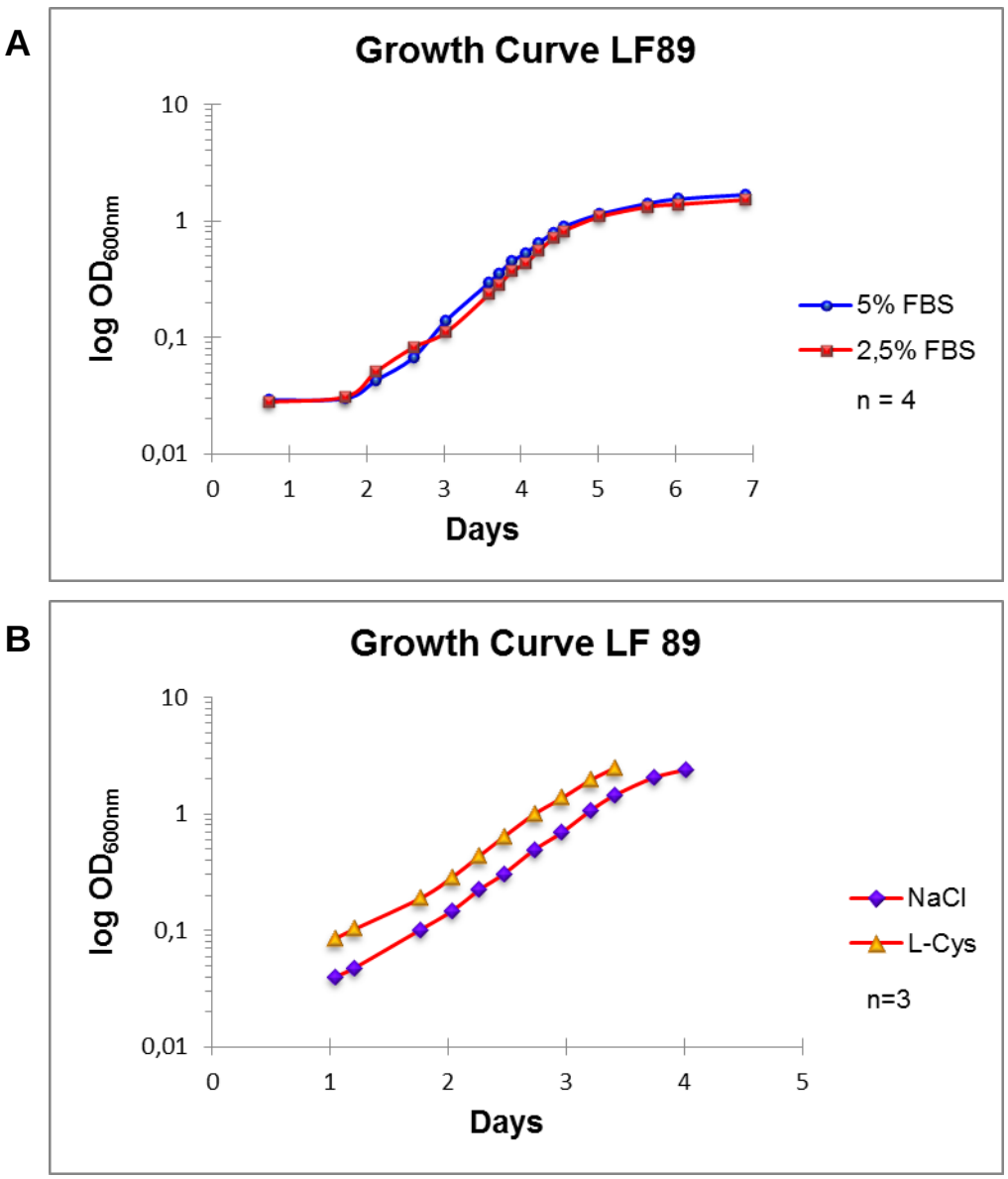

Figure 6: Growth curves for LF89: Optimization of liquid culture medium. (A) Comparison of the growth curves by varying the concentration of 5% (blue) FBS to 2.5% (red). **(B)** Comparison of the growth curves using 2.5% FBS and varying the concentration of sodium chloride at 0.8% (diamonds) or L-cysteine at 0.05% (triangles).

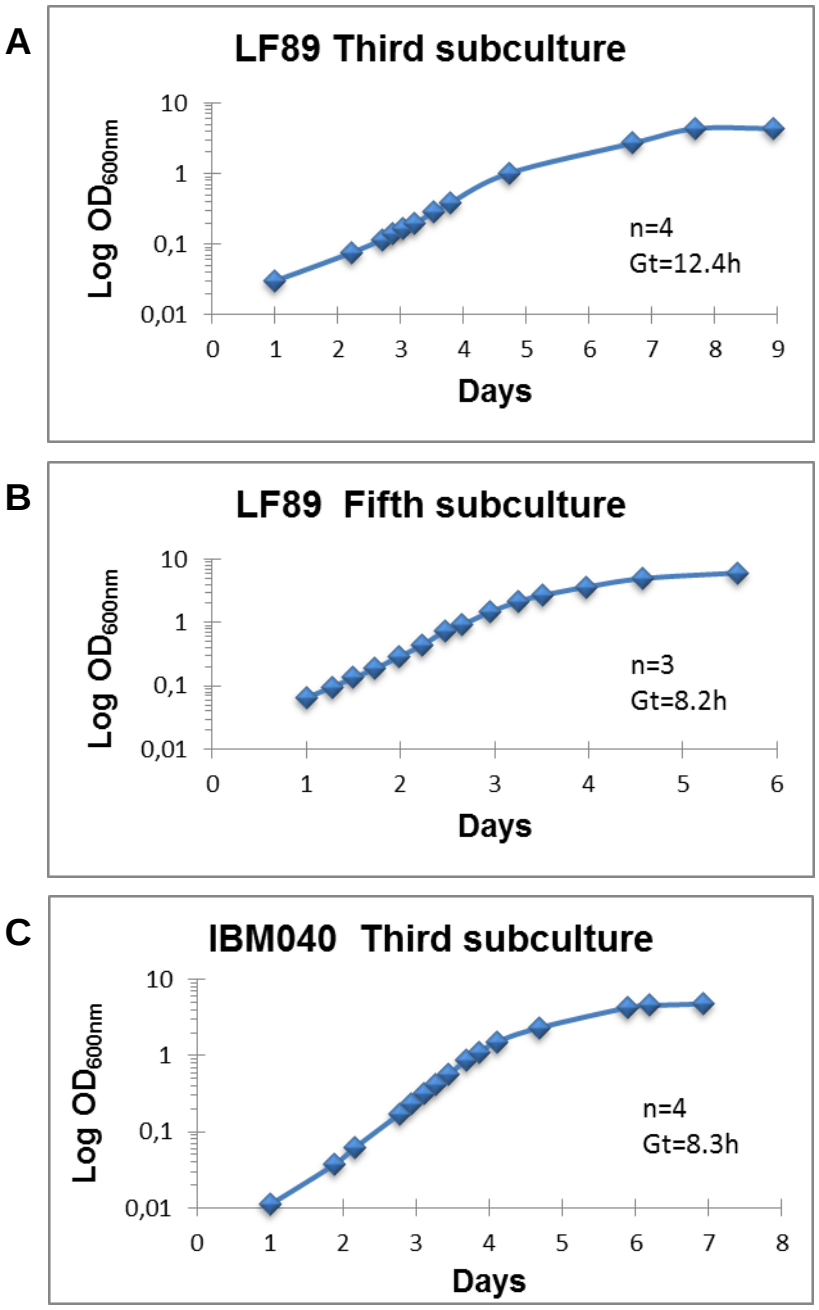

Figure 7: Growth curves in optimized culture medium for LF89 and IBM040. **(A)** Third subculture of reference strain. **(B)** Fifth subculture of reference strain. **(C)** Third subculture of IBM040 strain. From each growth curve the generation time (Gt) was calculated. The experiment was performed in triplicate and quadruplicate, as indicated in the legend of each graph.

3.1.2. Identification of optimal conditions of infection in cell line SHK1

The cell line SHK1 was derived from a macrophage-enriched cell culture, from Atlantic salmon head kidney. Characterisation of a long-term cell line SHK1 showed, proliferating cells appeared after several weeks in culture, but did not resemble macrophages. The cells were flattened and elongated with a variable morphology. Subcultured cells had a more homogeneous morphology. The cells reacted strongly with monoclonal antibodies (MAbs) directed against Atlantic salmon peripheral blood leucocytes but not with a MAb specific for Atlantic salmon polymorphonuclear leucocytes. The SHK-1 cells did not endocytose protein ligands which are recognised by the scavenger, mannose- and galactose-specific receptors. The cell line did phagocytose the fish pathogen*Aeromonas salmonicida*to some extent, but no bactericidal activity was observed over a period of 72 h. Of this profile of phenotypic and functional properties, it was concluded that the SHK-1 cell line is derived from leucocytes that have some of the properties of a macrophage (Dannevig *et al*., 1997).

The infection of cell line SHK1 with strain LF89 was evaluated by immunocytochemistry performing infection kinetics for 9 days. SHK1 cell line was infected with approximately 50 bacteria per cell, which were obtained from the cell culture supernatant infected with the reference strain displaying between 90-95% cytopathic effect. 24h post-infection, the culture medium was changed to remove any bacteria that failed to infect cells.

The results showed a progressive increase in the proportion of infected cells in the course of time, indicating that early lysis of infected cells allow the release of bacteria that replicate within the cell, which allows the infection of practically all of the cells cultured (Figure 8). Notably, the ninth day after the infection, in the cell culture supernatant a lot of bacteria were found which were released through lysis of cells containing them, so the amount of cells that remained attached to culture surface was lower than evaluated at the beginning of

infection.

In order to obtain bacteria from the inside of the cell line infection conditions were adjusted, thus was used for bacteria grown in liquid medium in the exponential growth phase, to perform the infection at different MOI from 10 to 400 bacteria per cell and evaluated for immunocytochemistry on the third day after infection resulting in that a higher MOI 200 reduced the percentage of infected cells (Table 3). It was also observed that increasing the MOI, infected cells had higher numbers of bacteria per cell, which leads to the assumption that the process of cell lysis would be faster than normal. This could lead to the decrease in the proportion of infected cells should be to lysis of cells infected with initially increased bacterial load.

By repeating this assay with some modifications that included the synchronization of the cell line through the incubation with L15 culture medium 1% FBS for 48h prior to infection and evaluating the results on the second day after infection a slight improvement in MOI of 10 and 50 was obtained, however there was a clear increase in the percentage of cells infected at MOI 100, reaching a 42% infected cells which was finally chosen for subsequent infections (Table 4). Interestingly when assessing the infectiousness at MOI 100 of a culture at the beginning of the stationary phase 32% of infected cells were obtained, a value smaller than that obtained by using bacteria in exponential growth.

To assess whether this change was the result of culture viability $TCID_{50}$ was calculated, in infected plates with serial dilutions of *P. salmonis* in exponential and stationary phase. The same result was obtained in both conditions $4{,}645x10^{6}TCID_{50}$, indicating that post-exponential cultures would be no more virulent than bacteria in exponential growth phase, which diverges from several reports indicating that pathogenic bacteria found in the post-stage of exponential growth, would present increased virulence phenotypes associated with the lack of nutrients in the culture medium.

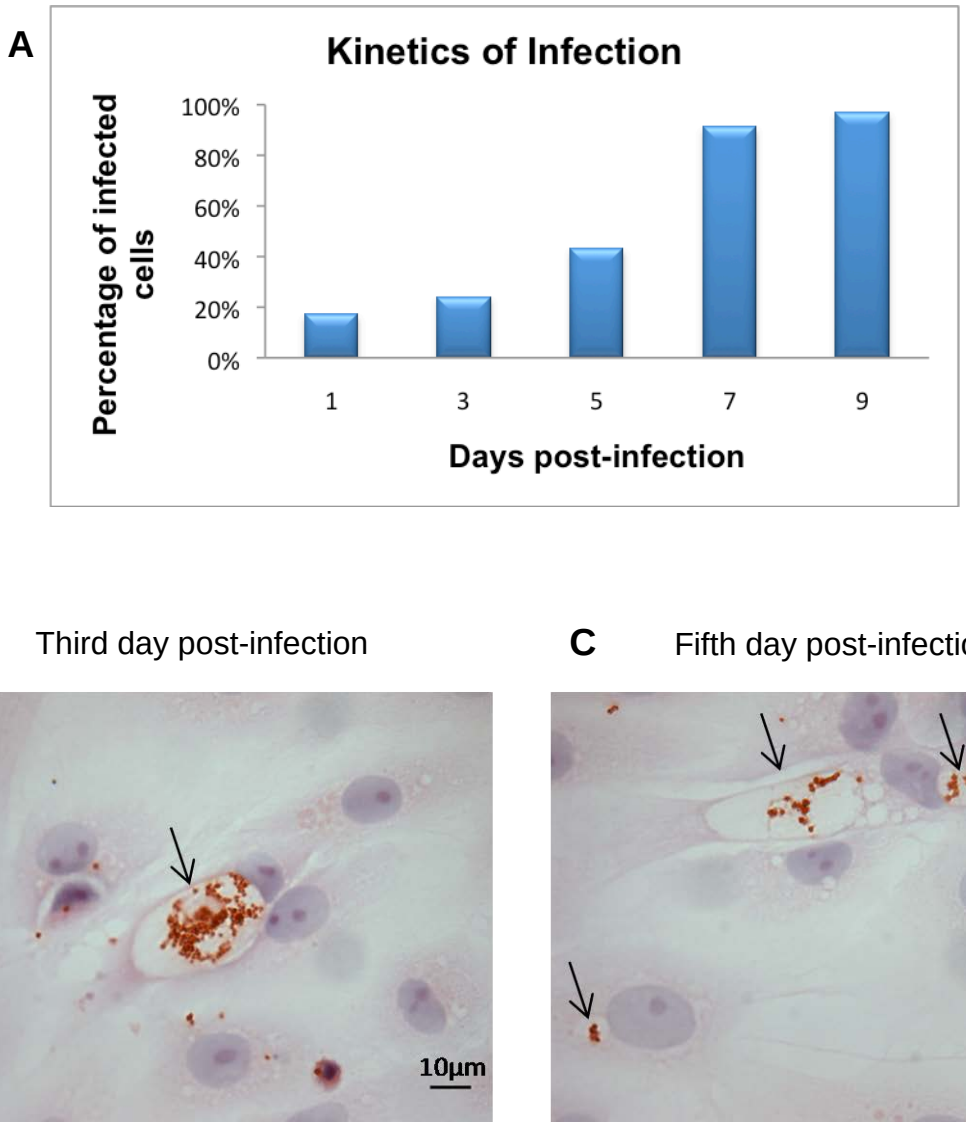

Figure 8: Evaluation of infection progress of of *P. salmonis* LF89 strain in SHK1 cell line. (A) Percentage of infected cells in the course of time. **(B, C)** Immunocytochemistry of *P. salmonis* in the cell line SHK1 infected with MOI 50, arrows indicate the bacteria into vacuoles of the cell. **(B)** Third day post-infection. **(C)** Fifth day post-infection.

Table 3: Percentage of SHK1 cells infected with the reference strain, using different multiplicities of infection evaluated by immunocytochemistry on the third day after infection.

MOI	Percentage of infected cells
10	8,8%
50	13%
100	20,6%
200	22,4%
300	14,7%
400	12,4%

Table 4: Percentage of SHK1 cells infected with the reference strain, using different multiplicities of infection evaluated by immunocytochemistry on the second day after infection.

MOI	Percentage of infected cells
10	9,7%
50	17,9%
100	41,2%

3.1.3. Purification of bacteria from an infected cell line

With the aim of assess differences in gene expression at the protein level of the reference strain when this is grown intracellularly or in culture cell-free was necessary to mount a purification system of the bacteria when it is grown intracellularly.

Purification of bacteria growing within the cell line SHK1 was performed by isopycnic centrifugation as described in Section 2.2.2.1 of materials and methods, thus able to obtain a band in the upper third of the gradient which corresponds with the bacteria of interest. This was checked by Gram stain and IFAT, which showed positive reaction, confirming the identity of *P. salmonis* (Figure 9).

Purification of bacteria growing within the cell line was possible thanks to the identification of the optimal conditions of infection of the cell line SHK1, because efficiency in the purification of the bacteria is low, therefore it is important to begin the purification with the highest percentage of infected cells with *P. salmonis*.

Clearly bacteria were obtained without apparent contamination of the cell line, pivotal condition for proper evaluation at the protein level of expression profiles of reference strain comparing both culture conditions. This constituted one of the main objectives of this thesis.

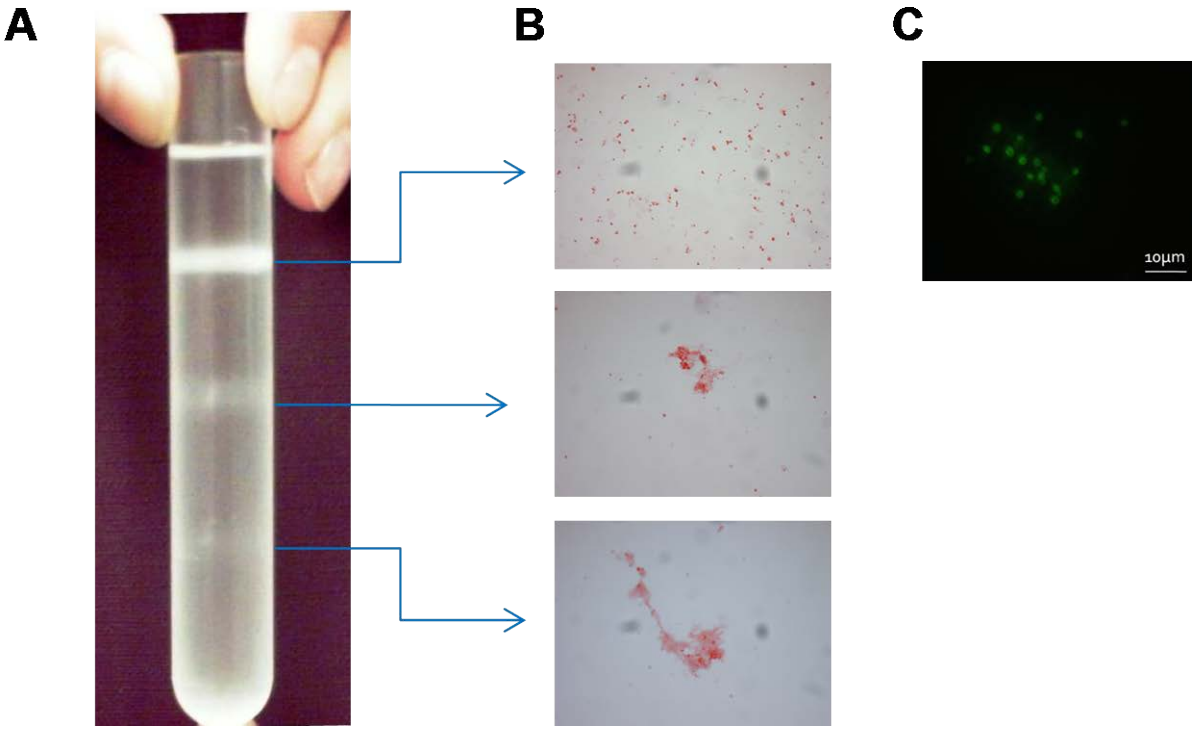

Figure 9: Purification of *P. salmonis* using OptiPrep gradients. (A) Iodixanol gradient in balance, white band in the upper third, corresponding to *P. salmonis*. **(B)** Gram staining of the three bands observed. **(C)** IFAT, upper third band (SRS Fluorotest indirect BiosChile).

3.2. PART 2: PROTEOMIC ANALYSIS OF LF89 STRAIN CULTIVATED IN CELL-FREE MEDIUM VERSUS GROWN IN SHK1 CELL LINE

3.2.1. Differential expression of proteins in *P. salmonis*, grown in a cell-free medium or in SHK-1 cell line.

The ability to sense and respond to the environment is essential for survival of all living organisms. Bacterial pathogens are of particular interest due to their ability to sense and adapt to the diverse range of conditions they encounter, both *in vivo* and in environmental reservoirs (Runkel *et al*., 2013). During the cycling from host to non-host environments, pathogenic bacteria encounter a variety of environmental insults ranging from temperature fluctuations, nutrient availability and changes in osmolarity, to the presence of antimicrobial peptides and reactive oxygen/nitrogen species. Such fluctuating conditions impact on various areas of bacterial physiology including virulence, growth and antimicrobial resistance (Runkel *et al*., 2013).

A key component of the success of any bacterial pathogen is the ability to recognize and mount a suitable response to the discrete chemical and physical stresses elicited by the host. Such responses occur through a coordinated and complex programme of gene expression and protein activity (Runkel *et al*., 2013).

Due to the ability of pathogenic bacteria to modify gene expression would compare the proteomes of the reference strain when it is subjected to two culture conditions: bacteria grown in a liquid culture medium previously described and bacteria grown in SHK1 cell line, where they reside in large vacuoles within cells.

To obtain total protein from bacteria grown in cell line it was necessary to mount a purification system of *P. salmonis*, which achieved a fraction enriched in bacteria, eliminating components of the cell line (protocol in section 3.1.3). Total proteins derived from both culture systems described above, were used for 2D-

DIGE assays as described in Materials and Methods in Section 2.2.2.8 and four experimental replicates were performed (Figure 10).

With 2D-PAGE technique, a spot on the gel was identified corresponding to a single protein, which allowed to assess the relative abundance of each spot on two related samples, followed by isolation and sequencing of a spot of interest.

Figure 10 A shows the electrophoretic fractionation in the second dimension where proteins from bacteria grown in cell line were labeled with G-dye200 prior to electrophoretic fractionation as well as proteins obtained from bacteria grown in liquid medium labeled with G-dye300, which are displayed in green and red respectively. Because fluorescence dyes differ slightly in their binding preference to each protein, dye-swaps was included to the experiment (Figure 10 B).

It is noteworthy that an internal control was included, a third sample composed of equal parts of two of the analyzed samples was labeled with G-dye100 and mixed with samples previously labeled with G-dye200 and G-dye300, prior to electrophoretic fractionation.

The results were analyzed with the Progenesis SameSpot software, which identified 226 Spots that showed significant difference (ANOVA p value <0.05) between two groups of samples. Intensity and extentio of 98 spots increased and 128 spots decreased in samples of bacteria grown in the cell line with respect to bacteria cultivated in liquid medium (Figure 11). Noteworthy, in this evaluation spots were considered that were present in both samples, these were assigned a relative value with respect to changes in expression level (Annex 1).

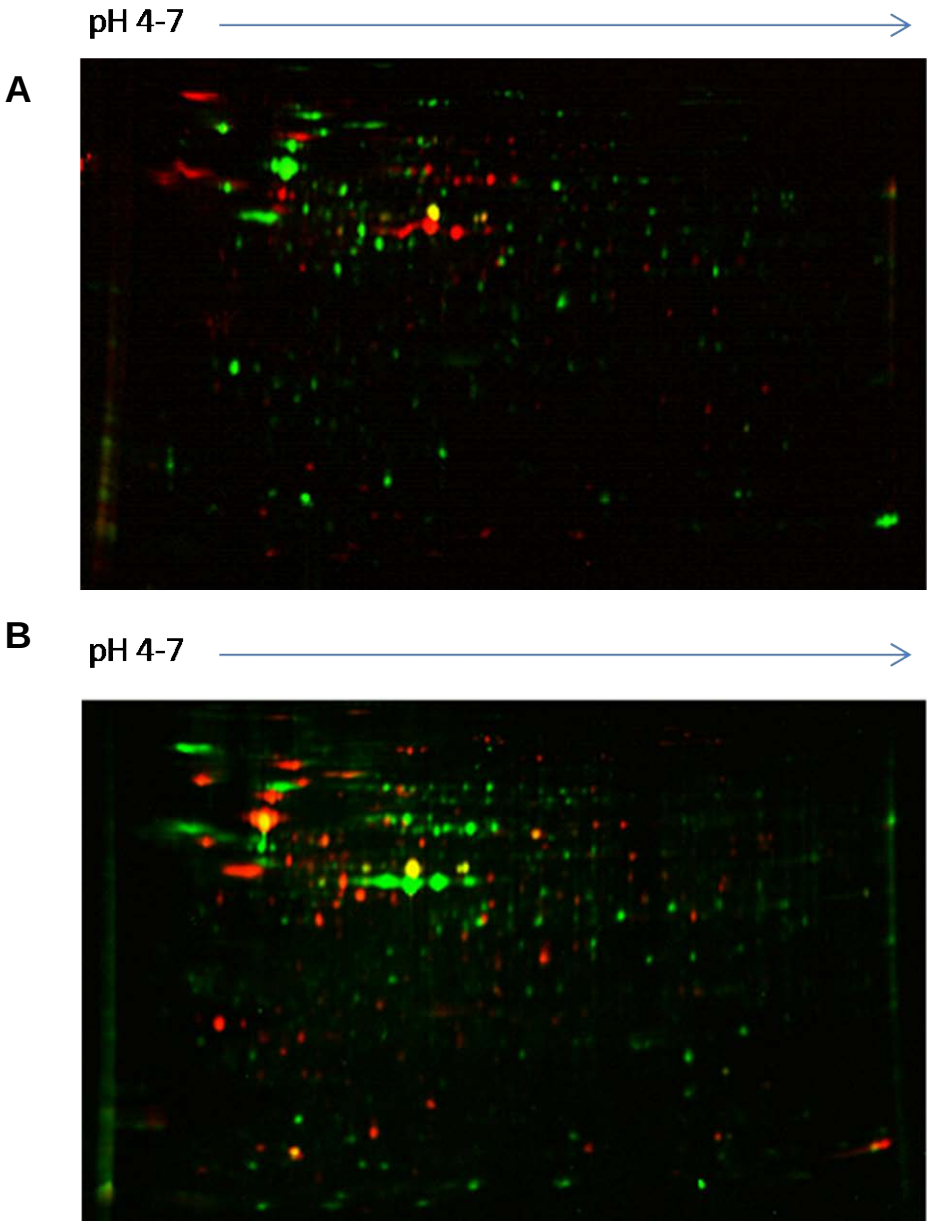

Figure 10: Comparative analyses of proteoms of LF89 cultivated in cell-free medium versus bacteria grown in SHK1 cell line by 2D-DIGE. (A) Green, G-dye200: Total proteins obtained from bacteria grown in SHK1 cell line and purified by isopycnic centrifugation. Red G-dye300: Total proteins obtained from bacteria grown in liquid medium **(B)** dye-swaps.

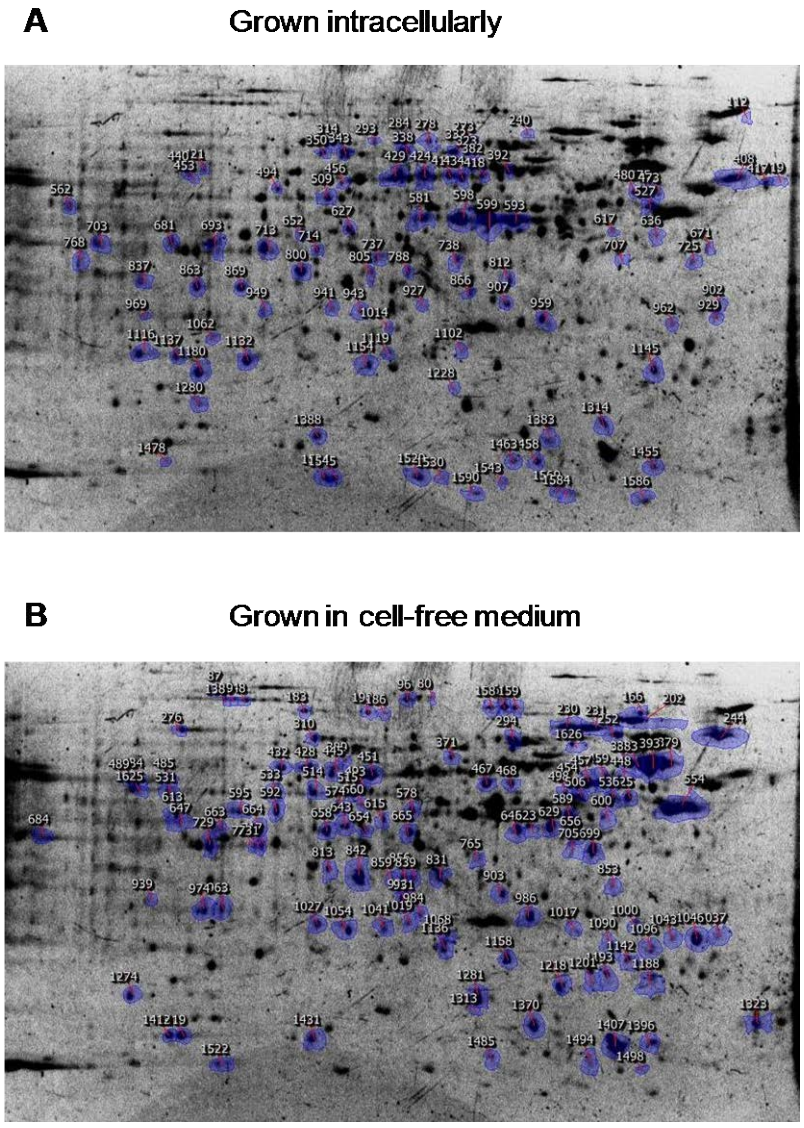

Figure 11: Analysis of 2D-DIGE with the Progenesis SameSpots software. Spot highlights that have statistically significant differences between LF89 grown in liquid medium or in SHK1 cell line. ANOVA p-value ≤ 0.05 **(A)** spot highlights that have greater intensity in samples from bacteria purified by isopycnic centrifugation, regarding bacteria obtained from liquid culture**. (B)** Spot highlights with less intensity in samples from bacteria purified by isopycnic centrifugation, regarding samples obtained from liquid culture**.**

3.2.2. Proteins sequencing of pre-selected spot, identification and classification according to functionality

The results of assessing differential expression of proteins in 2D-DIGE (Annex 1) were chosen at random 70 spot for isolation from the gel and subsequent sequencing. It was possible to identify proteins from 50 spots, obtaining as a result the identification of 69 protein sequences corresponding to *P. salmonis* (Annex 2). Because in several spots more than one protein was identified, which could be due to either isolation errors when the spot was extracted from the gel or to lack of resolution in the gel.

Among the 69 protein sequences was found that in some cases there are two sequences associated with the same protein as in the case of ClpP (Protease subunit of ATP-dependent Clp proteases) GPMI (Phosphoglyceromutase) and PyrC (Dihydroorotase and related cyclic amidohydrolases), GATA (Asp-tRNAAsn/Glu-tRNAGln amidotransferase A subunit). Both sequences identified were found in the same spot.

On the other hand, there was also a case where a protein sequence was identified in different positions in the gel, ie several spot identified correspond to the same protein, as is the case of GroEL in which four spots were associated with this protein and this could be due to modifications in the protein that affect it's migration in the gel. (Annex 2, Table 5). From 69 identified amino acid sequences, nine correspond to repeat sequences.

Table 6 summarize proteins, which were grouped according to the classification of Clusters of Orthologous Groups of proteins (COGs), also indicating how many proteins of a particular group were increased in intracellular living conditions.

Clusters of Orthologous Groups of proteins (COGs) were delineated by

comparing protein sequences encoded in 43 complete genomes, representing 30 major phylogenetic lineages. Each COG consists of individual proteins or groups of paralogs from at least 3 lineages and thus corresponds to an ancient conserved domain (http://www.ncbi.nlm.nih.gov/COG/)

Table 5: Proteins identified in more than one spot in 2D-DIGE analyses.

Gen	Associated Spot
Gro EL	4
ClpP	3
AtpA	2
Adk	2
Mdh	2
OmpR	2
Pnp	2

Table 6: Differentially expressed *P. salmonis* proteins identified by 2D-DIGE and mass spectroscopy grouped by the COGs classification.

Code	Description	N° associated proteins	N° of proteins increased intracellularly
C	Energy production and conversion	11	3
E	Amino acid transport and metabolism	1	-
EF	Amino acid transport and metabolism and Nucleotide transport and metabolism	2	-
F	Nucleotide transport and metabolism	12	-
G	Carbohydrate transport and metabolism	5	1
H	Coenzyme metabolism	2	1
I	Lipid metabolism	1	-
J	Translation, ribosomal structure and biogenesis	9	3
K	Transcription	3	1
L	DNA replication, recombination and repair	1	1
M	Cell envelope biogenesis, outer membrane	2	-
NO	Cell motility and secretion and Posttranslational modification, protein turnover, chaperones	3	-
O	Posttranslational modification, protein turnover, chaperones	13	-
OC	Posttranslational modification, protein turnover, chaperones and Energy production and conversion	1	-
P	Inorganic ion transport and metabolism	1	-
QR	Secondary metabolites biosynthesis, transport and catabolism and General function prediction only	1	-
T	Signal transduction mechanisms	2	1
TK	Signal transduction mechanisms and Transcription	1	-
	without classification	1	-

3.2.3 Validation of differential expression of pre-selected genes by RT-PCR in real time

For quantitative real time reverse transcription PCR (RT-PCR) assays is necessary to have constitutively expressed genes suitable for each study group. Accurate normalization is an absolute prerequisite for correct measurement of gene expression. The most common normalization strategy involves standardization to a single constitutively expressed control gene. However, in recent years, it has become clear that no single gene is constitutively expressed in all cell types and under all experimental conditions, implying that the expression stability of the intended control gene has to be verified before each experiment (Andersen *et al*., 2004). For these reasons it was proposed the goal of identifying genes that could be used as normalizers genes in qPCR assays several genes were tested, which was chosen based on several available publications.

The genes selected for test their stability are 16S ribosomal RNA (*16S*), RNA polymerase sigma factor (*rpoD*), RNA polymerase sigma factor (*rpoS*), DNA gyrase A subunit (*gyrA*), recombinase A (*recA*), recombination protein F (*recF*), pyrroline-5-carboxylate reductase (*proC*), transcription termination factor (*rho*), pyruvate kinase (*pykA*), signal recognition particle protein (*ffh*), glutamine synthetase (*glnA*), serine hydroxymethyltransferase (*glyA*), and replicative DNA helicase (*dnaB*) (Takle *et al.*, 2007; Nielsen and Boye, 2005; Savli *et al*., 2003; Vandecasteele *et al.,* 2001).

In general for all the primers used in qPCR optimal annealing temperature was verified through gradient PCR, covering temperature ranges from 50-65°C (Annex 3).
A variation in the Ct obtained by varying the concentration of primers was tested and the presence of a single peak in melting curves obtained was veryfied. These trials use genomic DNA of strain LF89 as template (Annex 6).

In the assays to evaluate gene expression through qPCR, cDNA was used as template, which was synthesized from total RNA samples of bacteria submitted to the diverse culture conditions previously described, in samples the RNA integrity was verified, performing an electrophoretic fractionation in denaturing conditions of an aliquot of each sample, as described in section 2.2.3.3. Some examples of the obtained results are shown in Annex 4. To exclude genomic DNA contamination in RNA samples, PCR to the RNA sample was performed, and as a result was expected to get only an amplification product in the positive control of reaction, in which using genomic DNA strain LF89 as template (Annex 5). In the case of RNA samples that showed amplification was repeated the treatment with DNAse I and PCR for verification was repeated. If genomic DNA contamination persisted, the sample was discarded.

The genes stability was tested in cultures of strain LF89 in exponential growth phase, using three variations of the culture medium, which are the previously described culture medium under conditions optimized by varying the pH from 7.0 to 6.0 and the culture medium to pH 7.0 with higher concentration of NaCl 1.8% (Figure 12). The results were analyzed with the NormFinder program (Andersen *et al*., 2004), delivering as a result that *16S* gene was the most stable followed by *rpoD* and as best combination of genes to be used simultaneously *ffh* and *rpoS*, which together deliver a stability value of 0.178, better than 0.406 obtained for the *16S* gene of (Table 7).

Choosing the best combination to use was realized by NormFinder program, considering the variation of each gene expression in both within the groups of samples to be analyzed such as the variation between the groups analyzed, in this case the three groups corresponding to the three variations of the culture medium (Figure 12).

In the particular case of the *16S* gene it was necessary to make a 1:10 dilution of cDNA used for template in qPCR reaction. Using the cDNA undiluted Ct was not obtained in many of the samples. On checking melting curves, all samples

exhibited a dissociation curve with a single peak. By fractionating this sample on an agarose gel, in all samples, bands of the expected size with great intensity were observed.

Although the dilution of cDNA was performed, the Ct values obtained were in the range of 9 to 12, which were lower than those Ct obtained using other genes of interest to be evaluated, which generally had Ct values between 19 and 26 for all genes analyzed, and therefore were discarded in subsequent analyzes.

Six genes that to proteome level showed differential expression regarding culture conditions, intracellular or cell-free medium were assessed by qPCR, and three genes were tested to analyze the stability of its expression in the described conditions, which were *rpoD*, *pykA* and *recA*.

The choice of genes to be evaluated was based on available information regarding their putative functions related to virulence in pathogens. Below outline some of the described functions of the genes selected to be evaluated by qPCR.

Csr system is composed of CsrA protein and two small non-coding regulatory RNAs (ncRNAs). Csr systems play in the pathogenesis of certain bacteria and in the establishment of successful infections in animal hosts. Csr systems appear to control the 'switch' between different physiological states in the infection process (Lucchetti-Miganeh *et al*., 2008).

Clp proteolytic complexes are essential for virulence and survival under stress conditions in several pathogenic bacteria. ClpP, the catalytic core of the Clp proteolytic complex, is involved in regulating a multitude of processes including, stresses response, regulation of biofilm formation, antibiotic resistance and virulence. ClpX is the ATPase subunit, is involved in contribute to virulence controlling the activity of major virulence factors in *Staphylococcus aureus* (Frees *et al*., 2003).

Defense against host-derived reactive oxygen species has been proposed as critical for intracellular replication, catalase maintain a critically low level of H_2O_2 compatible with proper phagosome trafficking in *Legionella pneumophila* (Bandyopadhyay *et al*., 2003).

Nucleoside diphosphate kinase (Ndk) is an important enzyme that generates nucleoside triphosphates (NTPs) or their deoxy derivatives by terminal phosphotransfer from an NTP such as ATP or GTP to any nucleoside diphosphate or its deoxy derivative. As NTPs, particularly GTP, are important for cellular macromolecular synthesis and signalling mechanisms, Ndk plays an important role in bacterial growth, signal transduction and pathogenicity (Chakrabarty, 1998). An example is the Mycobacterial Ndk, is a putative virulence factor that inhibits phagosome maturation and promotes survival of *Mycobacteria* within the macrophage (Sun *et al*., 2010).

Polynucleotide phosphorylase (PNPase) is one of the major exoribonucleases in bacteria and plays important roles in mRNA degradation, tRNA processing, and small RNA (sRNA) turnover (Zeng *et al*., 2010). In Yersinia spp PNPase independently controls virulence factor expression levels and export (Rosenzweig *et al*., 2007).

Succinyl-CoA synthetase, prevents the conversion of succinyl-CoA to succinate, and has been shown in *Salmonella Typhimurium* ΔsucCD mutant strain is attenuated for virulence (Bowden *et al*., 2010).

Stability analysis of gene expression was performed with NormFinder program result being that the *recA* gene is more stable gene expression among the tested genes (Table 8). Surprisingly *clpX* gene showed stability equal to *recA* considering that protein level *clpX* expression decreases in cell line. The program also provides a stability value for the best combination of genes, which is choose the most stable pair of genes, in this case corresponds to *rpoD* and *pykA*, which together have a stability value of 0.052.

In figure 13 can be observed that there is a considerable variation of gene expression between the two groups of samples analyzed. The choice of the best combination of genes is based on the variation between groups is opposed in each gene and that the difference in the variation is of similar magnitude being chosen genes, as for *pykA* and *rpoD*.

The results of qPCR were evaluated by the method of $2^{-\Delta\Delta CT}$ (Pfaffl, 2001) and were used as normalizer genes *pykA* and *rpoD* (Figure 14).

Statistical analysis showed that only *pnp*, *csrA*, *ndk* genes had significant differences comparing intracellular growth conditions with free living growth. However the results for *ndk* differ from those obtained in the proteomic analysis, which can be explained as there are different mechanisms that regulate gene expression, in some cases it may stabilize or increase the degradation rate of a specific messenger RNA depending on whether there is an increased or decrease demand of RNA product.

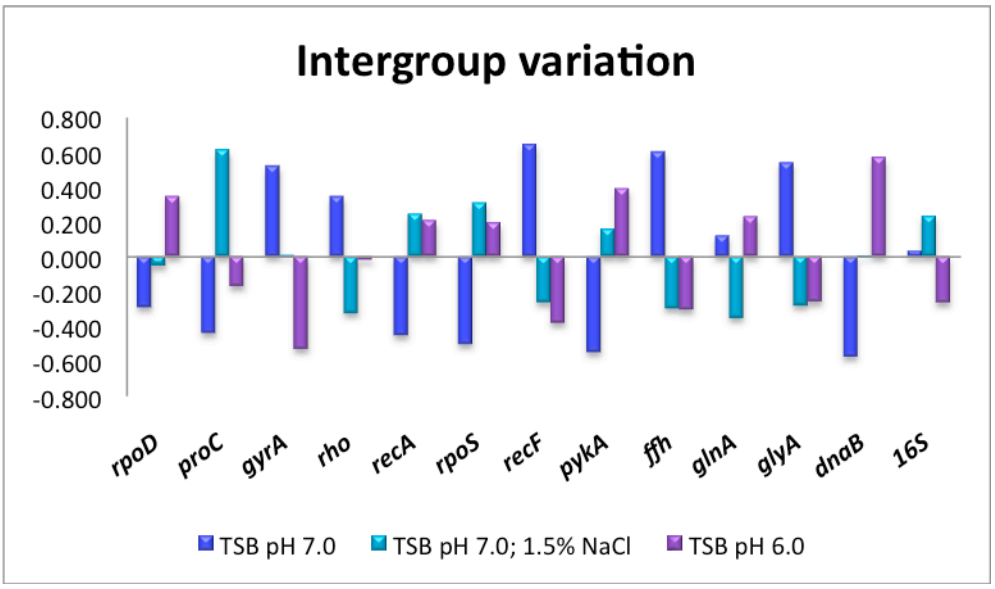

Figure 12: Intergroup variation of gene expression in LF89, growth on three liquid culture conditions.

Table 7: Stability of gene expression in strain LF89 grown in three different liquid culture conditions, evaluated by NormFinder program. In bold highlights the best combination of two genes that provide the best value for stability 0.178.

Gene name	Stability value
16S	0,406
rpoD	0,427
gyrA	0,434
recA	0,461
rpoS	**0,468**
glnA	0,471
pykA	0,531
proC	0,547
ffh	**0,566**
dnaB	0,566
recF	0,655
rho	0,664
glyA	0,675

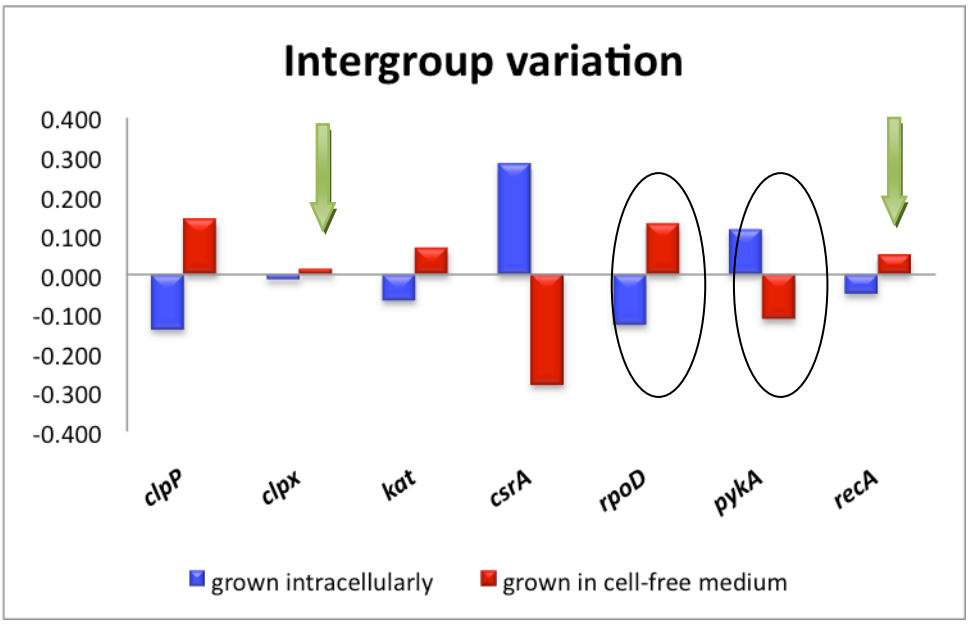

Figure 13: Intergroup variation of gene expression in LF89, growth in intracellular and liquid culture condition calculated by NormFinder program. Arrow indicates the most stable gene expression, in ovals highlights the best combination of two genes.

Table 8: Stability of gene expression in LF89 grown in two different culture conditions, evaluated by the NormFinder program. In bold highlights the best combination of two genes that provide the best value for stability 0.052.

Gene name	Stability value
recA	0,076
clpX	0,076
pykA	**0,174**
rpoD	**0,181**
kat	0,189
clpP	0,212
csrA	0,354

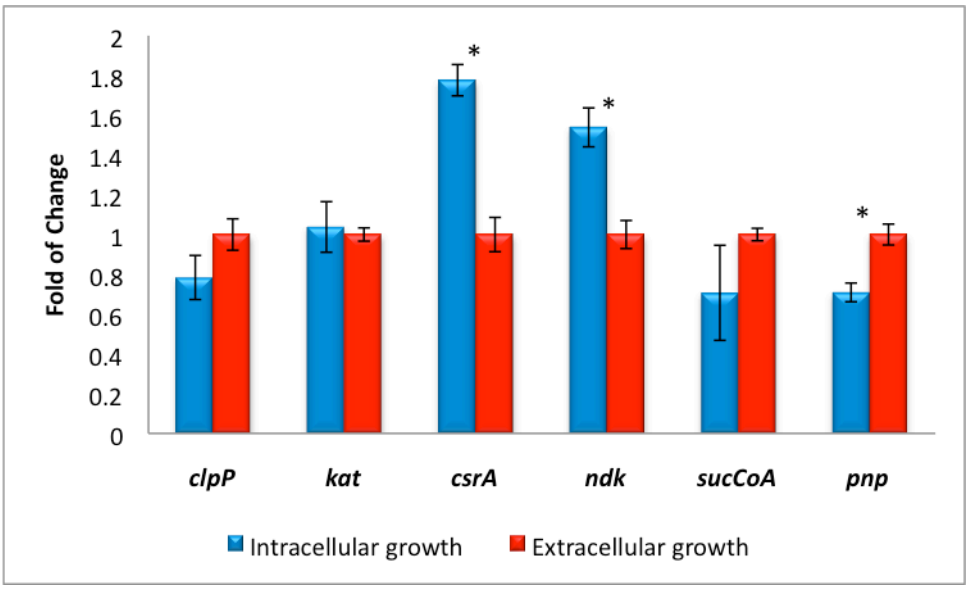

Figure 14: Transcription level of differentially expressed genes, preselected through the results of 2D-DIGE assay. Housekeeping genes *rpoD* and *pykA*. (*) p≤0.05.

3.3. PART 3: PROTEOMIC ANALYSIS OF TWO STRAINS OF *P. salmonis*, LF89 (REFERENCE STRAIN) AND IBM040 (VIRULENT STRAIN)

3.3.1. Evaluation of differential expression of proteins between two strains of *P. salmonis*, grown in a cell-free medium through 2D gels

With the aim to identify proteins that could be related to pathogenicity, differential protein expression between two strains of *P. salmonis* was evaluated. The reference strain LF89 is currently attenuated in virulence due to the large number of years It has maintained in cell lines without any contact with their natural host. The virulent strain IBM040, which in field trials has shown to be highly efficient in infection of salmon, achieving high levels of mortality. IBM040 strain was isolated recently. Both LF89 strain as IBM040 strain were isolated from kidney of *O. kisutch* affected by the SRS, in fish farms located in the X Region de los Lagos, Chile.

Infection assays in SHK1 cell line, using MOI of 50 showed that the pathogenic strain IBM040 produces cytopathic effect of cell line on the second day post infection, and the progression of the infection happens in a shorter time period than that obtained at perform the infection of the cell line with the reference strain LF89, in identical conditions of infection.

Differences in progression rates of infection between the two strains under study, could be evidence of differences in pathogenicity levels of both strains, although not been demonstrated whether differences in pathogenicity is the product of an more efficiently entry to the host cell, a intracellular replication speed greater or both processes.

Both strains were grown in culture medium described previously, and they were collected in the exponential growth phase between OD_{600nm} of 0.3-0.6, for the extraction of total proteins, as described in section 2.2.2.4. In Figure 15 the result

of 2D-PAGE can be appreciated. Four experimental replicates for each strain under study were performed and analyzed using Progenesis SameSpots software.

The analysis showed that 127 spots had significant statistically differences, 168 spots varying in intensity more than two times between the two samples groups tested. Of all spots detected 41 have statistically significant differences (ANOVA $p \leq 0.05$) and changed their expression level more than two times. 16 Spots that are increased in strain IBM040 and 25 spots are decreased in this strain compared to the expression levels of LF89 strain, which are highlighted in Figure 16.

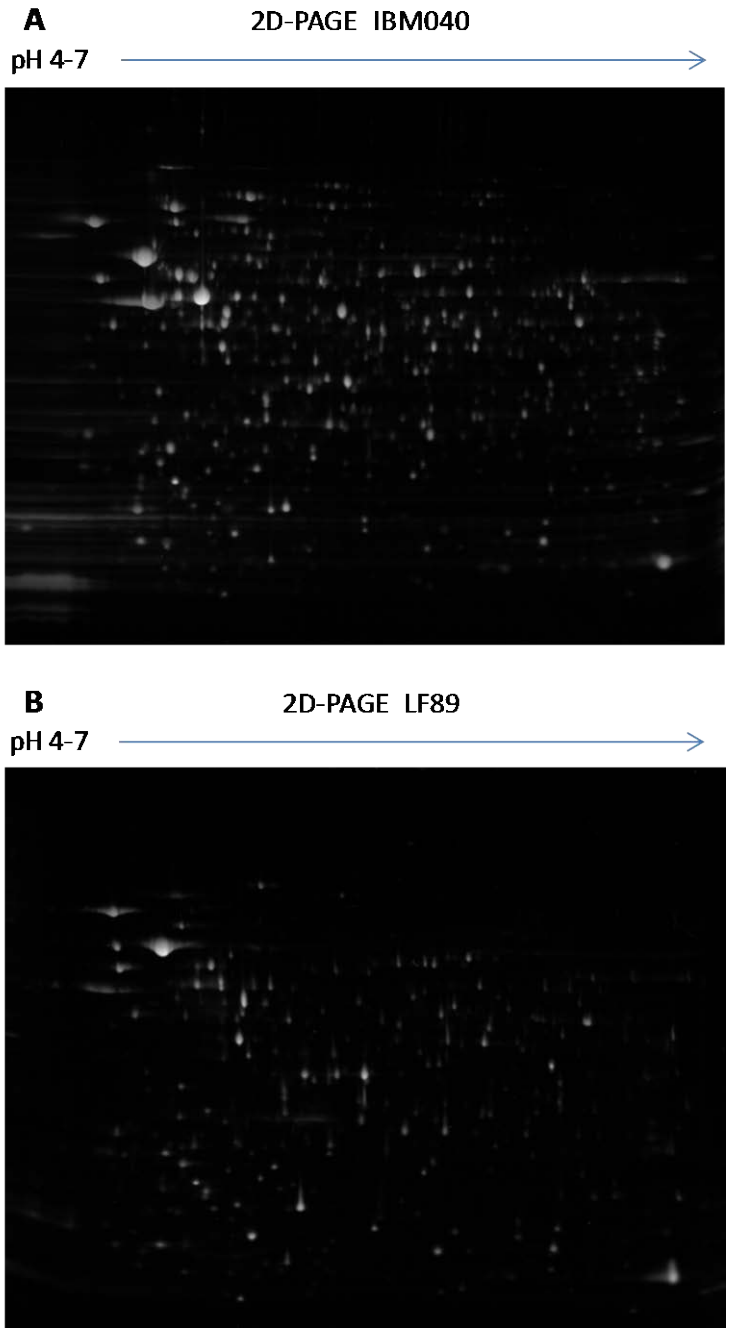

Figure 15: *P. salmonis* total proteins in 2D-PAGE. Bacteria were grown in liquid medium and total proteins were extracted in exponential grown phase**. (A)** Strain IBM040. **(B)** Strain LF89.

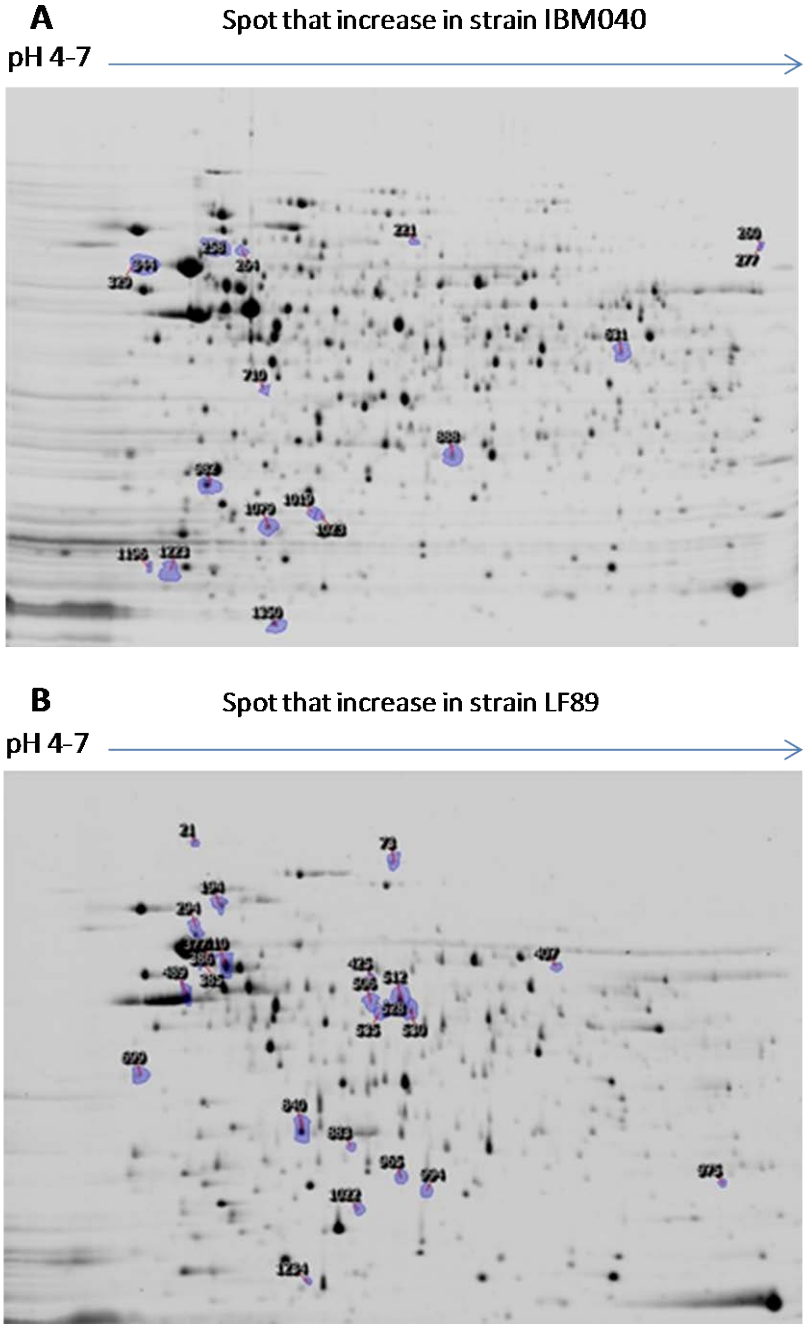

Figure 16: Analysis of 2D-PAGE with Progenesis SameSpots software. Spot highlights have significant statistically differences between IBM040 and LF89 strains. Anova p-value ≤ 0.05 and have a Max fold Change ≥ 2. **(A)** Spot highlights that increase in strain IBM040. **(B)** Spot highlights that decrease in strain IBM040.

3.3.2. Protein sequencing of pre-selected spots, identification and classification according to functionality

From the results obtained in the differential expression analysis carried out in the previous section were selected for sequencing 26 spots through mass spectroscopy, were identified a total of 32 amino acid sequences and four of these amino acid sequences are repeated. In this analysis there are two proteins were found in more than one spot, like citrate synthase (gltA) and 30S ribosomal protein S1 (rpsA) that was identified in two and four Spots respectively (Annex 8).

Table 9 summarizes the proteins identified, which are grouped according to the COGs classification, also indicating how many proteins of a particular group are increased in strain IBM040.

3.3.3. Differential expression validation of genes pre-selected by RT-PCR in real time

Seven genes that showed differential expression in proteome level between both strains in study were assessed by qPCR, and three genes were tested to analyze the stability of its expression, which were *rpoD*, *rpoS* and *recA*.

Below outline some of the described functions of the genes selected to be evaluated by qPCR.

Bacterial *purL* gene, encoding 5-phosphoribosyl N-formylglycinamide amidotransferase (EC 6.3.5.3), responsible for the conversion of 5-phosphoribosyl N-formylglycinamide (FGAR) to 5- phosphoribosyl N-formylglycinamidine (FGAM) in the purine biosynthesis pathway, mutations of *pur* genes often confer marked attenuations in virulence as exemplified by *purL* mutants in *Brucella abortus.* In *Francisella tularensis purL* mutant was found considerably attenuated when mice were inoculated with 10^7 CFU (Kadzhaev *et al*., 2009).

Results obtained by Bowden *et al*., 2010, suggest that disruption of the TCA cycle increases the ability of *S. Typhimurium* to survive within resting and activated murine macrophages. In contrast, an epithelial cell infection model showed that the *S. Typhimurium ΔgltA* strains had reduced net intracellular replication compared to the wild-type suggest that *Salmonella* may encounter environments within the host where a complete TCA cycle is advantageous.

The Dot/Icm System, the major virulence mechanism of phylogenetically related pathogens *Legionella pneumophila* and *Coxiella burnetii*, is responsible for their intracellular survival and multiplication, conditions that may also apply to *P. salmonis* (Gómez *et al*., 2013). DotB has been proposed to play a role in assembly of the T4SS, and export of substrates (Sexton *et al*., 2005).

The Escherichia coli RfaH, a paralog of the general transcription factor NusG that modulates general transcriptional pausing and termination in prokaryotes, is required for the expression of operons directing synthesis and export of the toxin haemolysin, the lipopolysaccharide core, and the F-factor sex pilus (Bailey *et al*., 1996).

Previous reports have shown that several crucial genes for virulence, including cellulase and hypersensitive response and pathogenicity (hrp) genes, were regulated by mutations in the *aroK* gene in Xanthomonas oryzae (Park *et al*., 2009).

Also has been shown, the expression of the *Moraxella catarrhalis* gene *mcmA* by Escherichia coli increases adherence to epithelial cells 100-fold. Furthermore, the disrupting *mcmA* gene decreases *M. catarrhalis* adherence to laryngeal and lung cells, which are relevant to pathogenesis by the bacterium (Lipski *et al*., 2007).

30S ribosomal protein S1 (rpsA), is an ‘atypical’ ribosomal protein weakly

associated with the 30S subunit that has been implicated in translation, transcription and control of RNA stability (Delvillani *et a*l., 2011).

Stability analysis of gene expression was performed with NormFinder program, including twelve genes in study, the result being that the *rpoD* gene is more stable gene expression among the tested genes (Table 10).

In figure 17 can be clearly seen that there is considerable variation of gene expression between the two groups of samples to be analyzed. The best combination choice of genes is based on the variation between groups that is opposed in each gene as for *mcmA* and *nusG*, and the difference in the variation is of similar magnitude being chosen genes. The stability value for the best combination of two genes is 0.086.

Since the *mcmA* and *nusG* belong to the genes that we want to measure, it was decided to use as a normalizer gene *rpoD*. The results of qPCR were evaluated by the method of $2^{-\Delta\Delta CT}$ (Pfaffl*,* 2001) (Figure 18).

Statistical analysis showed that four genes had significant differences, comparing the two strains of *P. salmonis*, *purL*, *nusG*, *gltA* and *dotB*. However the results for *dotB* and *gltA* differ from those obtained in the proteomic analysis.

Table 9: Differentially expressed proteins from *P. salmonis* pathogenic versus references strain identified by mass spectroscopy grouped by the COGs classification.

Code	Description	N° associated proteins	N° of proteins increased in IBM040
F	Nucleotide transport and metabolism	1	-
C	Energy production and conversion	3	-
E	Amino acid transport and metabolism	3	1
J	Translation, ribosomal structure and biogenesis	2	1
JE	Translation, ribosomal structure and biogenesis and Amino acid transport and metabolism	1	-
K	Transcription	2	1
L	DNA replication, recombination and repair	2	-
M	Cell envelope biogenesis, outer membrane	1	-
N	Cell motility and secretion	1	-
O	Posttranslational modification, protein turnover, chaperones	5	2
OC	Posttranslational modification, protein turnover, chaperones and Energy production and conversion	1	1
P	Inorganic ion transport and metabolism	1	-
QR	Secondary metabolites biosynthesis, transport and catabolism and General function prediction only	1	1
R	General function prediction only	3	1
S	Function unknown	1	-

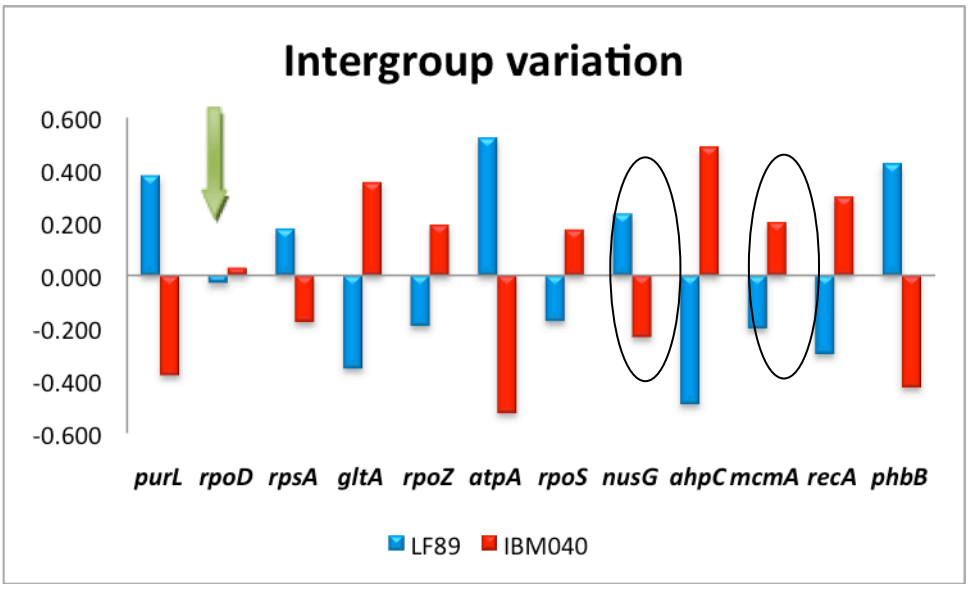

Figure 17: Intergroup variation of gene expression, between LF89 and IBM040 growth in liquid medium. Arrow indicates the gene of most stable expression**,** in ovals highlights the best combination of two genes.

Table 10: Stability of gene expression of LF89 and IBM040 both grown in liquid medium, evaluated by NormFinder program.

Gene name	Stability value
rpoD	0,237
mcmA	0,270
rpoZ	0,316
nusG	0,342
rpsA	0,381
rpoS	0,412
recA	0,417
gltA	0,417
purL	0,503
phbB	0,590
ahpC	0,644
atpA	0,723

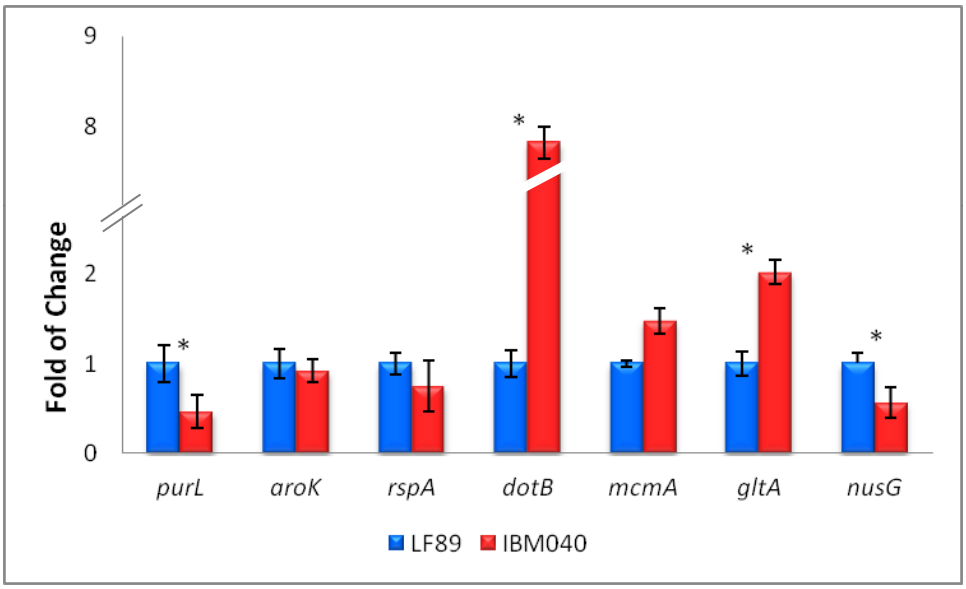

Figure 18: Transcription level of differentially expressed genes, preselected through the results obtained in 2D-PAGE assay. Housekeeping genes *rpoD*. (*) p≤0.05.

3.4. PART 4: DIFFERENTIAL GENE EXPRESSION OF GENES LIKELY INVOLVED IN PATHOGENICITY OF THE STRAIN IBM040 (PATHOGENIC) VERSUS THE REFERENCE STRAIN LF89 IN LIQUID CULTURE MEDIUM

3.4.1. Gene Identification that are potentially involved in infection processes or intracellular replication, through transcriptome sequencing of strains LF89 and IBM040

To perform the sequencing of *P. salmonis* transcriptomes, total RNA from both strains grown in liquid culture medium was extracted in its exponential growth phase. One RNA sample from each strain was subjected to sequencing with illumia system as described in Section 2.2.3.4.

The results were analyzed using the CLC Genomics wokbench 6 Software, using the default parameter settings. Sequences were assembled on the coding sequences of IBM040 strain, which were obtained of *P. salmonis* partial genome.

In the experiment the assembly with the default mapping parameters was run, allowing for a maximum of two mismatches, minimum exon coverage fraction: 0.2, Maximum number of hits for a read: 10 and the RPKM as the reporting expression value was chosen.

RPKM is defined as:

$$\text{Mapped reads millons} \times \text{exon length (kb)}$$

RPKM (Read Per Kilobase of exon Model value) seeks to normalize for the different number of mapped reads between samples.

In order to obtain mappings from different samples for comparison, also the second dataset was mapped. Annex 9 shows a summary table of Total number of

reads obtained and mapped reads in both samples.

For the expression analysis in the Workbench, set up an experiment using the transcriptómic assembled data. The LF89 and the IBM040 RNASeq samples were selected for a two group comparison of unpaired samples and this tool produces a new table showing the LF89 vs IBM040.

To identify differential expression, a statistical analysis was performed. The test on proportions Kal's Z-test was chosen, which is the test for comparing a single sample against another single sample.

By adding filters to the table view, fold change ≥3 and p≤0.01, the results shown in table 11 were obtained. Highlights in bold indicate genes that were subsequently evaluated by qPCR to validate their differential expression.

Table 11: Evaluation of differentially expressed genes from *P. salmonis* pathogenic (IBM040) versus references strain (LF89), identified by transcriptome sequencing analyses. In green highlights the increased expression values in strain IBM040, in yellow highlights the increased expression values in strain LF89. Bold, genes were analyzed by qPCR.

Feature ID	Gene name	Gene product	Gene Function	RPKM IBM040	RPKM LF89	Fold Change ≥3
S40C0462_2	0	hypothetical protein	0	4250,37	0,15	-27462,32
S40C0496_2	0	hypothetical protein	COG: Predicted permeases	5219,15	0,29	-18012,02
S40C0514_1	0	phage head-tail adaptor	COG: Bacteriophage head-tail adaptor	141,84	1,41	-100,60
S40C0145_11		K07488 transposase	COG: Transposase and inactivated derivatives	1067,07	11,99	-88,97
S40C0540_1	0	hypothetical protein	0	498,23	17,18	-29,00
S40C0228_4	0	K02050 sulfonate/nitrate/taurine transport system permease protein	0	4215,78	175,55	-24,01
S40C0055_6	**acrB**	multidrug efflux protein	COG: Cation/multidrug efflux pump	2311,90	100,92	-22,91
S40C0055_4		hypothetical protein predicted by Glimmer/Critica		6025,92	274,77	-21,93
S40C0055_5	**acrA**	acriflavin resistance periplasmic protein	COG: Membrane-fusion protein	2288,03	116,37	-19,66
S40C0041_2		hypothetical protein predicted by Glimmer/Critica	COG: Predicted permeases	327,62	18,81	-17,41
S40C0289_2	0	hypothetical protein	0	306,63	21,55	-14,23
S40C0436_3	0	integrase catalytic subunit	COG: Transposase and inactivated derivatives	109,24	7,81	-13,98
S40C0416_1	0	ISSod1, transposase OrfB	COG: Transposase and inactivated derivatives	21,24	1,59	-13,36
S40C0466_1	0	transposase	COG: Transposase and inactivated derivatives	15,27	1,20	-12,75
S40C0228_3	0	K02050 sulfonate/nitrate/taurine transport system permease protein	COG: ABC-type anion transport system, duplicated permease component	1813,82	150,10	-12,08
S40C0241_4	0	hypothetical protein predicted by Glimmer/Critica	0	54,56	5,08	-10,74

Feature ID	Gene name	Gene product	Gene Function	RPKM IBM040	RPKM LF89	Fold Change ≥3
S40C0241_3	0	hypothetical protein predicted by Glimmer/Critica	0	116,47	10,85	-10,74
S40C0241_2	**ssb**	single-stranded DNA-binding protein	COG: Single-stranded DNA-binding protein	64,64	6,20	-10,43
S40C0055_3		transcriptional regulator	COG: Predicted transcriptional regulators	1624,59	182,45	-8,90
S40C0233_1	**vasA**	K11896 type VI secretion system protein ImpG	COG: Uncharacterized protein conserved in bacteria	17,72	2,11	-8,41
S40C0507_3	tnp2	transposase	COG: Transposase and inactivated derivatives, IS30 family	180,68	22,63	-7,98
S40C0233_5	**vasQ**	K11901 type VI secretion system protein ImpB	COG: Uncharacterized protein conserved in bacteria	413,95	53,08	-7,80
S40C0228_2	0	ABC transporter, ATP-binding protein	COG: ABC-type spermidine/putrescine transport systems, ATPase components	125,71	16,35	-7,69
S40C0055_1		hypothetical protein predicted by Glimmer/Critica	COG: Uncharacterized protein conserved in bacteria	134,58	17,84	-7,54
S40C0233_2	0	K11896 type VI secretion system protein ImpG	COG: Uncharacterized protein conserved in bacteria	16,44	2,24	-7,35
S40C0425_1	tnp2	transposase	COG: Transposase and inactivated derivatives, IS30 family	138,64	19,15	-7,24
S40C0514_4	0	hypothetical protein predicted by Glimmer/Critica	0	10,86	1,61	-6,76
S40C0498_1	0	hypothetical protein predicted by Glimmer/Critica	0	48,03	9,70	-4,95
S40C0594_3	0	K03305 proton-dependent oligopeptide transporter, POT family	COG: Dipeptide/tripeptide permease	13,58	2,84	-4,78
S40C0103_10		hypothetical protein	COG: Amino acid transporters	466,98	100,67	-4,64
S40C0112_8	mutM	formamidopyrimidine-DNA glycosylase (EC:3.2.2.23)	COG: Formamidopyrimidine-DNA glycosylase	86,19	19,04	-4,53
S40C0041_7		K07019	COG: Predicted hydrolase of the alpha/beta-hydrolase fold	298,55	67,29	-4,44
S40C0001_15	rplE	50S ribosomal protein L5	COG: Ribosomal protein L5	2042,72	469,11	-4,35
S40C0185_5	0	K01091 phosphoglycolate phosphatase	COG: Predicted phosphatases	28,16	6,52	-4,32
S40C0105_2		hypothetical protein predicted by Glimmer/Critica		25,85	6,05	-4,27

Feature ID	Gene name	Gene product	Gene Function	RPKM IBM040	RPKM LF89	Fold Change ≥3
S40C0001_28	rpsJ	30S ribosomal protein S10	COG: Ribosomal protein S10	2726,41	671,08	-4,06
S40C0031_14	rnc	ribonuclease III (EC:3.1.26.3)	COG: dsRNA-specific ribonuclease	606,23	149,61	-4,05
S40C0004_12		hypothetical protein		2614,52	646,02	-4,05
S40C0127_1	deoB	phosphopentomutase (EC:5.4.2.7)		274,92	68,02	-4,04
S40C0415_2	0	hypothetical protein predicted by Glimmer/Critica	0	65,49	16,53	-3,96
S40C0082_8		hypothetical protein predicted by Glimmer/Critica		58,33	15,01	-3,89
S40C0114_3		K09117 hypothetical protein	COG: Uncharacterized conserved protein	1650,77	433,87	-3,80
S40C0389_4	0	hypothetical protein predicted by Glimmer/Critica	0	736,87	197,95	-3,72
S40C0134_6		hypothetical protein predicted by Glimmer/Critica		435,00	117,38	-3,71
S40C0103_9	yhcM	K06916	COG: Predicted ATPase	188,16	50,87	-3,70
S40C0005_1		hypothetical protein predicted by Glimmer/Critica	COG: Lauroyl/myristoyl acyltransferase	144,73	39,82	-3,63
S40C0582_1	0	integrase catalytic region	COG: Transposase and inactivated derivatives	83,86	23,11	-3,63
S40C0078_6	fhp	K05916 nitric oxide dioxygenase	COG: 2-polyprenylphenol hydroxylase and related flavodoxin oxidoreductases	301,92	83,99	-3,59
S40C0151_3	0	glycosyl transferase group 1	COG: Glycosyltransferase	963,79	274,79	-3,51
S40C0053_8	gatA	aspartyl/glutamyl-tRNA amidotransferase subunit A (EC:6.3.5.-)	COG: Asp-tRNAAsn/Glu-tRNAGln amidotransferase A subunit and related amidases	2971,04	863,76	-3,44
S40C0151_5	lgtG	glycosyl transferase group 1	COG: Glycosyltransferase	221,38	65,40	-3,39
S40C0001_18	rpsQ	30S ribosomal protein S17	COG: Ribosomal protein S17	719,90	214,54	-3,36
S40C0001_13	rplF	50S ribosomal protein L6	COG: Ribosomal protein L6P/L9E	4044,71	1206,69	-3,35
S40C0327_1	0	K03317 concentrative nucleoside transporter, CNT family	COG: Nucleoside permease	614,64	195,94	-3,14

Feature ID	Gene name	Gene product	Gene Function	RPKM IBM040	RPKM LF89	Fold Change ≥3
S40C0001_14	rpsH	30S ribosomal protein S8	COG: Ribosomal protein S8	5436,85	1738,92	-3,13
S40C0001_16	rplX	50S ribosomal protein L24	COG: Ribosomal protein L24	2590,43	842,74	-3,07
S40C0112_9		hypothetical protein predicted by Glimmer/Critica		8,41	0,00	8,41
S40C0450_1	0	LysR family transcriptional regulator	COG: Transcriptional regulator	82,39	0,00	82,39
S40C0462_3	0	hypothetical protein	0	285,30	0,00	285,30
S40C0475_3	0	hypothetical protein predicted by Glimmer/Critica	0	7,90	0,00	7,90
S40C0551_1	**ahpC**	K03386 peroxiredoxin (alkyl hydroperoxide reductase subunit C)	COG: Peroxiredoxin	42,84	0,00	42,84
S40C0551_2	ahpD	K04756 alkyl hydroperoxide reductase subunit D	COG: Uncharacterized conserved protein	19,03	0,00	19,03
S40C0554_2	oppB	oligopeptide transporter permease	COG: ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	8,21	0,00	8,21
S40C0334_3	0	hypothetical protein predicted by Glimmer/Critica	0	11,39	34,37	3,02
S40C0300_1	0	hypothetical protein predicted by Glimmer/Critica	0	180,45	545,55	3,02
S40C0354_2	0	hypothetical protein predicted by Glimmer/Critica	0	33,93	102,65	3,03
S40C0026_11		hypothetical protein predicted by Glimmer/Critica	COG: Outer membrane protein and related peptidoglycan-associated (lipo)proteins	73,61	223,25	3,03
S40C0567_2	0	hypothetical protein predicted by Glimmer/Critica	0	18,52	56,41	3,05
S40C0398_3	tnp2	transposase	COG: Transposase and inactivated derivatives, IS30 family	11,92	36,38	3,05
S40C0012_8	pyrB	aspartate carbamoyltransferase catalytic subunit	COG: Aspartate carbamoyltransferase, catalytic chain	205,89	628,38	3,05
S40C0303_1	0	hypothetical protein predicted by Glimmer/Critica	0	8,43	25,75	3,05

Feature ID	Gene name	Gene product	Gene Function	RPKM IBM040	RPKM LF89	Fold Change ≥3
S40C0150_2	fadL	K06076 long-chain fatty acid transport protein	COG: Long-chain fatty acid transport protein	16,79	51,34	3,06
S40C0285_2	pepB	K07751 PepB aminopeptidase	COG: Leucyl aminopeptidase	19,36	59,18	3,06
S40C0183_4	ycgM	fumarylacetoacetate (FAA) hydrolase	COG: 2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway)	252,57	781,13	3,09
S40C0280_1	0	hypothetical protein	COG: Uncharacterized protein involved in formation of curli polymers	372,10	1152,68	3,10
S40C0169_7	0	hypothetical protein	0	88,15	275,44	3,12
S40C0540_2	uspA	K06149 universal stress protein A	COG: Universal stress protein UspA and related nucleotide-binding proteins	12390,89	38718,98	3,12
S40C0550_3	0	hypothetical protein predicted by Glimmer/Critica	0	10,85	33,96	3,13
S40C0242_3	0	K07337 hypothetical protein	COG: Collagen-binding surface adhesin SpaP (antigen I/II family)	654,46	2049,63	3,13
S40C0299_2	0	hypothetical protein predicted by Glimmer/Critica	0	15,89	49,78	3,13
S40C0413_3	sun	RNA methylase, NOL1/NOP2/sun family	COG: tRNA and rRNA cytosine-C5-methylases	16,81	53,40	3,18
S40C0274_5	0	hypothetical protein predicted by Glimmer/Critica	0	342,23	1087,47	3,18
S40C0066_3		hypothetical protein predicted by Glimmer/Critica		29,08	92,76	3,19
S40C0022_4	icmJ	IcmJ		11,87	38,04	3,20
S40C0045_9	anmK	anhydro-N-acetylmuramic acid kinase	COG: Predicted molecular chaperone distantly related to HSP70-fold metalloproteases	54,31	177,58	3,27
S40C0466_2	0	hypothetical protein predicted by Glimmer/Critica	0	7,84	25,78	3,29
S40C0012_6	carB	K11541 carbamoyl-phosphate synthase / aspartate carbamoyltransferase	COG: Carbamoylphosphate synthase large subunit (split gene in MJ)	458,31	1515,72	3,31

Feature ID	Gene name	Gene product	Gene Function	RPKM IBM040	RPKM LF89	Fold Change ≥3
S40C0360_3	0	hypothetical protein predicted by Glimmer/Critica	0	5,40	17,88	3,31
S40C0183_3	0	K03415 two-component system, chemotaxis family, response regulator CheV	COG: Chemotaxis signal transduction protein	23,64	78,37	3,32
S40C0510_1	0	hypothetical protein	COG: Transposase and inactivated derivatives	6,26	20,86	3,33
S40C0097_2		hypothetical protein predicted by Glimmer/Critica		5,33	18,08	3,39
S40C0177_3	0	hypothetical protein predicted by Glimmer/Critica	0	60,31	204,84	3,40
S40C0347_2	0	hypothetical protein predicted by Glimmer/Critica	0	3473,66	11816,36	3,40
S40C0143_2	**dotA**	DotA	0	5,49	18,73	3,41
S40C0073_6		hypothetical protein	COG: ABC-type amino acid transport/signal transduction systems, periplasmic component/domain	47,89	163,82	3,42
S40C0591_2	0	transposase IS3/IS911 family protein	COG: Transposase and inactivated derivatives	88,66	306,90	3,46
S40C0427_3	0	hypothetical protein predicted by Glimmer/Critica	COG: ATPases involved in chromosome partitioning	22,66	78,72	3,47
S40C0022_3	icmP	IcmP		408,71	1438,53	3,52
S40C0012_3		hypothetical protein predicted by Glimmer/Critica		9,25	32,57	3,52
S40C0051_1		hypothetical protein predicted by Glimmer/Critica		721,73	2558,09	3,54
S40C0133_2	holB	DNA polymerase III subunit delta' (EC:2.7.7.7)	COG: DNA polymerase III, gamma/tau subunits	9,09	32,51	3,58
S40C0203_3	cyoC	cytochrome o ubiquinol oxidase subunit III	COG: Heme/copper-type cytochrome/quinol oxidase, subunit 3	50,29	179,92	3,58
S40C0412_2	0	hypothetical protein predicted by Glimmer/Critica	0	44,06	158,14	3,59

Feature ID	Gene name	Gene product	Gene Function	RPKM IBM040	RPKM LF89	Fold Change ≥3
S40C0140_4	0	hypothetical protein predicted by Glimmer/Critica	0	7,05	25,35	3,60
S40C0334_1	0	hypothetical protein predicted by Glimmer/Critica	0	44,82	161,41	3,60
S40C0012_14		hypothetical protein		16,23	58,66	3,61
S40C0133_3		hypothetical protein predicted by Glimmer/Critica	COG: Tfp pilus assembly protein PilZ	48,59	176,08	3,62
S40C0099_3	ddl	K01921 D-alanine-D-alanine ligase	COG: D-alanine-D-alanine ligase and related ATP-grasp enzymes	59,99	218,54	3,64
S40C0214_5	merA	K00520 mercuric reductase	COG: Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component, and related enzymes	10,66	40,59	3,81
S40C0150_5	0	hypothetical protein predicted by Glimmer/Critica	0	7,47	28,71	3,84
S40C0431_2	0	hypothetical protein predicted by Glimmer/Critica	0	56,55	219,17	3,88
S40C0622_1	0	hypothetical protein predicted by Glimmer/Critica	0	11,94	46,33	3,88
S40C0022_8	dotA	DotA		29,29	113,77	3,88
S40C0125_7		guanine deaminase (EC:3.5.4.3)	COG: Cytosine/adenosine deaminases	6,73	26,30	3,91
S40C0022_6	icmB	hypothetical protein		75,60	298,63	3,95
S40C0522_2	0	hypothetical protein predicted by Glimmer/Critica	0	11,22	44,58	3,97
S40C0246_3	yccA	K06890	COG: Integral membrane protein, interacts with FtsH	3750,34	15071,08	4,02
S40C0091_3		transporter	COG: Opacity protein and related surface antigens	64,46	260,80	4,05
S40C0048_3		hypothetical protein predicted by Glimmer/Critica		25,27	102,82	4,07

Feature ID	Gene name	Gene product	Gene Function	RPKM IBM040	RPKM LF89	Fold Change ≥3
S40C0375_1	0	hypothetical protein predicted by Glimmer/Critica	0	14,55	59,97	4,12
S40C0203_6	cyoA	K02297 cytochrome o ubiquinol oxidase subunit II	COG: Heme/copper-type cytochrome/quinol oxidases, subunit 2	173,55	717,60	4,13
S40C0362_2	0	hypothetical protein predicted by Glimmer/Critica	0	10,00	41,70	4,17
S40C0113_2		hypothetical protein predicted by Glimmer/Critica		28,20	118,24	4,19
S40C0391_1	0	transposase	0	5,65	23,76	4,20
S40C0011_1	**ggt**	gamma-glutamyltransferase (EC:2.3.2.2)	COG: Gamma-glutamyltransferase	213,28	922,06	4,32
S40C0022_5		hypothetical protein predicted by Glimmer/Critica		12,98	56,13	4,33
S40C0254_1	0	transposase	0	5,51	23,98	4,35
S40C0274_2	cheA	K03407 two-component system, chemotaxis family, sensor kinase CheA	COG: Chemotaxis protein histidine kinase and related kinases	5,98	26,72	4,46
S40C0376_3	0	hypothetical protein predicted by Glimmer/Critica	COG: Putative salt-induced outer membrane protein	312,94	1402,65	4,48
S40C0012_12	clpB	K03695 ATP-dependent Clp protease ATP-binding subunit ClpB	COG: ATPases with chaperone activity, ATP-binding subunit	1411,74	6379,09	4,52
S40C0204_1	0	hypothetical protein predicted by Glimmer/Critica	0	2,94	13,55	4,61
S40C0022_11	**icmL**	IcmL		149,42	693,03	4,64
S40C0214_6	0	K00520 mercuric reductase	COG: Uncharacterized conserved protein	7,81	36,34	4,65
S40C0192_6	0	K03415 two-component system, chemotaxis family, response regulator CheV	COG: Chemotaxis signal transduction protein	27,10	128,46	4,74
S40C0012_11	clpB	protein disaggregation chaperone	COG: ATPases with chaperone activity, ATP-binding subunit	1923,50	9143,11	4,75
S40C0063_13		hypothetical protein predicted by Glimmer/Critica		37,95	180,51	4,76

Feature ID	Gene name	Gene product	Gene Function	RPKM IBM040	RPKM LF89	Fold Change ≥3
S40C0375_2	0	hypothetical protein predicted by Glimmer/Critica	0	281,47	1368,84	4,86
S40C0012_5		hypothetical protein	COG: Carbamoylphosphate synthase small subunit	502,90	2499,39	4,97
S40C0192_7	cheV	K03415 two-component system, chemotaxis family, response regulator CheV	COG: FOG: CheY-like receiver	21,88	109,11	4,99
S40C0277_1	nucA	DNA/RNA non-specific endonuclease	COG: DNA/RNA endonuclease G, NUC1	783,52	3910,12	4,99
S40C0048_10		hypothetical protein predicted by Glimmer/Critica		9,99	50,45	5,05
S40C0017_5		hypothetical protein predicted by Glimmer/Critica		244,60	1244,81	5,09
S40C0427_4	0	K03496 chromosome partitioning protein	COG: ATPases involved in chromosome partitioning	166,66	869,81	5,22
S40C0477_1	0	hypothetical protein predicted by Glimmer/Critica	0	4,62	24,28	5,26
S40C0122_1		hypothetical protein predicted by Glimmer/Critica		3,93	20,79	5,29
S40C0342_2	0	hypothetical protein predicted by Glimmer/Critica	0	34,97	185,63	5,31
S40C0248_6	yccA	K06890	COG: Integral membrane protein, interacts with FtsH	514,69	2739,49	5,32
S40C0473_1	0	K11891 type VI secretion system protein ImpL	COG: Uncharacterized protein conserved in bacteria	35,69	191,97	5,38
S40C0400_1	0	hypothetical protein	0	61,38	333,49	5,43
S40C0237_1	0	hypothetical protein predicted by Glimmer/Critica	COG: Flagellar basal body-associated protein	68,45	374,94	5,48
S40C0099_6		hypothetical protein predicted by Glimmer/Critica		4,62	26,67	5,78
S40C0425_4	0	hypothetical protein predicted by Glimmer/Critica	0	75,70	464,69	6,14
S40C0635_1	0	hypothetical protein predicted by Glimmer/Critica	0	235,31	1447,75	6,15

Feature ID	Gene name	Gene product	Gene Function	RPKM IBM040	RPKM LF89	Fold Change ≥3
S40C0172_1	0	hypothetical protein predicted by Glimmer/Critica	0	4,02	24,84	6,18
S40C0018_11	flhF	flagellar biosynthesis regulator FlhF		4,08	25,82	6,32
S40C0473_2	0	hypothetical protein predicted by Glimmer/Critica	COG: Uncharacterized protein conserved in bacteria	8,07	51,13	6,34
S40C0425_2	0	hypothetical protein predicted by Glimmer/Critica	0	18,15	117,30	6,46
S40C0167_4	0	hypothetical protein predicted by Glimmer/Critica	0	28,15	187,01	6,64
S40C0493_2	0	hypothetical protein predicted by Glimmer/Critica	COG: Serine/threonine protein kinase	8,77	58,34	6,65
S40C0260_3	yeiE	putative DNA-binding transcriptional regulator	COG: Transcriptional regulator	15,50	106,27	6,85
S40C0193_3	0	hypothetical protein predicted by Glimmer/Critica	0	30,51	210,70	6,91
S40C0249_4	0	hypothetical protein predicted by Glimmer/Critica	COG: P pilus assembly protein, porin PapC	142,27	990,89	6,96
S40C0022_7		hypothetical protein predicted by Glimmer/Critica		238,89	1695,35	7,10
S40C0499_2	impJ	K11893 type VI secretion system protein ImpJ	COG: Uncharacterized protein conserved in bacteria	35,88	257,91	7,19
S40C0187_1	0	hypothetical protein predicted by Glimmer/Critica	COG: Ribosome-associated protein Y (PSrp-1)	1051,19	7586,87	7,22
S40C0499_1	0	K11906 type VI secretion system protein VasD	COG: Uncharacterized protein conserved in bacteria	137,36	996,08	7,25
S40C0170_7	0	hypothetical protein predicted by Glimmer/Critica	0	34,23	251,57	7,35
S40C0553_3	0	hypothetical protein predicted by Glimmer/Critica	COG: ATP-dependent transcriptional regulator	130,25	971,74	7,46
S40C0091_2		transporter		473,42	3958,70	8,36

Feature ID	Gene name	Gene product	Gene Function	RPKM IBM040	RPKM LF89	Fold Change ≥3
S40C0052_1		hypothetical protein predicted by Glimmer/Critica		4,56	38,36	8,40
S40C0397_1	0	hypothetical protein predicted by Glimmer/Critica	0	5,85	51,12	8,74
S40C0425_3	0	hypothetical protein predicted by Glimmer/Critica	0	145,18	1281,96	8,83
S40C0279_5	0	hypothetical protein predicted by Glimmer/Critica	0	131,33	1159,68	8,83
S40C0048_6		hypothetical protein predicted by Glimmer/Critica		23,38	225,84	9,66
S40C0249_5	**vgrE**	rhs element Vgr protein	COG: Uncharacterized protein conserved in bacteria	22,25	227,33	10,22
S40C0119_4	glsA	K01425 glutaminase	COG: Glutaminase	193,69	2186,75	11,29
S40C0397_2	0	hypothetical protein	COG: Phosphatidylserine/phosphatidylglycerophosphate/ cardiolipin synthases and related enzymes	24,92	296,38	11,89
S40C0470_2	0	hypothetical protein predicted by Glimmer/Critica	0	24,62	342,76	13,92
S40C0359_1	0	hypothetical protein	0	13,75	193,07	14,04
S40C0193_1	0	hypothetical protein	0	226,37	3261,53	14,41
S40C0048_5		hypothetical protein predicted by Glimmer/Critica		1,42	23,08	16,28
S40C0500_1	0	hypothetical protein predicted by Glimmer/Critica	0	26,58	495,33	18,63
S40C0052_2		hypothetical protein predicted by Glimmer/Critica		12,41	250,23	20,17
S40C0320_4	0	hypothetical protein predicted by Glimmer/Critica	COG: Uncharacterized low-complexity proteins	8,81	227,77	25,84
S40C0493_1	0	hypothetical protein predicted by Glimmer/Critica	0	3,06	108,03	35,32

3.4.2. Validation of differential expression of genes pre-selected by RT-PCR in real time

Ten genes that their transcriptomic level showed differential expression between both strains in study were assessed by qPCR, and four genes were tested to analyze their stability expression, which were *rpoD*, *rpoS*, *recA* and *pykA*.

Below outline some of the functions of the genes selected to be evaluated by qPCR.

AcrA can function as a periplasmic adaptor protein (PAP) in several resistance nodulation division (RND) tripartite efflux pumps family, of which AcrAB-TolC is considered the most important. This system confers innate multiple antibiotic resistance. Disruption of *acrB* impairs the ability of *Salmonella Typhimurium* to colonize and persist in the host (Blair *et al*., 2009). The global consequence of disruption of AcrAB-TolC efflux pump in *Salmonella enterica* includes reduced expression of SPI-1 and other attributes required to infect the host (Webber *et al*., 2009).

Vas A, VasQ and Vgr are component of Type VI secretion systems (T6SSs), which are transenvelope complexes specialized in the proteins transport directly into target cells. These systems are versatile as they can target either eukaryotic host cells and therefore modulate the bacteria-host interaction and pathogenesis or bacterial cells and therefore facilitate access to a specific niche (Cascales and Cambillau, 2012).

IcmL, IcmP and DotA are part of T4SS, essential for intracellular replication of *Legionella pneumophila* and *Coxiella burnetii.* Segal *et al*., 1998, showed that i*cmL* is one of genes are required for macrophage killing in *Legionella pneumophila*.

Central to the survival of pathogenic microorganisms is the ability to withstand the stress conferred by reactive oxygen metabolites due to that are

important defense mechanisms expressed by infected mammals, in particular by the oxidative burst of phagocytic cells. These defense mechanisms include enzymes that detoxify reactive oxygen species, such as alkyl hydroperoxide reductase (AhpC), which contributes to virulence in *F. tularensis* (Kadzhaev *et al*., 2009).

The γ-glutamyltranspeptidase activity of *F. tularensis* allows utilization of glutathione (GSH, gamma-glutamyl-cysteinyl-glycine) and gamma-glutamyl-cysteine dipeptide as cysteine sources to ensure intracellular growth. Mutant strain showed impaired intracellular multiplication and was strongly attenuated for virulence in mice (Alkhuder *et al*., 2009).

Stability analysis of gene expression was performed with NormFinder program, including ten genes in study, the result being that the *rpoD* gene is more stable gene expression, follow by *recA* (Table 12).

In figure 19 can be clearly seen that there is considerable variation of gene expression between the two groups of samples to be analyzed. The best combination of two genes is *acrA-pykA* with a stability value of 0.090.

Unfortunately none of the four tested genes to be used as housekeeping showed in the "intergroup variation" a deviation in the opposite direction with respect to the other genes tested to be used as housekeeping.

Since the *acrA* gene, belong to the genes that we want to measure, it was decided to use as a normalizer gene *rpoD*. The results of qPCR were evaluated by the method of $2^{-\Delta\Delta CT}$ (Pfaffl, 2001) (Figure 20).

Statistical analysis showed that eight genes had significant differences comparing the two strains of *P. salmonis*, *acrA*, *acrB*, *icmL*, *icmP*, *vasA*, *vasQ*, *dotA*, and *ahpC*. However the results for *ahpC*, *acrA* and *acrB*, coincide with those obtained in the transcriptome analysis.

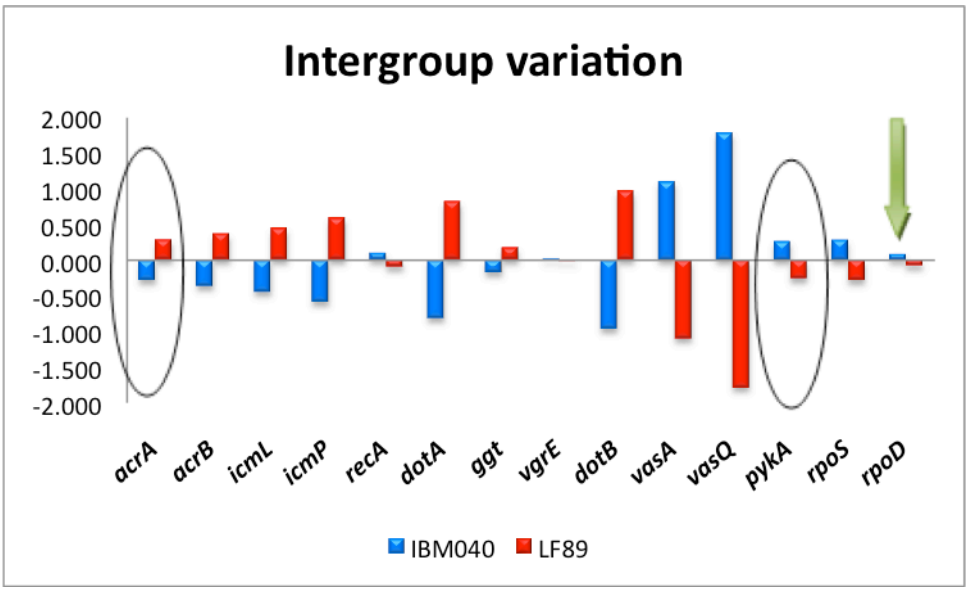

Figure 19: Validation of intergroup variation of gene expression between LF89 and IBM040 strains growth in liquid medium. The arrow indicates the gene of most stable expression, in ovals highlights the best combination of two genes.

Table 12: Validation of gene expression stability in LF89 and IBM040 both grown in liquid culture medium evaluated by NormFinder program.

Gene name	Stability value
rpoD	0,312
recA	0,319
vgrE	0,326
ggt	0,346
pykA	0,370
acrA	0,401
rpoS	0,508
acrB	0,514
icmL	0,581
icmP	0,695
dotA	0,837
dotB	1,157
vasA	1,339
vasQ	1,987

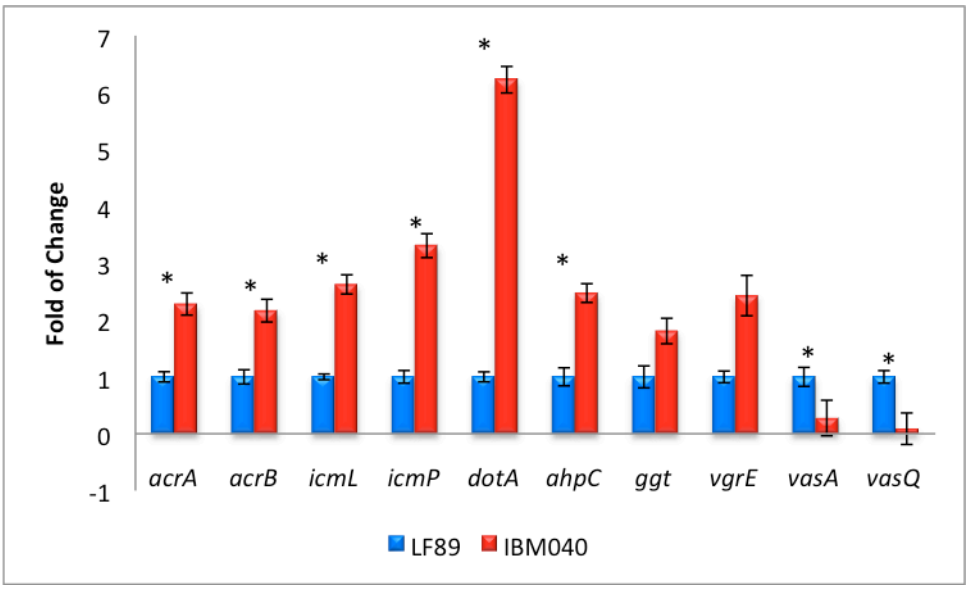

Figure 20: Transcription levels of *P. salmonis* preselected genes through the results obtained in transcriptome sequencing analysis. Housekeeping gene *rpoD*. (*) p≤0.05.

4. DISCUSSION

The gene expression analysis requires the optimization of all steps involved in the process leading to the end of the data collection. Therefore, when designing an analysis aimed at detecting differences in gene expression level, an important point to consider is the intrinsic variation that affects any experiment that includes the study of biological samples mutually independent. Is important to establish that the results obtained in this thesis represent the differences in gene expression at the time in which metabolic samples were obtained.

In order to ensure that the samples of both pathogenic strains corresponded to the same growth phase, a liquid culture medium for optimal growth of *Piscirickettsia salmonis* was developed.

Liquid culture media have several advantages, among them are the possibility to measure optical densities (OD) to quantify quickly and simply the amount of bacteria present in the culture, which is a great advantage for studies that use measurement of this parameter, such as the calculation of generation time and conducting quantitative antibiograms among many others (Forbes *et al*., 1998), As previously described the use of blood as a supplement does not allow performing these types of tests, where *P. salmonis* reaches a value of 0.25 OD_{620} after 13 days of incubation (Gomez *et al*., 2009).

Iron is an essential nutrient for bacteria and iron uptake mechanisms are essential for the virulence of several gram-negative pathogens, including *Neisseria* spp, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Yersinia pestis* and *Helicobacter pylori*. The mechanisms by which gram-negative pathogens obtain iron from the host are, in general, relatively well understood. They include secretion of low-molecular-weight iron chelators, called siderophores,

which scavenge iron from host iron-binding proteins such as transferrin, and secreted haemophores, which acquire iron from haemoglobin and haemin. Alternatively, proteins containing iron or haem can bind to specific receptors on the bacterial outer membrane. Independent of the source of the captured iron, transport into the bacterial cytoplasm is often dependent on cytoplasmic membrane ABC transporters (Brown *et al*., 2001).

Considering the importance of iron for pathogenic bacteria and that iron is a major component in the haem group of hemoglobin, was decided to use iron salts replacing the "blood or hemoglobin" commonly used in solid culture media for this pathogen, obtaining good results in the liquid culture of *P. salmonis.*

Having succeeded in formulating a liquid medium, we first noted the use of $FeCl_3$ as an iron source by replacing addition of blood in the culture medium. Reduction of the concentration of FBS at 2.5% in liquid medium did not reveal any difference in the growth of *P. salmonis*, relative to the same medium supplemented with 5% FBS. Furthermore cultures were performed in the same medium without the addition of fetal bovine serum, but despite achieving bacterial growth, this was slower than in previous formulations. Several microorganisms such as *Helicobacter pylori* are very difficult to culture *in vitro*, particularly in liquid media. However, for this pathogen growth has been demonstrated in several nutrient-rich media, fetal calf serum (FCS) is one of the most commonly used supplements (Vega *et al*., 2003). Also has been shown that FBS is one of the critical components in the composition of a culture medium formulated for intracellular pathogen *Coxiella burnetii* (Omsland *et al*., 2009).

Optimization of this culture medium, to adjust the concentrations of NaCl and L-cysteine made it possible to obtain higher densities in the culture of this pathogen as well as expanding the exponential growth phase, up to 2.5 OD_{600nm}. Importantly in successive subcultures in liquid medium, a decrease in the generation time was observed, which is indicative of adaptation of the bacteria to the culture medium.

In the growth curves performed, the optical densities in which the culture was in exponential growth phase was determined and due to variations in the results, depending on how many successive subcultures were carried out, it was decided to use in all cases cultures not exceeding 0.5 OD_{600nm}, in order to ensure that the samples corresponded to the same phase of growth and thus represent equivalent metabolic states, validating comparison.

As supplementary information to develop a liquid culture medium, a solid medium was formulated, yielding successfully growth of *P. salmonis*, this time on plates. Growth on this solid medium began to be noticeable after 4 days in seeded segments with plenty, at 9 days of cultivation single colonies could clearly visualize, corresponding to times lower than those observed for the growth of the same strain in solid culture media supplemented with sheep blood.

Both in the liquid as well as solid culture media, in addition to LF89, different strains of the bacterium *P. salmonis* were seeded, which were provided by the companies, Marin-Harvest and Aquagestión (Puerto Montt, Chile), obtaining a similar growth in all of them. The bacteria also maintained infectivity when the cell cultures (SHK-1) were inoculated, after bacteria were subjected to successive subcultures in both liquid and solid media. Similar results were obtained in artificial solid media supplemented with blood (Mikalsen *et al*., 2008; Mauel *et al*., 2008). This would indicate that the new liquid and solid medium presented here would not affect the infectivity of the microorganism, allowing their use in research and diagnostics.

The identification of optimal conditions for infection in cell line was a prerequisite for achieving recovery of bacteria from inside the SHK1 cells, necessary for the analysis of comparative proteomic analyses of reference strain LF89 grown in a culture medium free of cells or in the cell line.

The increase of bacteria found inside the cell line, when the cell culture was

synchronized through a decrease in SBF to 1%, 48h prior to infection, is a similar result to that obtained by Gomez *et al*. (2010) It was shown that culture media containing 1% FBS during the previous 48h achieved a synchronizing condition where 90% of cells were found in G_0/G_1 stage. The cell line UFL-Ag-286 from embryos of *Anticarsia gemmatalis*, previously deprived from FBS proved to be a better host for Multinucleopolyhedrovirus of *A. gemmatalis* (AgMNPV) propagation (Gomez *et al*., 2010).

A key component of the success of any bacterial pathogen is the ability to recognize and mount a suitable response to the discrete chemical and physical stresses elicited by the host. Such responses occur through a coordinated and complex programme of gene expression and protein activity, involving a range of transcriptional regulators, sigma factors and two component regulatory systems (Runkel *et al*., 2013). Due to the ability of pathogenic bacteria to modify gene expression in the present study the proteome of the reference strain when it is subjected to two culture conditions was compared: bacteria grown in a liquid culture medium previously described and bacteria grown in SHK1cell line, where they reside in large vacuoles within cells.

One of the most outstanding results corresponded to the huge difference in the patterns observed in the 2D-DIGE images, between the two groups of samples to be analyzed, indicating that there is a wide variation in gene expression levels of the bacteria when it is subjected to the two types of culture. Is important to note that in the sequences obtained through mass spectrometry, proteins of *Salmo salar* were identified, indicating the presence of contamination with proteins of the cell line in samples obtained from intra cellular growth, however the percentage of protein contaminants did not exceed 15%.

Within most remarkable results when performing this analysis was the identification of 69 protein sequences. Among the amino acid sequences identified was found that in some cases there are two sequences associated with the same protein and were founded in the same spot. When performing sequence

alignments, low identity between sequences was observed and to perform the analysis of functional domains in all cases was verified the sequences correspond to the same superfamily of proteins. These data point to the existence of errors in the annotation of genes in the genome of *P. salmonis*, since two different proteins should not have exactly the same name.

It is important to note that the genome annotation was performed automatically and still has not been done the manually cure to confirm correct annotation of each gene. It is noteworthy that *P. salmonis* genome, has not been achieved circularize therefore corresponds to a partial genome of this bacterium, probably due to technical problems in the sequencing strategy.

On the other hand, there was also a case where a protein sequence was identified in different positions in the gel, ie several spot identified correspond to the same protein (Annex 2, Table 5), this could be due to modifications in the protein that affect their migration in the gel this because when performing a search in the predicted coding sequences in the partial genome of strain LF89, only one sequence was found associated with each of these proteins.

Note that in the classification of proteins according to functionality (Table 6) eleven proteins were found associated with “energy production and conversion”, of which three had increased expression in intracellular living conditions. This might indicate an adaptation of the bacteria in nutrient bioavailability during intracellular growth conditions.

Thirteen proteins associated with “posttranslational modification, protein turnover, chaperones” were found, all decreased expression in intracellular growth conditions, 9 of these are described as chaperones, which normally are increased in conditions of stress, which may indicate that the conditions intracellular life in this bacterium could represent the normal condition of growth and that a liquid culture correspond to cellular stress environment.

In a third group 12 proteins were associated, corresponding to “nucleotide transport and metabolism”. It is known that the nucleotide may be associated in the involvement of signaling pathways that could induce virulence in this bacterium.

Importantly, the enzyme Succinate dehydrogenase/fumarate reductase (SdhA) showed decreased expression in intracellular living conditions. In the last time this enzyme has become important in several investigations related to its function and association with virulence as demonstrated in *Salmonella* enterica serovar *typhimurium* where double mutants are avirulent in immunogenic BALB/c mice (Mercado-Lubo *et al*., 2008). As in *Legionella pneumophila*, mutants lacking the Dot/Icm, substrate SdhA, were severely impaired for intracellular growth within mouse bone marrow macrophages, with the defect absolute in triple mutants lacking sdhA and its two paralogs (Laguna *et al*., 2006). This information makes this enzyme an interesting target for future studies.

In this thesis, it was proposed the evaluation of gene expression through qRT-PCR of some preselected genes, for which it is necessary first to define constitutive gene expression in the study conditions, to be used as normalizing genes.

When performing a quantitative reverse transcription-PCR (RT-qPCR) analysis, several parameters need to be controlled to obtain reliable quantitative expression measures. These include variations in initial sample amount, RNA recovery, RNA integrity, efficiency of cDNA synthesis, and differences in the overall transcriptional activity of the cells analyzed (Andersen *et al*., 2004).

An important point to consider in the analysis of qPCR is the integrity of the RNA, due to Bacterial mRNA is characterized by being extremely labile and has a short half-life (less than 2 minutes). Furthermore, small changes in the environment of the bacteria can trigger rapid transcriptional response which results in the alteration of the representation of the transcripts within the population of

mRNA (Bandyra *et al*., 2013). These factors are a major source of variability in the comparison studies focused on gene expression and considered when designing this study. Therefore, the RNA samples were obtained following a purification protocol which contemplates a short centrifugation, and the rapid lysis and mRNA stabilization with a reagent based on guanidine thiocyanate.

Accurate normalization is an absolute prerequisite for correct measurement of gene expression. For RT-qPCR, the most commonly used normalization strategy involves standardization to a single constitutively expressed control gene. However, the task of identifying these genes is not trivial. It is composed of two steps: first, to identify which genes are likely candidates; and second, to verify the stability of these candidates (Andersen *et al*., 2004).

To identifying genes that could be used as normalizers genes in qPCR assays several genes were chosen based on publications available. To verify the stability of these candidates genes, NormFinder program was used, which is an algorithm for identifying the optimal normalization gene among a set of candidates. It ranks the set of candidate normalization genes according to their expression stability in a given sample set and given experimental design. The algorithm is rooted in a mathematical model of gene expression and uses a solid statistical framework to estimate not only the overall expression variation of the candidate normalization genes, but also the variation between sample subgroups of the sample set. Notably, “NormFinder” provides a stability value for each gene, which is a direct measure for the estimated expression variation enabling the user to evaluate the systematic error introduced when using the gene for normalization (Andersen *et al*., 2004).

The results confirmed genes of greater stability are: 16S, RpoD, RecA, PykA, and due to variations intergroup obtained, confirming the need to reassess, housekeeping genes in each experimental design and validate their choice.

To validate the differential expression of preselected gene, we first proved

the stability of gene expression of the three most stable genes previously tested and it was concluded that the best pair to be used as normalizing genes are *rpoD* and *pykA*. Today the use of more than one gene normalizer is becoming more evident as previous studies have shown that all genes are subject to control gene expression when study conditions vary. For a gene to be valid as a control gene, its expression should not vary in the tissues or cells under investigation. The ideal internal control gene is universally valid, with a constant expression level across all thinkable tissue samples, cells, experimental treatments, and designs. Unfortunately, literature shows that as of yet, no such housekeeping gene has been Found (Andersen et al., 2004).

When analysis of gene expression stability was performed the RecA gene had more stable gene expression among the tested genes (Table 8). Surprisingly *clpX* gene showed stability equal to *recA* considering that protein level ClpX expression decreases in cell line growth. On the other hand it is described that Clp proteases are the most widespread energy-dependent proteases in bacteria. Their two-component architecture of protease core and ATPase rings results in an inventory of several Clp protease complexes that often coexist (Kress *et al*., 2009). Almost all bacteria contain ClpXP chaperone –protease, this makes ClpXP the most ubiquitous of the Clp protease (Kress *et al*., 2009). Clpx is the ATPase subunit and by itself works as a chaperone, whereas in association whith the ClpP protease, it is believed to eliminate, misfolded, aggregated, and dysfunctional proteins targets. (Dziedzic *et al*., 2010). Also has been shown the ClpX regulates dynamics of FtsZ assembly by blocking the reassembly of FtsZ in an ATP independent manner and that ClpX expression is elevated during intracellular growth, conditions known to delay FtsZ ring assembly in *Mycobacterium tuberculosis,* which is crucial for initiation of the cell division process in eubacteria (Dziedzic *et al*., 2010). This background we could indicate that if *P. salmonis* division was active in intracellular growth conditions, one could understand the protein level decrease observed in the 2D-DIGE analysis.

Because little is known about *P. salmonis*, finding a housekeeping gene for

the evaluation in qPCR assay constituts an important contribution to knowledge about this bacterium.

Of the six genes that proteome level showed differential expression regarding culture conditions, intracellular or cell-free medium were assessed by qPCR, three showed significant differences in their expression; *csrA*, *pnp* and *ndk*. However the results for *ndk* differ from those obtained in the proteomic analysis (Table 13).

The table 13 summary the results obtained in the comparison of the two culture conditions of strain LF89, both proteomic and qPCR analysis, which shows that three of the tested genes showed no significant differences, which could be given by external factors affecting the stability of the bacterial RNA, at the time of extraction of RNA, particularly samples obtained from bacteria grown on cell line, because these showed higher variability between replicas made, is important to consider that in these samples the extraction procedure is slightly different, since the first, cells containing the bacterium are washed, then loosened from the growth bracket, centrifuged briefly and then lysed. Despite having no significant differences in gene expression variation, it can be seen that there is a tendency toward decreased expression under intracellular conditions, which coincides with the observed in proteomic analysis.

In cells, mRNA and protein levels are fine-regulated to adjust continuously to cellular needs. Recently, several large-scale studies in prokaryotes showed weak correlations between mRNA and protein abundances highlighting the significant importance of post-transcriptional regulations. Post-transcriptional regulations involve dynamic adaptation of mRNA and protein turnover and also modulation of the efficiency of mRNA translation into protein. mRNA and protein stabilities are function of both sequence determinants and decay processes (Picard *et al., 2009).*

The importance of Csr (carbon storage regulator) post-transcriptional

systems is gradually emerging; these systems control a variety of virulence-linked physiological traits in many pathogenic bacteria. Csr system is composed of CsrA protein and two small non-coding regulatory RNAs (ncRNAs) CsrB and CsrC. CsrA is an RNA-binding protein that could prevent translation of target mRNA by binding to a site near the Shine–Dalgarno sequence, thus blocking ribosome binding and facilitating mRNA decay. CsrA has also been shown to acts as a positive regulator by stabilizing and subsequently increasing the translation of certain target mRNAs (Lucchetti-Miganeh *et al*., 2008). In *Campylobacter jejuni* CsrA mediates oxidative stress responses, biofilm formation, and host cell invasion (Fields *et al*., 2008). Also has been shown to CsrA and CsrB are required for the post-transcriptional control of the virulence-associated effector protein AvrA of *Salmonella* enterica. (Kerrinnes *et al*., 2011) and is involved in controlling the expression of the *Salmonella* SPI-1 and SPI-2 virulence regulons through HilD (Martínez *et al.*, 2011). CsrA also been involved in post-transcriptional regulation of stationary phase activation of *Legionella pneumophila* Icm/Dot effectors (Rasis *et al*., 2009).

Polynucleotide phosphorylase (PNPase) is one of the major exoribonucleases in bacteria and plays important roles in mRNA degradation, tRNA processing, and small RNA (sRNA) turnover (Zeng *et al*., 2010). In Yersinia spp PNPase independently controls virulence factor expression levels and export (Rosenzweig *et al*., 2007) furthermore it has been shown that PNPase downregulates the transcription of T3SS structural and effector genes of the phytopathogenic bacterium *Dickeya dadantii* (Zeng *et al*., 2010) which is an essential virulence factor for many bacterial pathogens. Also has been shown that PNPase is a global regulator of virulence and persistency in *Salmonella* enterica, since when conducted a single point mutation in the gene for polynucleotide phosphorylase bacterial invasion and intracellular replication were affected, which determines the alternation between acute or persistent infection in a mouse model for *Salmonella enterica* infection. For many pathogens, the ability to regulate their replication in host cells is a key element in establishing persistency (Clements *et al*., 2002).

Nucleoside diphosphate kinase (Ndk) is a ubiquitous small protein (15 kDa) found in virtually all organisms, from eukaryotes to prokaryotes (Sun *et al*., 2010). Ndk is an important enzyme that generates nucleoside triphosphates (NTPs) or their deoxy derivatives by terminal phosphotransfer from an NTP such as ATP or GTP to any nucleoside diphosphate or its deoxy derivative. As NTPs, particularly GTP, are important for cellular macromolecular synthesis and signalling mechanisms, Ndk plays an important role in bacterial growth, signal transduction and pathogenicity (Chakrabarty, 1998). An example is the Mycobacterial Ndk Initially, was described as an intracellular nucleotide pool balance mediator because it has several enzymatic properties such as autophosphorylation and GTPase activity, as well as phosphotransfer activities. More importantly Ndk is now known to be secreted by mycobacteria and in vitro analyses have demonstrated that Mtb Ndk possesses GAP activity towards Rho GTPases. Sun *et al*. (2010) showed that recombinant Mtb Ndk dephosphorylates Rab7-GTP and also Rab5-GTP in a cell-free biochemical assay consistent with the finding that phagosomes containing a BCG strain with knocked-down Ndk, matures at higher rate leading ultimately to increased intracellular killing (Sun *et al*., 2010).

This evidence might lead us to think that to be a secreted protein, it could explain the increased transcriptional level of expression of this protein, and the protein level decreased when *P. salmonis* is grown intracellularly.

Table 13: Summary of the results obtained in the comparison of the two culture conditions of LF89 strain. Bold; genes that match the increase or decrease of expression in both analysis.

Gene	Variation in intracellular growth conditions	
	Proteomic analysis	**qPCR analysis**
clpP	decreases	no significant difference
kat	decreases	no significant difference
csrA	increases	increases
ndk	decreases	increases
sucCoA	decreases	no significant difference
pnp	decreases	decreases

Within most outstanding results when performing proteomics analysis of the differential expression of proteins between the two strains of *P. salmonis* is the identification of 28 protein sequences, that in 2D-PAGE analysis showed varying intensity more than two times between the two samples groups tested, which is clear evidence of the differences in gene expression levels between these two strains which differ in pathogenicity.

It is important to note that we identified two proteins that previously were considered hypothetical, since being identified through mass spectroscopy, it provides evidence that confirms their existence.

In this analysis we saw that there are 2 proteins were found in more than one spot, it is the case of citrate synthase (gltA) was identified on two spot and 30S ribosomal protein S1 (rpsA) that was identified in 4 Spots (Annex 8). This could be due to modifications in the protein that affect their migration in the gel this

because when performing a search in the predicted coding sequences in the partial genome of strain LF89 and IBM040, only one sequence was found associated with each of these proteins.

S1 is an ‘atypical’ ribosomal protein weakly associated with the 30S subunit that has been implicated in translation, transcription and control of RNA stability (Delvillani *et a*l., 2011). The multiple functions associated with this protein could be explained through modifications that affecting their putative function which would lead to the results, detecting this protein in 4 different positions in the gel.

In the classification of sequenced proteins there is a relatively homogeneous distribution of proteins, however one group has more than 3 proteins which is, “Posttranslational modification, protein turnover, chaperones”, which could be indicative of Adaptability to the culture medium, considering that both bacterial strains showed differences in their replication rate in the liquid culture medium.

To validate the differential expression of preselected gene, when both strains LF89 and IBM040 are subjected to identical culture conditionswe first proved the stability of gene expression of the three most stable genes previously tested and it was concluded that the best genes is *rpoD*. This result is consistent with the description of the principal sigma factor gene, also called housekeeping sigma factor, considering that both bacteria were in identical culture conditions and that RNA extraction was performed in both cases exponential growth phase.

Statistical analysis showed that four genes had significant differences when comparing the two strains of *P. salmonis*; *purl*, *nusG*, *gltA* and *dotB*. However the results for *dotB* and *gltA* differ from those obtained in the proteomic analysis. The table 14 summary the results obtained in the comparison of the two strains of *P. salmonis*, both proteomic and qPCR analysis, which shows that three of the tested genes showed no significant differences. These results are not surprising since several large-scale studies in prokaryotes showed weak correlations

between mRNA and protein abundances, highlighting the significant importance of post-transcriptional regulations (Picard *et al*., 2009). It is important to note that protein concentrations in cells depend on the rates of degradation and dilution by growth of both mRNA and protein but also on the translational rate (Picard *et al*., 2009)

Citrate synthase (gltA) is involved in tricarboxylic acid (TCA) cycle. An incomplete TCA cycle has been found in a surprisingly large number of bacterial pathogens including Helicobacter pylori, Haemophilus influenzae and Streptococcus mutans. The primary role of the TCA cycle is to provide NADH which is used by bacterial cells for ATP synthesis via the electron transport chain (ETC). However, the TCA cycle also plays a key role in the synthesis of intermediates for anabolic pathways; specifically 2-ketoglutarate, oxaloacetate and succinyl-CoA are starting points for the synthesis of glutamate, aspartate and porphyrin respectively. Results obtained by Bowden *et al*., 2010, suggest that disruption of the TCA cycle increases the ability of *S. Typhimurium* to survive within resting and activated murine macrophages, In contrast, an epithelial cell infection model showed that the *S. Typhimurium ΔgltA* strains had reduced net intracellular replication compared to the wild-type suggest that Salmonella may encounter environments within the host where a complete TCA cycle is advantageous.

DotB has been proposed to play a role in assembly of the T4SS, and export of substrates (Sexton *et al*., 2005). The Dot/Icm (T4SS) System, the major virulence mechanism of phylogenetically related pathogens *Legionella pneumophila* and *Coxiella burnetii*, is responsible for their intracellular survival and multiplication, conditions that may also apply to *P. salmonis* (Gómez *et al*., 2013). DotB is a hexametric ATPase in a ring shape, the activity of which is essential to Dot/Icm-dependent activities. In *L. pneumophila* the majority of DotB was found to be cytoplasmic, while small amounts of DotB were recovered in inner membrane fractions (Nagai *et al*., 2011).

Bacterial *purL* gene, encoding 5-phosphoribosyl N-formylglycinamide amidotransferase, is the responsible for the conversion of 5-phosphoribosyl N-formylglycinamide (FGAR) to 5- phosphoribosyl N-formylglycinamidine (FGAM) in the purine biosynthesis pathway. Mutations of *pur* genes often confer marked attenuations in virulence as exemplified by *purL* mutants in *Brucella abortus* (Alcantara *et al.,* 2004). In *Francisella tularensis purL* mutant was found to be considerably attenuated in mice (Kadzhaev *et al*., 2009).

The Escherichia coli *rfaH*, a paralog of the general transcription factor *nusG* that modulates general transcriptional pausing and termination in prokaryotes, is required for the expression of operons directing synthesis and export of the toxin haemolysin, the lipopolysaccharide core, and the F-factor sex pilus (Bailey *et al*., 1996).

For genes *purL* and *nusG*, in both cases qPCR as proteomic analysis, showed less expression in strain IBM040 with respect to the strain LF89, which is different than expected if it is considered this genes as a virulence factor. The limited existing information related to the function of proteins of *P. salmonis*, indicates that it is essential to move forward to the realization of functional assays to clarify the true impact of a given gene in the processes of infection and levels of virulence of this pathogen.

It is important to mention that some of the differences obtained between proteomic and qPCR analysis, could also be attributed to slight differences in the liquid medium, because proteomics experiments were performed several months before qPCR experiments, a period in which small adjustments were made to the culture medium such as final pH adjustment to 7.0, as the bacteria grew with similar efficiencies in the culture medium without adjusting pH, which has pH around 6.2. We must also mention that due to the period of time that elapsed between the two experiments, the initial inoculum for the realization of the experiments could have changed due to multiple subcultures to which he was subjected during this period of time, probably achieving greater adaptation the

culture medium was maintained.

Table 14: Summary of the results obtained in qPCR and proteomics analysis of the comparison between the two strains of *P. salmonis*. Bold; genes that match the increase or decrease of expression in both analysis.

Gene	Variation in strain IBM040	
	Proteomic analysis	**qPCR analysis**
aroK	decreases	no significant difference
purL	decreases	decreases
rspA	decreases	no significant difference
dotB	decreases	increases
mcmA	decreases	no significant difference
gltA	Decreases	increases
nusG	Decreases	decreases

For the identification of genes that are potentially involved in infection processes or intracellular replication, transcriptome sequencing of strains LF89 and IBM40 was performed**.** The results were analyzed with CLC Genomics wokbench 6 Software. Sequences were assembled on the coding sequences of IBM040 strain, which were obtained of partial genome of *P. salmonis*.

For the assembly, was selected the option to include non specific match. The term non-specific matches refers to reads that can be mapped equally well to more than one location on the reference. Since it is not possible to tell which transcript such reads actually came from, the Workbench has to decide where to place them. In such cases, the Workbench first estimates the expression of each gene based only on reads that map uniquely to that gene. It then uses this information to weight the distribution of the reads that can be mapped equally well to more than one location. Alternatively assembly also was performed selecting a Maximum number of hits for a read to 1, where all reads matching in more than one position are excluded (Annex 9).

The importance of being aware of the potential effects of non-specific matches becomes even more evident when looking at the full data set where the proportion of non-specific matches is higher. Consider that with the full reference transcriptome, there is a greater chance of finding sequences that are represented more times, e.g. arising through gene duplications or horizontal gene acquisition. Non-specific matches can also be given by overlapping in ORF.

One of the pitfalls when including non-specific matches is that the number of unique matches can be too low to ensure a reliable distribution of the non-specific matches. One way of approaching this problem was to run the same data set with different settings, results are shown in Annex 10, where you can appreciate the high correlation in expression values between the two parameter settings during assembly, however are observed several genes showing higher expression values when including nonspecific match. On the other hand,

completely disregarded non-specific reads, may underestimate the expression levels of genes in gene families. For this reason it was decided to use the alignments including non specific match for comparison of the two transcriptomes.

Only one sample of each strain was sequenced for the transcriptome analysis, which gives us a hint in differential gene expression between these two strains so it is absolutely necessary to perform a validation of the results obtained in this experiment.

Ten genes that transcriptomic level showed differential expression between both strains in study were assessed by qPCR, and four genes were tested to analyze the stability of its expression, which were *rpoD*, *rpoS*, *recA* and *pykA*. The results indicate that *rpoD* is more stable gene expression, similar to results obtained in previous analysis, probably because the samples were analyzed under identical culture conditions, and in the exponential growth phase, when RNA was extracted.

Statistical analysis showed that eight genes had significant differences when comparing the two strains of *P. salmonis*. However, only the results for *ahpC*, *acrA* and *acrB*, coincide with those obtained in the transcriptome analysis. In the table 15 shown a summary of the results obtained in transcriptomic and qPCR analysis of the comparison between the two strains of *P. salmonis*.

It is important to mention that some of the differences obtained between transcriptomic and qPCR analysis, could also be attributed to slight differences in the liquid medium, because transcriptomic analysis was performed several months before qPCR experiments, a period in which small adjustments were made to the culture medium such as final pH adjustment to 7.0, as the bacteria grew with similar efficiencies in the culture medium without adjusting pH, which has pH around 6.2. It is important to mention this because results obtained by Gómez, *et al*., published this year, show that there is a strong dependence of pH on the expression of components of the system Dot/Icm.

Then genes *ahpC*, *acrA* an *acrB* shown increaded expression in the pathogenic IBM040 strain, compared to LF89 strain in both transcriptomic and qPCR analysis. Central to the survival of pathogenic microorganisms is the ability to withstand the stress conferred by reactive oxygen metabolites since these are important defense mechanisms expressed by infected mammals, in particular by the oxidative burst of phagocytic cells. These defense mechanisms include enzymes that detoxify reactive oxygen species, such as alkyl hydroperoxide reductase (AhpC), which contributes to virulence in *F. tularensis* (Kadzhaev *et al*., 2009). AcrA can function as the periplasmic adaptor protein (PAP) in several resistance nodulation division (RND) tripartite efflux pumps family, of which AcrAB-TolC is considered the most important. This system confers innate multiple antibiotic resistance. Disruption of AcrB impairs the ability of *Salmonella Typhimurium* to colonize and persist in the host (Blair *et al*., 2009). The global consequence of disruption of the AcrAB-TolC efflux pump in *Salmonella enterica* includes reduced expression of SPI-1 and other attributes required to infect the host (Webber *et al*., 2009).

IcmL, IcmP and DotA are part of T4SS, essential for intracellular replication of *Legionella pneumophila* and *Coxiella burnetii.* Segal *et al*., 1998, showed that IcmL is one of genes are required for macrophage killing in *Legionella pneumophila*. Also has been shown, DotA is secreted into the extracellular milieu from culture-grown *L. pneumophila* in a Dot/Icm-dependent fashion (Nagai and Kubori, 2011).

VasA, VasQ and Vgr are component of Type VI secretion systems (T6SSs), which are transenvelope complexes specialized in the transport of proteins or domains directly into target cells. These systems are versatile as they can target either eukaryotic host cells and therefore modulate the bacteria-host interaction and pathogenesis or bacterial cells and therefore facilitate access to a specific niche (Cascales and Cambillau, 2012).

Table 15: Summary of the results obtained in Transcriptomic and qPCR analysis of the comparison between the two strains of *P. salmonis*. Bold; genes that match the increase or decrease of expression in both analysis.

Gene	Variation in strain IBM040	
	Transcriptome analysis	**qPCR analysis**
icmL	decreases	increases
icmP	decreases	increases
acrA	Increases	increases
acrB	increases	increases
dotA	decreases	increases
ggt	decreases	no significant difference
vgrE	decreases	no significant difference
vasA	Increases	decreases
VasQ	increases	decreases
ahpC	increases	increases

4.1. FINAL REMARKS

This thesis identified expression genes that make up the T4SS, such as *dotA*, *dotB*, *icmL* y *icmP* through qPCR analyses. Also expression at the protein level of *dotB* gene was confirmed. Based on this new information collected in this thesis, together with the latest publications on the subject, it is proposed that *P. salmonis* uses an intracellular survival strategy similar to that described for *Legionella pneumophila.*

Adding to the recent publication of "Evidence of the Presence of a Functional Dot/Icm Type IV-B Secretion System in the Fish Bacterial Pathogen *Piscirickettsia salmonis*", which showed that the expression of four components of T4SS (*dotB*, *dotA*, *icmE* and *icmK*), is increased at 48h post infection of the phagocytic fish-derived RTS11 cell line. In addition was shown that *P. salmonis*-containing vacuoles do not fuse with lysosomes, indicating that there is a bacterium-driven interference in the endosomal maturation process that ensures bacterial survival, where the Dot/Icm secretion system could be responsible by delivering effectors proteins inside the host cell.

Here evidence was presented of differential expression of proteins SdhA and NdK. These proteins are secreted by the T4SS in bacteria related to *P. salmonis* and their function is associated with intracellular replication of bacteria. These observations suggest that *P. salmonis* has a functional T4SS, that might be responsible for its intracellular survival.

In order to demonstrate the functionality of T4SS and putative secreted proteins, knock out gene experiments could be carried out once *P. salmonis* be efficiently transformed. Up to now no successful transformation of *P. salmonis* has been reported.

It is important to emphasize the pivotal role of the TCA cycle, because of the results, several enzymes involved in the TCA cycle showed differential expression, which leads us to believe in the close regulation possessing these enzymes, due to the multiple roles associated with this cycle, especially the role in the synthesis of intermediates for anabolic pathways; specifically 2-ketoglutarate, oxaloacetate and succinyl-CoA are starting points for the synthesis of glutamate, aspartate and porphyrin respectively. It has been suggested that disruption of the TCA cycle increases the ability of *S. typhimurium* to survive within resting and activated murine macrophages, In contrast, epithelial cells are non-phagocytic cells and unlike macrophages cannot mount an oxidative and nitrosative defence response against pathogens, the *S. typhimurium* TCA cycle mutant strains showed reduced or no change in intracellular levels compared to wild-type. This evidence does point out that enzymes belonging to, TCA cycle, can have more than one function depending on the environment of the pathogen.

5. CONCLUSIONS

- A liquid culture system for the optimal growth of *P. salmonis*, which was key to the development of several objectives of this thesis was set up "*de novo*".

- The optimal conditions for SHK1 cell line infection with the reference strain were identified. This allowed purification of the bacteria from the infected cell line through isopycnic centrifugation without apparent contamination, thus enabling the following analyses.

- This thesis demonstrated differential gene expressions between LF89 and IBM040 strains. It also demonstrated differential gene expression when comparing growth under intracellular conditions with growth in liquid culture.

- The differential gene expression at the transcriptome level was demonstrated due to the identification of stable gene expressions which could be used as Housekeeping gene in qPCR assays.

- Differential expression of the *P. salmonis* proteome grown in cell line SHK1 was evaluated through 2D-DIGE. 98 spots were founded increase and 128 spots decrease compared to bacteria grown in cell-free medium. Additionally, we identified 56 proteins sequences by mass spectroscopy. In the validation by qPCR, three showed significant differences, *csrA, ndK* and *pnp.*

- In the comparative protein analysis of LF89 with IBM040 strains, 41 Spots that presented significant differential expression 28 amino acid sequences were identified. In the validation by qPCR, three showed significant differences *gltA*, *nusG* and *dotB*.

- The comparative analysis of the transcriptomes of IBM040 vs LF89 strains showed differential expression of 185 genes, many of which still have not been classified. Validation of differential expression by qPCR was confirmed in 8 of 10 analyzed genes. However only three genes (*acrA*, *acrB* and *ahpC*) showed coincidence with the observed variation in the sequencing results of transcriptome.

The results are a significant contribution to elucidate the effect of differences in gene expression and intracellular survival of this pathogenic bacterium. Future functional genomic studies, combined with the use of mutant strains, will allow a better understanding of the function of the genes described herein, and their relationship with pathophysiological processes of *P. salmonis.*

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7. ANNEXES

Annex 1: Analysis of 2D-DIGE with Progenesis SameSpot software. Spots showed differences between samples of proteins obtained from bacteria grown in cell line and bacteria grown in a liquid medium.

Experiment Design		
Condition	Liquid medium	In cell line
Replicates	4	4

Tags	
	Significant
	in cell line
	liquid medium

Spot #	Anova (p)	Fold	Tags	Average Normalised Volumes	
				Liquid medium	In cell line
593	9,03E-08	14,4		0,6	8,641
1463	5,57E-07	5,2		0,38	1,985
1529	6,05E-07	6,7		0,422	2,823
338	9,14E-07	4,1		0,321	1,324
1534	1,11E-06	4,2		0,614	2,553
1431	1,16E-06	6,3		4,251	0,675
869	1,30E-06	5,7		0,304	1,723
418	1,54E-06	10,3		0,406	4,187
863	1,55E-06	4,3		0,357	1,536
1586	2,12E-06	3,6		0,684	2,485
1388	2,51E-06	4,1		0,415	1,72
1458	2,56E-06	5,7		0,411	2,353
424	3,18E-06	10,9		0,468	5,095
681	5,87E-06	5,8		0,392	2,265
693	7,28E-06	3,6		0,662	2,378
813	8,06E-06	3,5		2,875	0,822
599	8,18E-06	16,3		0,648	10,542
1455	8,20E-06	6,3		0,465	2,928
907	9,01E-06	9,3		0,385	3,562
941	9,82E-06	3,7		0,453	1,682
1569	9,99E-06	7		0,413	2,872
598	1,07E-05	16,1		0,54	8,687
533	1,23E-05	6,4		2,797	0,436

Spot #	Anova (p)	Fold	Tags	Average Normalised Volumes	
				Liquid medium	In cell line
853	1,40E-05	2,5		3,089	1,23
1090	1,50E-05	4,9		4,709	0,968
1193	1,60E-05	2,6		2,127	0,832
475	1,78E-05	15,9		0,559	8,896
416	2,06E-05	13,5		0,314	4,241
531	2,14E-05	3,3		1,878	0,573
629	2,23E-05	14,1		9,801	0,694
627	2,25E-05	7,2		0,436	3,142
1625	2,49E-05	4,5		1,819	0,408
1396	3,55E-05	4,3		3,957	0,913
1046	3,66E-05	17,3		12,721	0,735
738	3,82E-05	4,4		0,617	2,723
589	4,01E-05	11,6		14,728	1,265
986	4,33E-05	3,9		2,793	0,708
1584	4,50E-05	6		0,424	2,561
699	4,58E-05	9		6,369	0,71
408	5,28E-05	17,7		0,298	5,274
473	5,88E-05	9,2		0,571	5,237
1314	5,91E-05	8,8		0,392	3,463
595	6,30E-05	3,2		2,02	0,631
273	6,88E-05	3		0,622	1,853
332	7,25E-05	4,8		0,361	1,718

Spot #	Anova (p)	Fold	Tags	Average Normalised Volumes	
				Liquid medium	In cell line
432	7,29E-05	7,1		3,908	0,549
393	7,38E-05	26,7		10,823	0,405
664	7,44E-05	4,5		2,225	0,493
428	7,83E-05	5,4		2,357	0,438
1281	8,04E-05	7,3		3,837	0,527
1370	8,12E-05	8,7		6,684	0,767
812	8,63E-05	5,6		0,412	2,319
451	8,64E-05	5,9		4,11	0,695
1419	8,74E-05	6,2		2,98	0,48
962	8,76E-05	4,8		0,443	2,12
613	9,18E-05	3,8		2,918	0,763
842	9,87E-05	8,1		4,83	0,598
467	9,95E-05	7		4,272	0,607
383	1,05E-04	14,7		8,55	0,582
379	1,07E-04	12		8,217	0,686
645	1,12E-04	7,2		6,15	0,852
525	1,14E-04	9,5		12,678	1,331
1280	1,19E-04	3,6		0,429	1,551
856	1,21E-04	4,4		2,927	0,661
1590	1,22E-04	4,3		0,427	1,821
656	1,25E-04	5,3		7,096	1,327
866	1,36E-04	4,9		0,435	2,147
1180	1,38E-04	4,8		0,381	1,833
457	1,44E-04	6		7,954	1,319
1116	1,53E-04	4,5		0,425	1,921
159	1,55E-04	4,9		4,014	0,813
623	1,61E-04	3,5		3,996	1,146
434	1,73E-04	16,3		0,308	5,015
489	1,80E-04	6,5		3,712	0,571
643	1,84E-04	2,2		2,319	1,037
943	1,91E-04	3,7		0,38	1,425
1383	1,98E-04	2,9		0,786	2,269
703	2,02E-04	3,6		0,469	1,7
382	2,16E-04	2,9		0,825	2,384
729	2,17E-04	5,8		3,998	0,693
592	2,28E-04	4		2,371	0,585
831	2,31E-04	2		1,639	0,811
902	2,49E-04	9,5		0,423	4,001
498	2,50E-04	4		4,304	1,079
484	2,56E-04	4,8		2,332	0,489

Spot #	Anova (p)	Fold	Tags	Average Normalised Volumes	
				Liquid medium	In cell line
343	2,89E-04	3,9		0,342	1,345
284	3,11E-04	6,7		0,375	2,51
963	3,16E-04	2,4		1,946	0,796
161	3,19E-04	9,3		6,883	0,737
671	3,24E-04	6,5		0,471	3,073
705	3,46E-04	3,1		2,83	0,909
1530	3,62E-04	3,6		0,502	1,811
456	3,65E-04	2,3		0,893	2,072
1132	3,76E-04	4,7		0,342	1,617
805	3,76E-04	3,9		0,482	1,878
202	3,84E-04	6,1		6,894	1,134
1136	3,95E-04	4,7		2,935	0,63
714	3,98E-04	4		0,44	1,782
1274	4,16E-04	2,7		1,48	0,541
578	4,19E-04	6,3		4,077	0,651
294	4,36E-04	4		4,336	1,079
252	4,39E-04	8,4		6,769	0,809
652	4,44E-04	3,3		0,62	2,065
158	4,49E-04	4,4		2,842	0,644
1494	4,55E-04	2,7		2,026	0,762
1626	4,57E-04	2		2,155	1,086
554	4,71E-04	6,8		8,579	1,256
240	4,82E-04	4,6		0,454	2,089
509	4,83E-04	2,5		0,747	1,879
1158	5,01E-04	2,7		1,619	0,598
1142	5,05E-04	4,3		4,158	0,957
713	5,48E-04	5,4		0,418	2,261
930	5,63E-04	3,9		2,536	0,656
323	5,71E-04	5		0,501	2,525
1043	5,78E-04	4,8		3,802	0,789
1096	5,78E-04	3,9		3,543	0,91
560	5,78E-04	2,4		1,823	0,768
480	6,17E-04	6,8		0,791	5,357
278	6,75E-04	4,6		0,442	2,042
112	6,79E-04	15,4		0,308	4,755
658	7,04E-04	3,7		2,115	0,578
929	7,42E-04	7		0,403	2,828
190	7,83E-04	5,4		2,882	0,534
1019	7,84E-04	3		2,239	0,755
493	8,37E-04	6,7		4,978	0,741

Spot #	Anova (p)	Fold	Tags	Average Normalised Volumes	
				Liquid medium	In cell line
506	9,30E-04	6,5		8,04	1,239
350	9,38E-04	4,4		0,445	1,975
949	9,56E-04	2,4		0,52	1,226
717	9,73E-04	2,9		2,599	0,884
1522	9,78E-04	2,5		1,574	0,619
1485	9,93E-04	2,4		1,706	0,712
96	0,001	3,5		1,999	0,573
1323	0,001	4,6		4,827	1,044
1054	0,001	3,1		1,998	0,64
927	0,001	3,8		0,517	1,94
138	0,001	3,1		1,677	0,545
468	0,001	2,1		2,862	1,359
684	0,001	1,8		1,152	0,647
1188	0,001	1,8		1,624	0,923
615	0,001	2,4		2,697	1,103
1478	0,001	3,6		0,584	2,096
231	0,001	5,4		2,836	0,521
1119	0,001	2,1		0,507	1,088
310	0,001	3,4		1,863	0,541
1201	0,001	2,1		1,848	0,88
663	0,001	2,1		2,076	0,98
903	0,001	1,7		1,996	1,147
230	0,001	3,6		3,266	0,902
636	0,001	2,8		1,073	3,053
1102	0,002	2,8		0,546	1,506
186	0,002	2,2		1,713	0,781
139	0,002	3,6		1,849	0,516
562	0,002	3,1		0,496	1,533
1407	0,002	7		2,302	0,327
80	0,002	3,3		2,091	0,64
837	0,002	2,6		0,447	1,147
417	0,002	12		0,428	5,12
380	0,002	4,3		3,074	0,716
984	0,002	2,9		2,195	0,758
392	0,002	3		0,992	2,933
371	0,002	2,6		2,126	0,823
536	0,002	4,5		6,593	1,481
788	0,002	2,9		0,473	1,367
1313	0,002	2,5		1,973	0,802
1228	0,002	2,4		0,448	1,071

Spot #	Anova (p)	Fold	Tags	Average Normalised Volumes	
				Liquid medium	In cell line
183	0,002	2,8		1,568	0,56
765	0,002	2,1		2,438	1,148
1545	0,002	3,9		0,441	1,73
725	0,002	5,1		0,373	1,916
421	0,002	2,8		0,569	1,617
454	0,003	2,5		2,741	1,098
768	0,003	2,9		0,476	1,387
731	0,003	2,9		2,099	0,73
617	0,003	5,3		0,862	4,527
969	0,003	2,6		0,523	1,335
148	0,003	3,3		1,619	0,496
419	0,003	9,4		0,434	4,067
931	0,003	1,7		1,237	0,734
1154	0,003	2,4		0,473	1,127
724	0,003	1,8		1,406	0,761
293	0,003	3,3		0,562	1,845
389	0,003	2,5		1,766	0,703
859	0,003	1,7		1,205	0,717
445	0,003	2,4		1,791	0,733
166	0,003	3,4		4,54	1,325
485	0,004	2,6		1,639	0,623
1062	0,004	1,8		0,527	0,967
1041	0,004	1,8		1,326	0,744
1068	0,004	2,5		1,951	0,767
448	0,004	2,8		5,43	1,946
244	0,004	4,1		5,471	1,328
276	0,004	2,6		1,267	0,484
1543	0,004	3,2		0,579	1,842
1014	0,005	2,4		0,561	1,371
314	0,005	3,2		0,446	1,422
707	0,005	5,3		0,535	2,836
515	0,005	1,5		1,204	0,805
1037	0,005	2,6		1,894	0,723
581	0,006	4,3		0,866	3,727
1137	0,006	2,3		0,386	0,903
527	0,006	2,4		1,691	4,122
1145	0,006	1,7		0,993	1,682
654	0,006	2,2		2,384	1,09
459	0,006	2,6		4,065	1,57
574	0,007	1,6		1,093	0,669

Spot #	Anova (p)	Fold	Tags	Average Normalised Volumes	
				Liquid medium	In cell line
959	0,007	1,7		0,811	1,385
453	0,007	2,6		0,561	1,447
974	0,007	2		1,597	0,793
800	0,008	1,7		0,974	1,629
600	0,008	2		3,559	1,762
440	0,009	2,5		0,499	1,224
87	0,009	2,7		1,488	0,555
1017	0,009	1,5		1,77	1,212
839	0,009	2		1,705	0,86
1000	0,01	2,7		2,477	0,908
737	0,01	1,4		1,006	1,382
665	0,01	2		3,84	1,874
939	0,01	1,6		1,135	0,707
1412	0,011	2,5		1,249	0,505
429	0,011	1,6		1,481	2,378
647	0,011	3,5		2,251	0,652
514	0,011	1,4		1,701	1,257
1498	0,012	1,7		1,44	0,858
1218	0,013	1,6		1,068	0,678
1027	0,013	2		1,393	0,71
494	0,015	1,6		0,457	0,732

Annex 2: Proteins identified by mass spectroscopy in 2D-DIGE assay. In Yellow stpots highlights that in 2D-DIGE assay, showed a decrease in intensity in samples of bacteria grown in cell line compared with that were growing in cell-free medium. In green spots highlights, that showed a increase in bacteria grown in cell line. In bold highlights the genes tested in qPCR.

Nº Spot	ID proteína	Score	Sequence coverage %	N° COGs	Code COGs	Gen	Gene Name or Family Name
96	S89C0005_2	745	17	COG0209	F	NrdA	Ribonucleotide reductase alpha subunit
166	S89C0001_15	105	4	COG0480	J	FusA	Translation elongation and release factors (GTPases)
166	S89C0011_2	441	20	COG0459	O	GroL	Chaperonin GroEL (HSP60 family)
166	S89C0244_1	418	16	COG0574	G	PpsA	Phosphoenolpyruvate synthase/pyruvate phosphate dikinase
230	**S89C0002_34**	1292	36	COG1185	F	**Pnp**	Polyribonucleotide nucleotidyltransferase (polynucleotide phosphorylase)
231	S89C0002_34	1348	38	COG1185	F	Pnp	Polyribonucleotide nucleotidyltransferase (polynucleotide phosphorylase)
244	S89C0046_3	1900	47	COG0443	O	DnaK	Molecular chaperone
252	S89C0019_11	1371	41	COG0326	O	HtpG	Molecular chaperone, HSP90 family
273	S89C0441_1	48	3	COG1190	J	LysU	Lysyl-tRNA synthetase class II

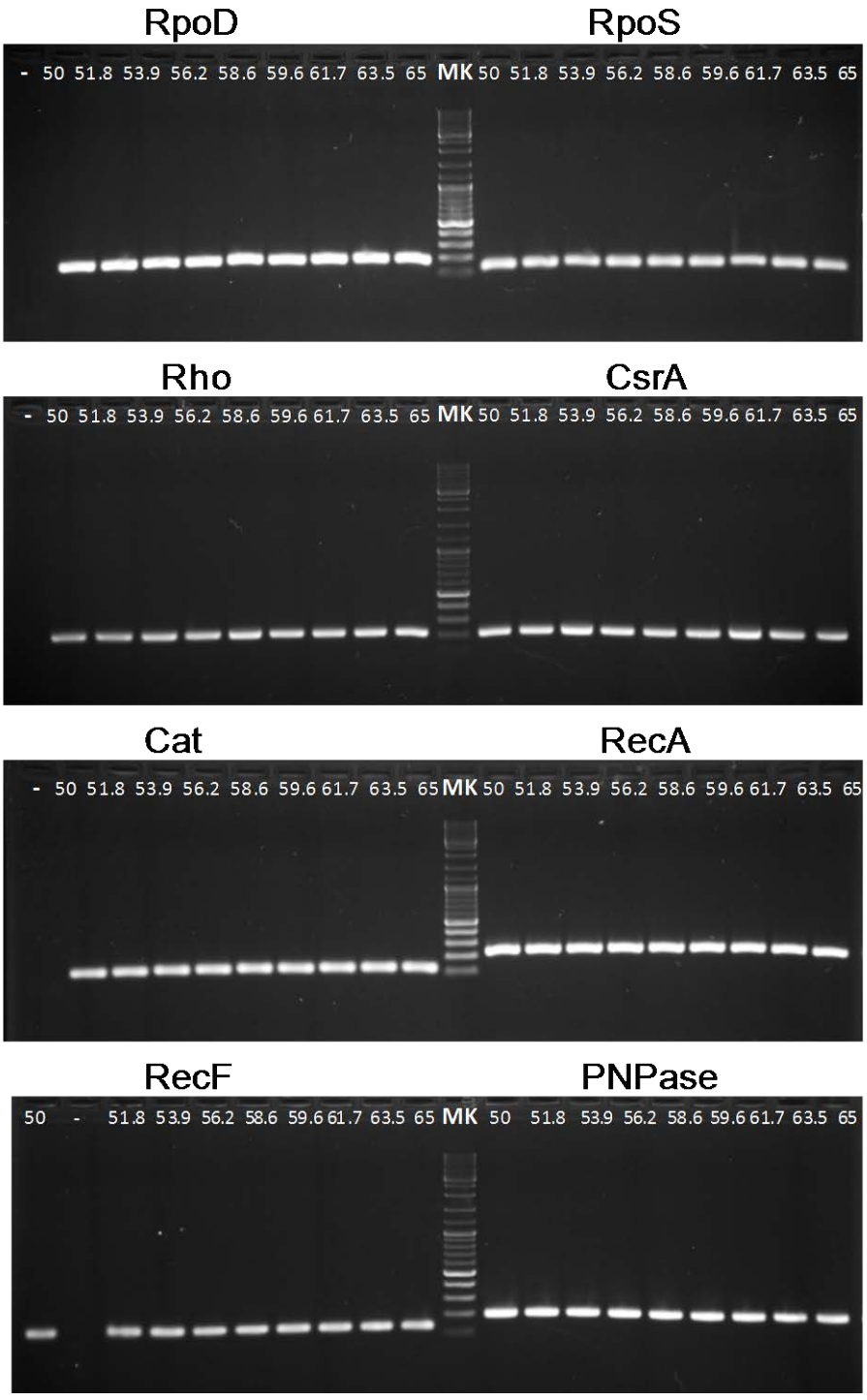
Nº Spot	ID proteína	Score	Sequence coverage %	N° COGs	Code COGs	Gen	Gene Name or Family Name
310	S89C0018_3	68	3	COG1053	C	SdhA	Succinate dehydrogenase/fumarate reductase, flavoprotein subunits
310	S89C0071_10	280	11	COG0441	J	ThrS	Threonyl-tRNA synthetase
393	S89C0011_2	2207	49	COG0459	O	GroL	Chaperonin GroEL (HSP60 family)
399	S89C0011_2	1273	39	COG0459	O	GroL	Chaperonin GroEL (HSP60 family)
424	S89C0013_14	156	8	COG0056	C	AtpA	F0F1-type ATP synthase alpha subunit
432	S89C0255_3	692	38	COG0753	P	KatE	Catalase
451	S89C0011_13	199	13	COG0138	F	PurH	AICAR transformylase/IMP cyclohydrolase PurH (only IMP cyclohydrolase domain in Aful)
451	S89C0013_14	1456	45	COG0056	C	AtpA	F0F1-type ATP synthase alpha subunit
456	S89C0013_14	336	13	COG0056	C	AtpA	F0F1-type ATP synthase alpha subunit
456	S89C0072_1	174	40	COG0154	J	GatA	Asp-tRNA^{Asn}/Glu-tRNAGln amidotransferase A subunit and related amidases
456	S89C0273_1	570	31	COG0154	J	GatA	Asp-tRNA^{Asn}/Glu-tRNAGln amidotransferase A subunit and related amidases

Nº Spot	ID proteína	Score	Sequence coverage %	N° COGs	Code COGs	Gen	Gene Name or Family Name
459	S89C0003_9	264	14	COG1220	O	HslU	ATP-dependent protease, ATPase subunit
459	S89C0013_16	815	37	COG0055	C	AtpD	F0F1-type ATP synthase beta subunit
459	**S89C0057_9**	88	3	COG1219	O	**ClpX**	ATP-dependent protease Clp, ATPase subunit
459	S89C0257_4	173	7	COG0696	G	GpmI	Phosphoglyceromutase
459	S89C0257_5	94	12	COG0696	G	GpmI	Phosphoglyceromutase
506	S89C0005_3	89	5	COG0208	F	NrdF	Ribonucleotide reductase beta subunit
506	S89C0011_2	168	9	COG0459	O	GroL	Chaperonin GroEL (HSP60 family)
506	S89C0018_7	84	7	COG0508	C	AceF	Dihydrolipoamide acyltransferases
531	S89C0024_11	103	19	COG0044	F	PyrC	Dihydroorotase and related cyclic amidohydrolases
531	S89C0024_12	175	15	COG0044	F	PyrC	Dihydroorotase and related cyclic amidohydrolases
562	S89C0326_3	141	8	COG1158	K	Rho	Transcription termination factor
565	S89C0001_44	752	42	COG0202	K	RpoA	DNA-directed RNA polymerase alpha subunit/40 kD subunit
565	S89C0028_15	324	16	COG0404	EF	GcvT	Glycine cleavage system T protein (aminomethyltransferase)
585	S89C0270_3	88	8	COG0114	C	FumC	Fumarase

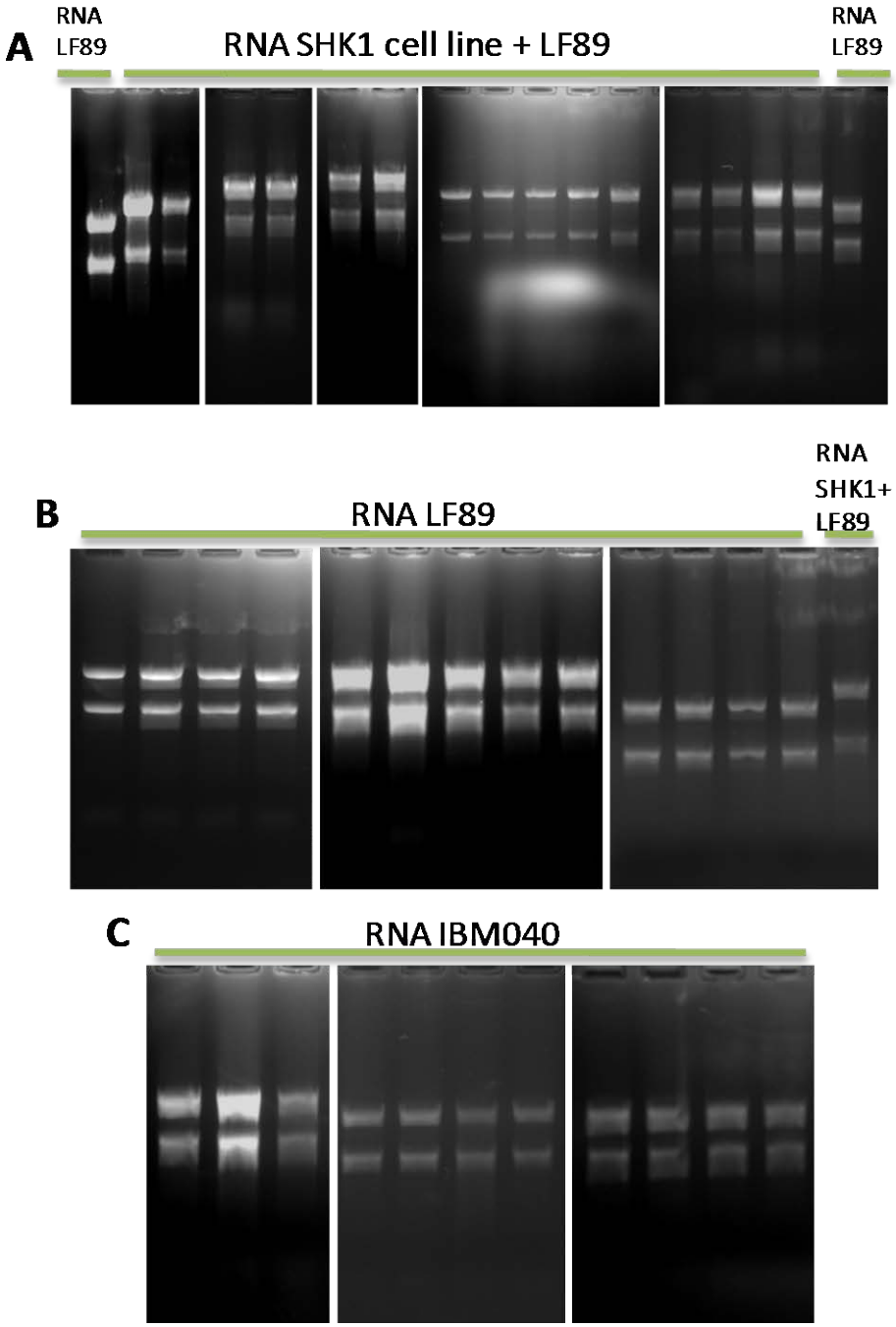
Nº Spot	ID proteína	Score	Sequence coverage %	N° COGs	Code COGs	Gen	Gene Name or Family Name
595	S89C0119_2	397	24	COG0205	G	PfkA	6-phosphofructokinase
599	S89C0118_4	70	6	COG0592	L	DnaN	DNA polymerase sliding clamp subunit (PCNA homolog)
664	S89C0005_1	95	18	COG4992	E	ArgD	Ornithine/acetylornithine aminotransferase
665	S89C0081_9	378	27	COG0505	EF	CarA	Carbamoylphosphate synthase small subunit
684	S89C0039_13	474	27	COG2885	M	OmpA	Outer membrane protein and related peptidoglycan-associated (lipo)proteins
699	S89C0032_10	801	49	COG0264	J	Tsf	Translation elongation factor Ts
703	S89C0116_5	327	17	COG0452	H	Dfp	Phosphopantothenoylcysteine synthetase/decarboxylase
713	S89C0070_8	41	5	COG0039	C	Mdh	Malate/lactate dehydrogenases
729	S89C0070_8	725	43	COG0039	C	Mdh	Malate/lactate dehydrogenases
729	S89C0137_3	115	6	COG0407	H	HemE	Uroporphyrinogen-III decarboxylase
842	**S89C0018_9**	720	37	COG0074	C	**SucD**	Succinyl-CoA synthetase alpha subunit
963	S89C0051_3	158	19	COG0745	TK	OmpR	Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain
963	S89C0074_3	206	16	COG0284	F	PyrF	Orotidine-5'-phosphate decarboxylase

Nº Spot	ID proteína	Score	Sequence coverage %	N° COGs	Code COGs	Gen	Gene Name or Family Name
963	S89C0170_2	186	19	COG0563	F	Adk	Adenylate kinase and related kinases
974	S89C0051_3	422	45	COG0745	T	OmpR	Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain
974	S89C0170_2	125	14	COG0563	F	Adk	Adenylate kinase and related kinases
1027	S89C0079_10	519	52	COG0625	O	Gst	Glutathione-S-transferase
1027	S89C0138_3	155	10	COG1207	M	GlmU	N-acetylglucosamine-1-phosphate uridyltransferase (contains nucleotidyltransferase and I-patch acetyltransferase domains
1046	S89C0023_7	789	62	COG0450	O	AhpC	Peroxiredoxin
1054	S89C0069_1	553	43	COG1028	QR	FabG	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)
1068	**S89C0038_12**	221	28	COG0740	NO	**ClpP**	Protease subunit of ATP-dependent Clp proteases
1068	**S89C0057_10**	260	19	COG0740	NO	**ClpP**	Protease subunit of ATP-dependent Clp proteases
1116	S89C0032_12	91	10	COG0233	J	Frr	Ribosome recycling factor
1136	**S89C0038_12**	362	43	COG0740	NO	**ClpP**	Protease subunit of ATP-dependent Clp proteases
1158	S89C0001_6	338	39	COG0250	K	NusG	Transcription antiterminator

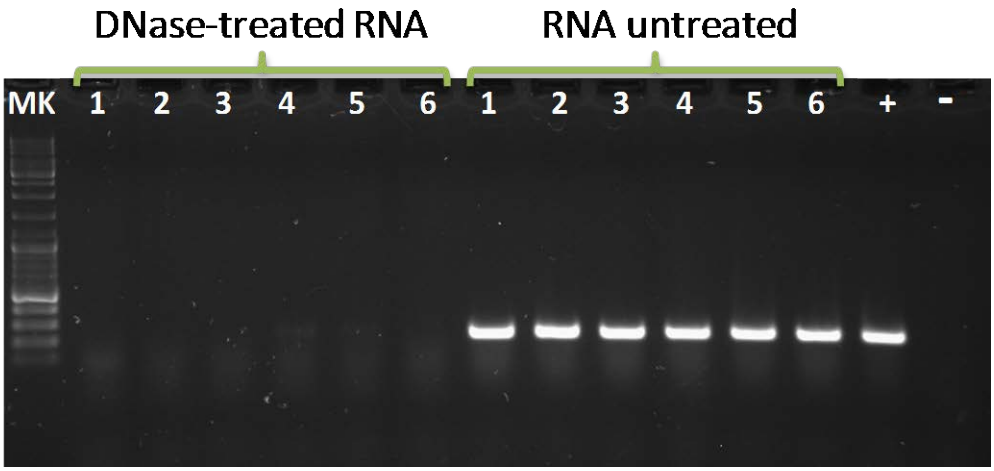
Nº Spot	ID proteína	Score	Sequence coverage %	N° COGs	Code COGs	Gen	Gene Name or Family Name
1274	S89C0248_3	238	31	COG0764	I	FabA	3-hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratases
1281	S89C0006_7	522	47	COG0359	J	RplI	Ribosomal protein L9
1323	S89C0001_10	298	50	COG0222	J	RplL	Ribosomal protein L7/L12
1370	**S89C0010_11**	485	38	COG0105	F	**Ndk**	Nucleoside diphosphate kinase
1383	S89C0154_5	199	51	COG0662	G	CpsB	Mannose-6-phosphate isomerase
1396	S89C0326_2	175	44	COG0526	OC	TrxA	Thiol-disulfide isomerase and thioredoxins
1407	S89C0011_3	265	53	COG0234	O	GroS	Co-chaperonin GroES (HSP10)
1431	S89C0229_3	309	50	-	-	-	Phasin family protein
1485	**S89C0064_4**	150	28	COG1551	T	**CsrA**	Carbon storage regulator (could also regulate swarming and quorum sensing)
1625	S89C0176_1	690	38	COG0104	F	PurA	Adenylosuccinate synthase



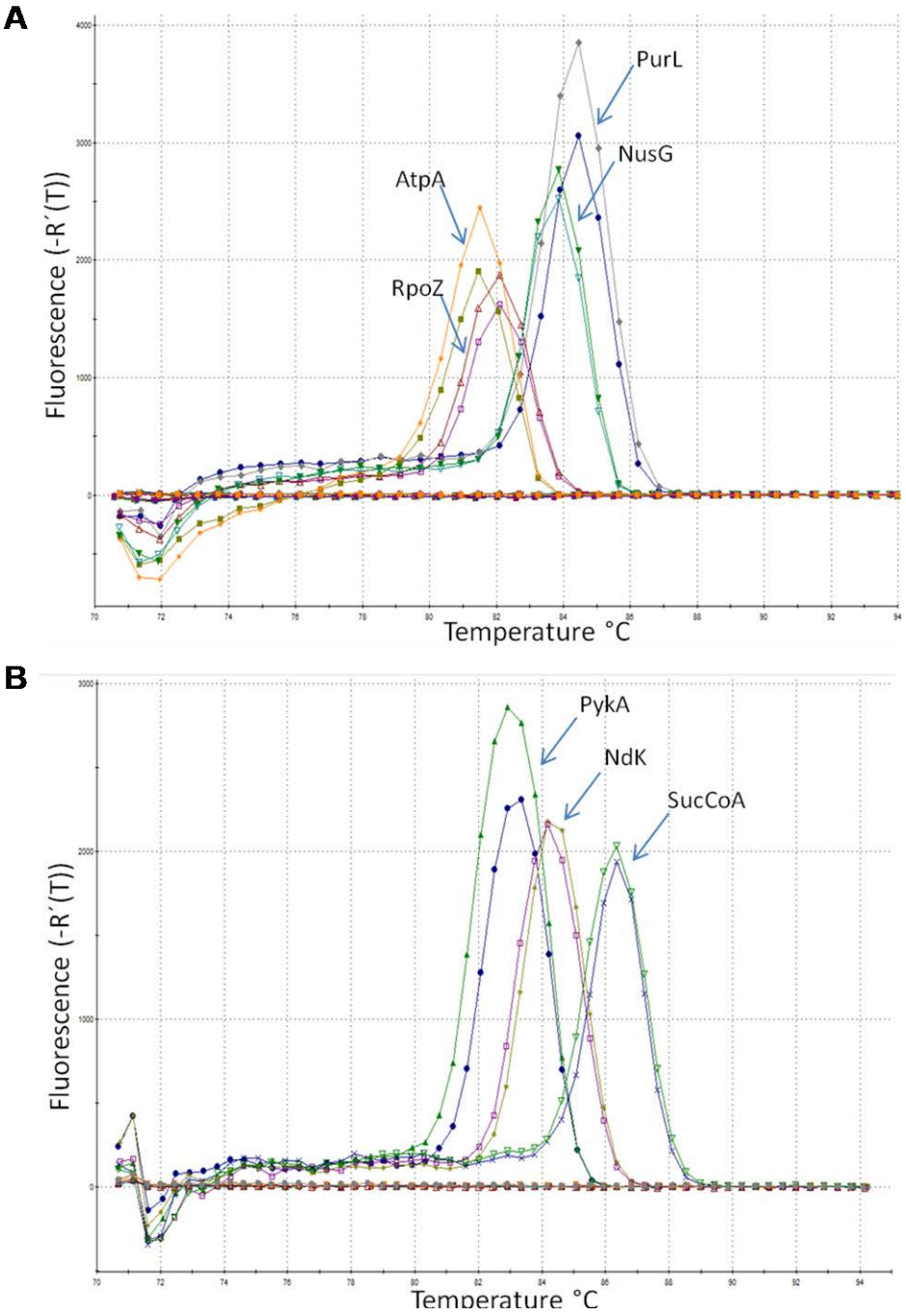
Annex 3: Analysis of the PCR products on gradient. Electrophoretic fractionation in 1% agarose gel. The numbers on the gel indicate the annealing temperature used in PCR. (MK) gene ruler ladder mix (Fermentas), (-): negative control.



Annex 4: Verification of integrity of RNA samples. Electrophoretic fractionation on 1% agarose gel in denaturing conditions. **(A)** RNA samples from cell line SHK1 containing strain LF89. **(B)** RNA samples LF89 strain grown in a liquid medium. **(C)** RNA samples IBM040 strain grown in a liquid medium.



Annex 5: Molecular verification of the absence of genomic DNA into RNA samples. Electrophoretic fractionation on 1% agarose gel of PCR product of RecA gene. (MK) gene ruler ladder mix (Fermentas). Numbers 1-6: RNA samples treated and untreated with DNase, (+) positive control genomic DNA of LF89. (-) negative control without template.



Annex 6: Dissociation curve of some genes used in qPCR. For each gene are two dissociation curves corresponding to the use of two primers concentrations of 0.2µM and 0.4µM.

Annex 7: Analysis of 2D-PAGE with Progenesis SameSpot software. Spots showed differences between samples of proteins obtained from strain IBM040 and LF89, both grown in a liquid medium.

Experiment Design		
Condition	IBM040	LF89
Replicates	4	4

Tags	
	Anova p-value ≤ 0.05
	Max fold change ≥ 2
	condition 1: LF89
	condition 2: IBM040

Spot #	Anova (p)	Fold	Tags	Average Normalised Volumes	
				Condition 1	Condition 2
258	3.69E-06	9.9		1.01E+06	9.90E+06
385	1.85E-04	4.8		7.41E+05	1.55E+05
410	2.72E-04	2.3		9.21E+06	3.95E+06
840	3.06E-04	3.6		4.25E+06	1.18E+06
294	4.56E-04	3.8		2.80E+06	7.44E+05
1079	4.75E-04	2.5		1.37E+06	3.41E+06
1223	5.12E-04	3		8.66E+05	2.58E+06
982	5.56E-04	2.4		3.67E+06	8.63E+06
1234	0.002	2.2		1.05E+05	4.79E+04
1022	0.003	2.7		4.37E+05	1.60E+05
699	0.003	2.2		1.25E+06	5.55E+05
965	0.003	2.2		6.90E+05	3.10E+05
512	0.004	2.8		8.47E+06	3.04E+06
386	0.004	5.6		3.58E+05	6.35E+04
535	0.005	2.9		5.24E+05	1.81E+05
530	0.006	2.5		2.27E+06	9.15E+05
506	0.008	2.7		1.36E+06	5.02E+05
1023	0.009	3.6		1.03E+05	3.68E+05
377	0.01	7.5		3.56E+05	4.74E+04
329	0.011	7.2		4.67E+04	3.36E+05
631	0.013	2.1		1.24E+06	2.58E+06

Spot #	Anova (p)	Fold	Tags	Average Normalised Volumes	
				Condition 1	Condition 2
277	0.013	3.3		1.68E+04	5.47E+04
21	0.013	2.9		1.73E+05	5.86E+04
975	0.013	2.1		1.40E+05	6.80E+04
260	0.015	2.7		1.70E+04	4.60E+04
1196	0.016	2.3		8.41E+04	1.96E+05
994	0.016	2.3		6.10E+05	2.69E+05
194	0.016	3.3		2.58E+06	7.71E+05
407	0.018	2.2		2.51E+05	1.13E+05
1019	0.019	2.9		1.08E+05	3.10E+05
888	0.02	2.1		2.69E+06	5.56E+06
528	0.021	2.1		6.19E+05	2.93E+05
1350	0.022	2.3		1.50E+06	3.53E+06
425	0.023	2.2		1.75E+05	7.81E+04
883	0.026	2		2.00E+05	9.88E+04
344	0.03	8		5.82E+05	4.63E+06
221	0.036	3.7		5.24E+04	1.96E+05
264	0.037	5.1		9.79E+04	4.96E+05
710	0.04	2.9		1.26E+05	3.67E+05
489	0.042	3		6.26E+06	2.11E+06
73	0.05	3		1.51E+06	5.13E+05

Annex 8: Proteins identified by mass spectroscopy in 2D-PAGE assay. In light blue spot highlights that in 2D-PAGE assay, showed less intensity in samples of IBM040 strain compared with LF89 strain. In lilac spot highlights, that showed a higher intensity in IBM040 strain. In bold highlights the genes tested in qPCR.

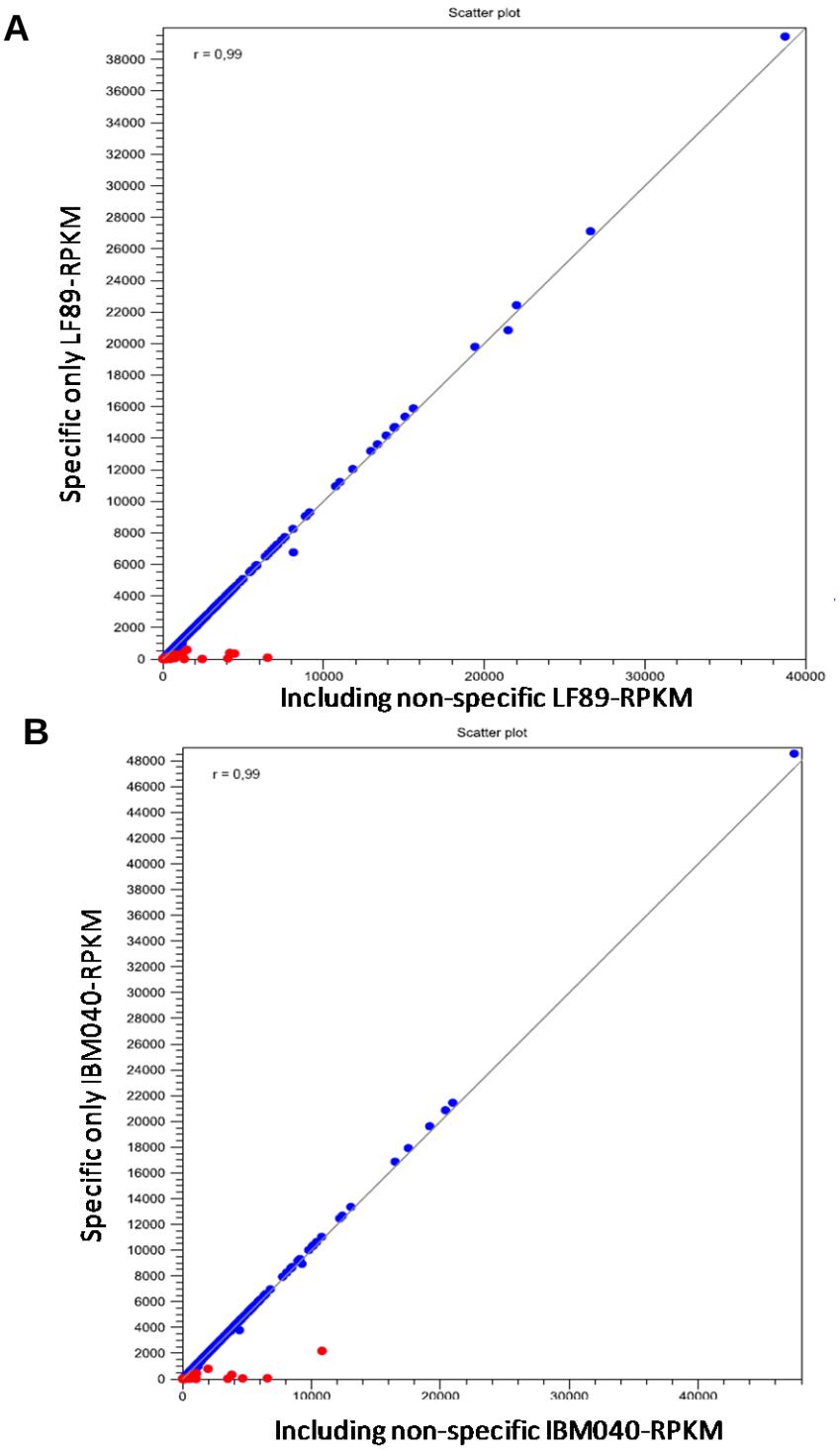
Nº Spot	ID gen	Score	Sequence Coverage %	N° COGs	Code COGs	Gen	Description
21	S40C0058_3	509	9	COG0046	F	**purL**	phosphoribosylformylglycinamidine synthase (EC:6.3.5.3)
73	S89C0112_3	1365	28	COG1026	R	**mcmA**	K06972
194	S89C0019_11	1589	40	COG0326	O	htpG	K04079 molecular chaperone HtpG
258	S89C0052_7	1318	37	COG0539	J	rpsA	30S ribosomal protein S1
294	S89C0052_7	1317	37	COG0539	J	rpsA	30S ribosomal protein S1
334	S89C0011_2	1776	46	COG0459	O	Grol	chaperonin GroEL
385	S89C0052_7	813	28	COG0539	J	rpsA	30S ribosomal protein S1
489	S89C0001_17	1038	51	COG0050	JE	tufB	K02358 elongation factor EF-Tu
506	S40C0009_1	462	26	COG0372	c	**gltA**	K01647 citrate synthase
512	S40C0009_1	1229	48	COG0373	C	**gltA**	K01647 citrate synthase
535	S89C0059_11	589	32	COG2805	N	**dotB**	defect in organelle trafficking protein DotB (ATPase)
631	S89C0006_13	571	35	COG0082	E	aroC	chorismate synthase (EC:4.2.3.5)
840	S89C0298_3	696	43	COG4322	S	-	hypothetical protein

Nº Spot	ID gen	Score	Sequence Coverage %	N° COGs	Code COGs	Gen	Description
865	S89C0118_4	611	22	COG0592	L	dnaN	K02338 DNA polymerase III subunit beta
888	S89C0069_1	837	58	COG1028	QR	**phbB**	acetoacetyl-CoA reductase (EC:1.1.1.36)
982	S89C0199_4	101	12	COG0652	O	cyp	K03767 peptidyl-prolyl cis-trans isomerase A (cyclophilin A)
994	S89C0021_9	96	5	COG0242	J	def	peptide deformylase (EC:3.5.1.88)
1022	S40C0402_2	764	60	COG0703	E	**aroK**	shikimate kinase I (EC:2.7.1.71)
1079	S89C0255_4	665	34	COG0517	R	-	signal-transduction protein
1223	S89C0326_2	182	44	COG0526	OC	trxA	K03671 thioredoxin 1
1234	S40C0447_2	133	39	-	L	-	DNA polymerase beta domain protein region
1350	S89C0470_2	143	26	-	K	rpoZ	DNA-directed RNA polymerase subunit omega (EC:2.7.7.6)
407a	S89C0013_14	323	14	COG0056	C	atpA	K02111 F-type H+-transporting ATPase subunit alpha
407b	S89C0255_3	273	20	COG0753	P	KatE	K03781 catalase
407c	S89C0039_10	136	13	COG0836	M	manC	K00971 mannose-1-phosphate guanylyltransferase
410a	S89C0052_7	1363	36	COG0539	J	rpsA	30S ribosomal protein S1
410b	S89C0057_9	1119	50	COG1219	O	clpX	ATP-dependent protease ATP-binding subunit ClpX
410c	S89C0013_16	682	33	COG0055	C	atpD	K02112 F-type H+-transporting ATPase subunit beta
530a	S89C0079_1	376	30	COG0536	R	obgE	GTPase ObgE

Nº Spot	ID gen	Score	Sequence Coverage %	N° COGs	Code COGs	Gen	Description
530b	S89C0277_4	308	26	COG2866	E	-	hypothetical protein
965a	S89C0001_6	200	39	COG0250	K	**nusG**	transcription antitermination protein NusG
965b	S89C0003_8	175	22	COG5405	O	hslV	ATP-dependent protease peptidase subunit

Annex 9: Summary assembled reads of data obtained in trancriptoma sequencing of strain LF89 and IBM040.

	Found Genes	**Total number of reads**	**Total number of mapped reads**	**Total number of unmapped reads**
LF89 specific only	2708	192398307	33947616	158450691
LF89 including non specific reads	2708	192398307	34597888	157800419
IBM040 specific only	2708	222174103	51053835	171120268
IBM040 including non specific reads	2708	222174103	52257170	169916933



Annex 10: Scatter plot of RPKM value between assembly of specific only vs including non specific reads. Points are highlighted in red representing sequences that vary in their expression value more than 2 times. (A) LF89. (B) IBM040.