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Study on pathogenicity of *Streptococcus parauberis* in olive flounder, *Paralichthys olivaceus*

by

Yoonhang Lee

Department of Aquatic Life Medicine

The Graduate school

Pukyong national university

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Study on pathogenicity of *Streptococcus parauberis* in olive flounder, *Paralichthys olivaceus*

넙치, *Paralichthys olivaceus*에 대한 *Streptococcus parauberis*의 병원성 연구

Advisor: Prof. Do Hyung Kim

by

Yoonhang Lee

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by

Yoonhang Lee

Approved by :

(Chairman) Prof. Hyun Do Jeong

(Member) Prof. Ki Hong Kim

(Member) Prof. Do Hyung Kim

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Study on pathogenicity of *Streptococcus parauberis* in olive flounder, *Paralichthys olivaceus*

Yoonhang Lee

Department of Aquatic Life Medicine, The Graduate school

Pukyong national university

Abstract

This study attempted to understand pathogenicity of *Streptococcus parauberis* by comparing genomes of five *S. parauberis* strains and global gene expression profiling of this pathogen exposed to olive flounder serum. In this study, serotyping of 75 *S. paraberis* strains was performed. Pathogenicity of 15 strains was evaluated in olive flounder by subcutaneous injection. Whole genome sequencing of three high and two low virulent strains was conducted using PacBio RSII (Pacific Biosciences) single-molecule real-time (SMRT) technology. Total RNA was extracted from samples of *S. parauberis*

SPOF3K cultured in the serum and BHIB (+1% NaCl) for 1, 2 and 4 h. RNA sequencing was performed using Illumina hiseq 2500 platform, and differential gene expression (DEG) was analyzed using Limma/Voom package. As a result, the genome of two strains of serotype Ia contained a 12 Kbp sized plasmid harboring tetracycline resistance and Internalin J gene, known as a virulence factor. Importantly, only serotype Ia strains were found to contain bacterial cellulose synthase, which would be of importance in bacterial biofilm formation and peptidoglycan O-acetyltransferase, which is reported to bring resistance against lysozyme. In our transcriptome analysis, in line with this, these genes were significantly up-regulated. Gene expression profiling showed fifteen important virulence- and nine stress-related genes significantly up-regulated in serum, indicating their crucial role in adapting hostile environment and in pathogenicity. In conclusion, our findings would provide a comprehensive view toward adaptive strategy and pathogenesis of *S. parauberis*, which would provide fundamental knowledge for further development of control measures in the future.

General Introduction

Olive flounder (*Paralichthys olivaceus*) is the most important fish species in South Korean aquaculture industry. Economic value of the fish is equivalent to 59% of total value for cultured fisheries production in 2016 (Statistics Korea., 2017). Previous studies (Cho et al., 2008; Jung et al., 2012; Kim et al., 2012 and Jee et al., 2014) have already shown that Streptococcosis is being one of the dominating bacterial diseases in olive flounder, causing huge economic losses. *Lactococcus garvieae*, *S. iniae,* and *S. parauberis* have been reported as the causative agent for Streptococcosis in olive flounder (Baeck et al., 2006 and Shin et al., 2006). However, recent studies revealed that *S. parauberis* has become a dominant etiological agent for Streptococcosis in Korean fish farms (Baeck et al., 2006 and Park et al., 2016).

S. parauberis has been previously regarded as *Streptococcus uberis* genotype II, before the current species name was first proposed by Williams and Collins (1990). Since its first isolation from turbot (*Scopthalmus maximus*) in Spain (Domeénech et al., 1996), this

pathogen has caused disease in different fish species worldwide (Baeck et al., 2006; Haines et al., 2013; Oguro et al., 2014 and Lazado et al., 2018). Although inactivated vaccines are commercially available and vaccination for streptococcosis has become common in Korea (Kwon et al., 2014 and Park et al., 2016), use of antibiotics is also a general option for the treatment of this disease. However, the efficacy of antibiotic therapy for the diesease has decreased with emergence of antibiotic resistant strains (Meng et al., 2009 and Park et al., 2009).

It has been proposed that strains of *S. parauberis* in Japan and Korea are composed of two serotypes (termed I and II) (Kanai et al., 2009 and Han et al., 2011). Meng et al. (2009) showed that strains of two different serotypes possessed different antibiotic resistant related genes (Meng et al., 2009). Also, previous studies (Han et al., 2011 and Woo et al., 2013) showed that *S. parauberis* strains are different in terms of pathogenicity: serotype II strains tend to be more virulent than serotype I. Recently, Kanai et al. (2015) have further divided *S. parauberis* serotype I to three subserotypes (Ia; Ib and Ic), which corresponded with pulse field gel electrophoresis typing. Tu et al.

(2015) reported that there are genetic differences in capsular polysaccharide (CPS) depending on serotypes, suggesting CPS gene would be important in determining the serotype.

Common clinical signs of streptococcosis are darkened skin, hemorrhages around the mouth, eyes, and pectoral fins, paled friable liver with hepatomegaly, ascetic fluid in the peritoneal cavity, and opaque eyes (Kang et al., 2007; Kim et al., 2007 and Won et al., 2013). Previous studies (Won et al., 2013 and Kim et al., 2017) found that *S. parauberis* can cause pericarditis and meningitis. However, the virulence mechanism of *S. parauberis* has not been fully understood.

The main objectives of this study were to understand virulence mechanisms of *S. parauberis* to obtain basic knowledge for the development of prevention and treatment strategies against streptococcosis caused by *S. parauberis*. Therefore, comparative genomic analysis using high and low virulent strains was performed to identify key virulence factors (**Chapter I**). Also, genome-wide transcriptome profiling using bacterial culture of *S. parauberis* in

olive flounder serum was performed to determine the mode pathogenicity of *S. parauberis* in host (**Chapter II**).

Chapter I. Identification of virulence factors of *Streptococcus parauberis* based on comparative genomic study

1. Introduction

The rapid development of sequencing techniques have completed tens of thousands of bacterial whole genome sequences worldwide (http://ftp.ncbi.nih.gov/genomes/). The profound set of genomic data is being increasing in size and significantly contributing to comprehensive insight into overall microbial knowledge: genome organization, identification, evolution, environmental adaptation, functional identification regarding on various cellular processes (Binnewies et al., 2006; Moran et al., 2008; Rocha et al., 2008 and Richter et al., 2009). Especially in clinical microbiology, the comparative genome analysis with pathogenic and low or nonpathogenic species / strains enabled us to better understand their virulence mechanisms and host adaptation strategies (Sudheesh et al., 2012 and Bertelli et al., 2013).

Recently, genome sequence of *S. parauberis* strains derived from fish and bovine sources in Asia, Europe and USA are being uploaded in public databases and there have been several studies showing genomic traits of this bacteria (Nho et al., 2011; Oguro et

al., 2014; Seo et al., 2015; Haines et al., 2016 and Liu et al., 2017). The comparative genome study using three *S. parauberis* strains derived from Korea and Japan demonstrated their genetically different characteristics especially in carbohydrate metabolism and presence of Clustered, regularly interspaced, short palindromic repeat (CRISPR) / Cas9 system (Nho et al., 2013).

In this chapter, we aimed to determine high and low virulent *S. parauebris* strains with experimental challenge test (*In vivo*) and survival test in olive flounder serum (*Ex vivo*) and identify important genomic difference including phylogenomic relationship and factors related to virulence and drug resistance based on comparative genome analysis of *S. parauebris* strains.

2. Materials and methods

2.1. Bacteria and culture conditions

A total of 75 *S. parauberis* stains were used in this study (Figure 1-1 and Table 1-1). Bacteria were isolated from olive flounder (*Paralichthys olivaceus*) and starry flounder (*Platichthys stellatus*) throughout the commercial aquaculture farms in South Korea from 1999 to 2018. The reference strain, KCTC3651 was purchased form Korean Collection for Type Culture (KCTC, Daejeon, South Korea). Bacterial isolates were identified as *S. parauberis* based on PCR assay using specific primer set (Spa 2152 : 5' – TTT CGT CTG AGG CAA TGT TG - 3', Spa 2870 : 5' – GCT TCA TAT ATC GCT ATA CT – 3') developed by Mata et al. (2004). Bacteria were cultured in Brain heart infusion broth or agar (BHIB; BHIA, BD, USA) supplemented with 1% NaCl (BN) at 28 °C for 24 hours. For the further use, bacteria were stored at -70 °C in BHIB containing 10 % glycerol.

Table 1-1. Bacterial strains used in this study

Strain name	Origin of strain		
	Region	**Host**	**Year**
KCTC3651	United Kingdom	Mastis sample	1990
KSP1	JeJudo	Olive flounder	2003
KSP2	JeJudo	Olive flounder	2003
KSP3	JeJudo	Olive flounder	2003
KSP4 [1), 2)]	JeJudo	Olive flounder	2003
KSP5	JeJudo	Olive flounder	2004
KSP6	JeJudo	Olive flounder	2004
KSP7	JeJudo	Olive flounder	2004
KSP8	JeJudo	Olive flounder	2004
KSP9	JeJudo	Olive flounder	2004
KSP10 [1), 2)]	JeJudo	Olive flounder	2004
KSP11	JeJudo	Olive flounder	2005
KSP12	JeJudo	Olive flounder	2005
KSP13	JeJudo	Olive flounder	2005
KSP14 [1)]	JeJudo	Olive flounder	2005
KSP15	JeJudo	Olive flounder	2005
KSP16	JeJudo	Olive flounder	2005
KSP17	JeJudo	Olive flounder	2005
KSP18	JeJudo	Olive flounder	2005
KSP19	JeJudo	Olive flounder	2005
KSP20 [1), 2)]	JeJudo	Olive flounder	2005
KSP21	JeJudo	Olive flounder	2005
KSP22 [1)]	Haenam	Olive flounder	2005

(Continued)

Strain name	Origin of strain		
	Region	Host	Year
KSP23	Haenam	Olive flounder	2005
KSP24	Wando	Olive flounder	2005
KSP25	Wando	Olive flounder	2005
KSP26	Wando	Olive flounder	2005
KSP27	Wando	Olive flounder	2005
KSP28 [1)]	Wando	Olive flounder	2005
KSP29	Wando	Olive flounder	2005
KSP30	Wando	Olive flounder	2005
KSP31	Wando	Olive flounder	2005
KSP32	Wando	Olive flounder	2005
KSP33	Wando	Olive flounder	2005
KSP34	Wando	Olive flounder	2005
KSP35	Wando	Olive flounder	2005
KSP36	Wando	Olive flounder	2005
KSP37	Wando	Olive flounder	2005
KSP38	Wando	Olive flounder	2005
KSP39	Wando	Olive flounder	2005
KSP40	Wando	Olive flounder	2005
KSP41	Wando	Olive flounder	2005
KSP42	Wando	Olive flounder	2005
KSP43	Wando	Olive flounder	2005
KSP44 [1)]	JeJudo	Olive flounder	1999
KSP45	JeJudo	Olive flounder	1999

(Continued)

Strain name	Origin of strain		
	Region	**Host**	**Year**
KSP46	JeJudo	Olive flounder	1999
KSP47 [1)]	Gyungsangbuk-do	Olive flounder	1999
PH0710 [1)]	Pohang	Starry flounder	2007
PH0711	Pohang	Starry flounder	2007
04151K	Geojedo	Olive flounder	2013
04162K [1)]	Geojedo	Olive flounder	2013
SPOF3K [1), 2)]	Geojedo	Olive flounder	2013
HFTC0023	Pohang	Olive flounder	2014
HFTC0059	Pohang	Olive flounder	2014
HFTC0060	Pohang	Olive flounder	2014
HFTC0063	Pohang	Olive flounder	2014
HFTC0064 [1,) 2)]	Pohang	Olive flounder	2014
HFTC0076	Youngduk	Starry flounder	2014
HFTC0083 [1)]	Youngduk	Starry flounder	2015
HFTC0086 [1)]	Uljin	Starry flounder	2015
HFTC0091	Uljin	Starry flounder	2015
HFTC0157 [1)]	Pohang	Starry flounder	2015
HFTC0237	Youngduk	Starry flounder	2015

(Continued)

Strain name	Origin of strain		
	Region	Host	Year
SPOF18J1	JeJudo	Olive flounder	2018
SPOF18J2	JeJudo	Olive flounder	2018
SPOF18J3	JeJudo	Olive flounder	2018
SPOF18J4	JeJudo	Olive flounder	2018
SPOF18J5	JeJudo	Olive flounder	2018
SPOF18J6	JeJudo	Olive flounder	2018
SPOF18J7	JeJudo	Olive flounder	2018
SPOF18J8	JeJudo	Olive flounder	2018
SPOF18J9	JeJudo	Olive flounder	2018
SPOF18J10	JeJudo	Olive flounder	2018
SPOF18J11	JeJudo	Olive flounder	2018

1) *S. parauberis* strains used for pathogenicity test

2) *S. parauberis* strains used for survival test in serum and whole genome sequencing

Table 1.2. Multiplex PCR primers for bacterial serotyping

Primer	Sequence (5' - 3')	Amplicon size (bp)
Ia-For	ATTGTTAGTCATTCAGTTGT	213
Ia-Rev	AATTATAGTCAACAGTCCAG	
Ib/Ic-For	ATTTCTACCAGGTTACTTTG	303
Ib/Ic-Rev	ACATCTCGAAACTTCATATT	
II-For	GAACTACTTAGGTTTAGCAT	413
II-Rev	AACTTGTAAATAGGATTGCT	

2.2. Bacterial serotyping

For bacterial serotyping, a multiplex PCR assay targeting polysaccharide polymerase gene (*wzy*) developed by Tu et al. (2015) was employed in this study. Bacterial DNA of 75 *S. parauberis* strains was prepared using AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea) according to the manufacture's instruction. For multiplex PCR, 10 pmole of three primer pairs, 1 μl of bacterial DNA and sterile distilled water up to total volume of 20 μl were added to AccuPower PCR PreMix (Bioneer). The primer sequence and amplicon size are demonstrated in Table 1-2. The amplification was conducted in MyCycler thermal cycler (Bio-Rad, Hercules, California, USA) with conditions as follows: 95 °C for 3 min, 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s and a final extension at 72 °C for 7 min. The amplified products were displayed by electrophoresis on ethidium bromide-stained 2% agarose gels (Bio-Rad). The serotypes of publically available genome sequences were determined using *in silico* PCR (http://insilico.ehu.es/).

2.3. Pathogenicity test

2.3.1. Fish

Olive flounder (~40 g) were purchased from aquaculture farm in Goheung, Korea and were acclimatized to the aerated and circulated conditions in Institute of Fisheries Science of Pukyong National University, Gigang, Korea.

For challenge experiment, fish were brought to the laboratory and acclimated in the seawater with continuous aeration. Half of the water volume was exchanged every day. After one-week acclimation, water temperature was raised 1 °C per a day until it reached 26 °C. Three fish were randomly sacrificed and confirmed not to be infected by infectious diseases prior to the challenge test.

2.3.2. Challenge test

Fifteen *S. parauberis* strains were selected with consideration of isolation source / year and serotype, and used for experimental challenge test (Table 1-1). Prior to the challenge experiment, *S. parauberis* strains were subcutaneously injected into olive flounder (One hundred microliter; 10^{10} CFU ml^{-1}) and were recovered from blood of dead or moribund fish within 24 hours post challenge.

After the bacterial pure culture in BN medium at 28 °C for 24 hours, they were washed once, resuspended in phosphate buffered saline (PBS; pH 7.4 at 25 °C). One hundred microliter of cell suspension of *S. parauberis* strain containing 10^5 CFU was subcutaneously injected into ten olive flounders housed in rectangular tanks (30 L: 50 cm x 35 cm x 30 cm). The water temperature was maintained at 26 °C, and half of the water volume was exchanged every day. The mortality was observed for 14 days and the spleen of dead fish was swabbed and plated in the BN medium to check the *S. parauberis* infection which was later confirmed by colony PCR using a specific primer set (Mata et al., 2004).

2.4. Bacterial survival test in olive flounder serum

Five strains were selected for bacterial survival test in olive flounder serum (Table 1-1). Bacteria were cultured in BN for 18 hours at 160 rpm at 26°C. Bacterial cells were harvested at early-stationary phase and washed with PBS by centrifugation for 10 min at 6000 rpm in room temperature. The bacterial pellet was re-suspended in PBS and adjusted to approximately 2 x 10^5 CFU ml^{-1}. Two milliliters of bacterial suspension was mixed with the same amount of BN medium or olive flounder serum, and they were incubated for four hours at 160 rpm at 26°C in triplicate. Olive flounder blood was drawn from healthy one-year-old fish weighing approximately 100g with minimum anesthesia using MS-222 (Sigma-Aldrich, Saint louis MO, USA) and serum was separated from the clot by centrifugation at 6500rpm at 4°C. At 0, 1, 3 and 6 hours post incubation (hpi), bacterial viable counts were measured by plate counting method: one hundred microliters of serially diluted cultures were plated onto BN at 26°C.

2.5. Comparative whole genome analysis

2.5.1. Genomic DNA preparation

In this study, five *S. parauberis* strains were selected for bacterial whole genome sequencing (Table 1-1). Bacterial strains were grown in BN medium 28 °C until it reaches late-exponential period. Genomic DNA was prepared using Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instructions. The quantity and purity of DNA samples were checked using Quant-iT PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA) and NanoVue Plus™ spectrophotometry (GE Healthcare Life Sciences, USA) respectively (DNA concentration > 200 ng; Total volume > 30 µl; UV A280/A280 > 1.8).

2.5.2. Whole genome sequencing

Whole genome sequencing was performed with PacBio RS II (Pacific Biosciences, USA) single-molecule real-time (SMRT) and the raw sequences were *de novo* assembled using PacBio SMRT Analysis 2.3.0. Gene prediction was conducted using tRNAscan-SE (Schattner et al., 2005) for tRNA search, Rfam (Griffiths-Jones et al., 2005) for rRNA and non-coding RNA search, and Prodigal (Hyatt et al., 2010) for CDS search. Predicted genes were functionally annotated by homology search against KEGG (Kanehisa et al., 2000), SEED (Gerdes et al., 2014), Swiss-Prot (Boeckmann et al., 2003), and eggNOG (Jensen et al., 2007) databases. The identification of virulence related genes were conducted using blastx analysis (e-value < e^{-10}) based on Virulence Factor Database (VFDB; http://www.mgc.ac.cn/vf/), which provides a comprehensive sequence data of virulence factors (Chen et al., 2015). Also, blastn analysis (With strict cutoff) based on Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca/) was used for verification of antibiotic resistance related genes (Jia et al., 2017).

2.5.3. Genome identification and phylogenomic analysis

Average Nucleotide Identity (ANI) of sequenced genome were calculated for bacterial genome identification using EDGAR version 2.3 platform (https://edgar.computational.bio.uni-giessen.de/) (Blom et al., 2016). The genome sequences that are hitherto-publically available were retrieved from the National Center for Biotechnology Information (NCBI) bacterial genome database (http://ftp.ncbi.nih.gov/genomes/) and used for our analysis (Table 1-3).

Phylogenetic analysis based on whole genome sequence of our strains and/or publically available strains were calculated to determine the phylogenomic distribution and relationship of *S. parauberis*. The core genes of multiple genome sequences were computationally calculated, aligned based on EDGAR version 2.3 platform which generated .nex file, that was subsequently used to construct phylogenetic tree by using Splitstree version 4 software (Huson., 1998). The binary analysis of pan-genome based on compared genomes were performed, which was further used to

construct a hierarchical clustering using one minus pearson correlation metric with a computational visualization web-based tool, Morpheus (https://software.broadinstitute.org/morpheus/).

2.5.4. Comparative genome analysis

Comparative pan-genome analysis of genome sequence of our strains and/or hitherto-publically available strains were performed with EDGAR version 2.2 platform. The genomic subsets, pan-genome, core-genome and singletons of genomes were retrieved by computational calculation of sequence similarity. The generated comparative genomic data was visualized for comprehensive interpretation by using this platform.

PCR analysis was performed on 75 *S. parauberis* strains for verification of gene presence / absents using specific primer sets (Table 1-4), designed based on genome sequences, using Primer3Plus (Untergasser et al., 2012) and in silico PCR at www.insilico.ehu.es/ (Bikandi et al., 2004). The genomic DNA preparation, PCR and electrophoresis were performed as mentioned above with PCR condition as follow: Pre-denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 60 s and final extension at 72°C for 7 min.

Table 1-3. Publically available *S. parauberis* genome sequence used in this study

N	Strain	Size (Mb)	Genes	Level	Isolate Region	Host
1	KCTC11537	2.14389	2222	Complete	Korea	Olive flounder
2	KCTC11980BP	2.13949	2144	Scaffold	Korea	Olive flounder
3	KRS02109	2.04654	2046	Contig	Japan	Olive flounder
4	KRS02083	2.17624	2234	Contig	Japan	Olive flounder
5	SK-417	1.95884	1957	Contig	Japan	Rock fish
6	T1	2.01196	2019	Contig	Spain	Turbot
7	N11	1.99927	2061	Contig	USA	Wild striped bass
8	PL23	2.02048	2069	Contig	USA	Wild striped bass
8	N198_2	1.97583	2021	Contig	USA	Wild striped bass
9	PL9	2.01223	2074	Contig	USA	Wild striped bass
10	RP15	2.01263	2074	Contig	USA	Wild striped bass
11	RP17	2.01642	2072	Contig	USA	Wild striped bass
12	RP25	2.03113	2086	Contig	USA	Wild striped bass
13	SP-llh	2.52126	2547	Scaffold	China	Dairy cow with mastitis
14	NCFD 2020	2.16448	2186	Complete	England	Dairy cow with mastitis

* N: Serial number of genome used in this study.

Table 1-4. Primers used for verification of gene presence / absent.

Primer	Target gene (Locus)	Sequence	Amplicon size (bp)	Reference
tetM-For	Tetracycline resistance gene *tetM* (SPK10_00587)	GTTAAATAGTGTTCTTGGAG	657	Aarestrup et al., 2000
tetM-Rev		CTAAGATATGGCTCTAACAA		
tetS-For	Tetracycline resistance gene *tetS* (SPSF3K_02213)	ATCAAGATATTAAGGAC	573	Charpentier et al., 1993
tetS-Rev		TTCTCTATGTGGTAATC		
ermB-For	Macrolide resistance gene *ermB* (SPSF3K_01188; SPSF3K_01176)	GTAAACAGTTGACGATATTCTCG	224	Nagai et al., 2001
ermB-Rev		CGTACCTTGGATATTCACCG		
ANT(6)-Ia-For	Aminoglycoside resistance gene ANT(6)-Ia (SPSF3K_01182)	AGGGGTCACGCGCAAATATT	512	Designed in this study
ANT(6)-Ia-Rev		CCCAACCTTCCACGACATCA		
mobA-For	Mobilization protein *mobA* (SPSF3K_02220)	ACAAATTGTCGGGCAGTTGC	544	Designed in this study
mobA-Rev		TAAACGCCTCGTACGAGCTC		
inlJ-For	Internalin J *inlJ* (SPSF3K_02212)	GCTGCTGATGTGACGGCTAA	588	Designed in this study
inlJ-Rev		CGCTGGTTTTTCTCCGGTTG		
BCS-For	Bacterial cellulose synthase (SPSF3K_02074)	ATGGCAAGCGGTTTAACACC	588	Designed in this study
BCS-Rev		ATGATGCAACAACCAAAAGCT		
oatA-For	Peptidoglycan O-acetyltransferase *oatA* (SPSF3K_01374)	TCGGGCTGATGATCGGAATG	595	Designed in this study
oatA-Rev		GCACCCCCTGGCTTCTTAAT		

2.6. Statistical analysis

The experimental data of viable bacterial counts in broth and serum (Figure 2-1) were analyzed using SPSS (20.0), with significant differences among data being tested by ANOVA. Duncan method was implemented to compare statistical difference among the group ($P < 0.05$).

3. Results

3.1. Bacterial serotyping

Results of serotyping of 75 *S. parauberis* strains are shown in Figure 1-1 and 1-2, and Table 1-5. Thirty-two, thirty-eight and four *S. parauberis* strains were identified as serotype Ia, Ibc and II, respectively. Among 38 serotype Ibc strains, 32 were isolated in 2005. Considering the distribution bias of serotype Ibc, serotype Ia was the most predominant serotype found in this study. Interestingly, only four strains isolated from 2003 to 2005 were typed as serotype II. The reference strain, KCTC3651, was not classified as any of serotypes based on PCR method used in this study, which corresponded with a previous study using micro-agglutination test (Woo et al., 2013).

Table 1-5. Serotypes of 75 *S. parauberis* strains used in this study

N.	Strain	Sero-type	N.	Strain	Sero-type	N.	Strain	Sero-type
1	KSP1	II	26	KSP26	Ibc	51	04151K	Ia
2	KSP2	Ia	27	KSP27	Ibc	52	04162K	Ia
3	KSP3	Ia	28	KSP28	Ibc	53	SPOF3K	Ia
4	KSP4	II	29	KSP29	Ibc	54	HFTC0023	Ia
5	KSP5	Ibc	30	KSP30	Ibc	55	HFTC0059	Ia
6	KSP6	Ibc	31	KSP31	Ibc	56	HFTC0060	Ia
7	KSP7	Ia	32	KSP32	Ibc	57	HFTC0063	Ia
8	KSP8	Ia	33	KSP33	Ibc	58	HFTC0064	Ia
9	KSP9	Ia	34	KSP34	Ibc	59	HFTC0076	Ibc
10	KSP10	II	35	KSP35	Ibc	60	HFTC0083	Ia
11	KSP11	Ibc	36	KSP36	Ibc	61	HFTC0086	Ia
12	KSP12	Ibc	37	KSP37	Ibc	62	HFTC0091	Ia
13	KSP13	Ibc	38	KSP38	Ibc	63	HFTC0157	Ia
14	KSP14	II	39	KSP39	Ibc	64	HFTC0237	Ia
15	KSP15	Ibc	40	KSP40	Ibc	65	SPOF18J1	Ia
16	KSP16	Ibc	41	KSP41	Ibc	66	SPOF18J2	Ia
17	KSP17	Ibc	42	KSP42	Ibc	67	SPOF18J3	Ia
18	KSP18	Ibc	43	KSP43	Ibc	68	SPOF18J4	Ia
19	KSP19	Ibc	44	KSP44	Ia	69	SPOF18J5	Ia
20	KSP20	Ibc	45	KSP45	Ia	70	SPOF18J6	Ibc
21	KSP21	Ibc	46	KSP46	Ia	71	SPOF18J7	Ibc
22	KSP22	Ibc	47	KSP47	Ia	72	SPOF18J8	Ia
23	KSP23	Ibc	48	KCTC 11537	-	73	SPOF18J9	Ia
24	KSP24	Ibc	49	PH0710	Ia	74	SPOF18J10	Ia
25	KSP25	Ibc	50	PH0711	Ia	75	SPOF18J11	Ibc

* N.: Serial number of strains; listed in the Figure 1-2.

Figure 1-1. Distribution of 75 *S. parauberis* serotypes according to (A) isolation year and (B) isolation region.

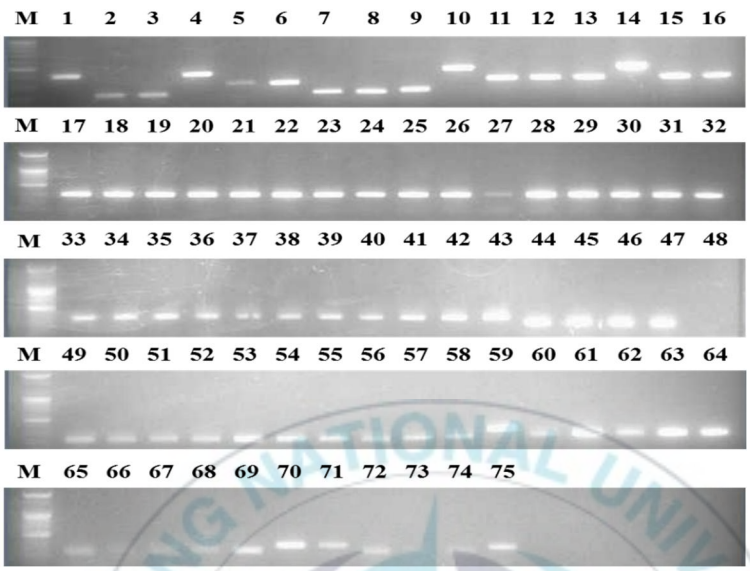

Figure 1-2. PCR results for serotyping of 75 *S. parauberis* strains: M, 100 bp DNA ladder; Numbers (1-75) are listed in column N. in Table 1-1.

3.2. Pathogenicity test

Data for pathogenicity test using 15 selected *S. parauberis* strains are shown in Figure 1-3. Average cumulative mortality was from 13.33% (KSP10) to 100% (HFTC0064). Fish in the control group (Negative control) did not show any mortality. There is a clear tendency that pathogenicity of serotype Ia strains is the highest, followed by Ibc and II. In particular, pathogenicity of serotype Ia strains isolated in recent years (between 2013 and 2015) was higher than other Ia strains.

Although starry flounder derived strain PH0710 isolated in 2007 showed comparatively lower degree of pathogenicity. In contrast, three strains (HFTC0083, HFTC0086 and HFTC0157) derived from starry flounder showed high pathogenicity.

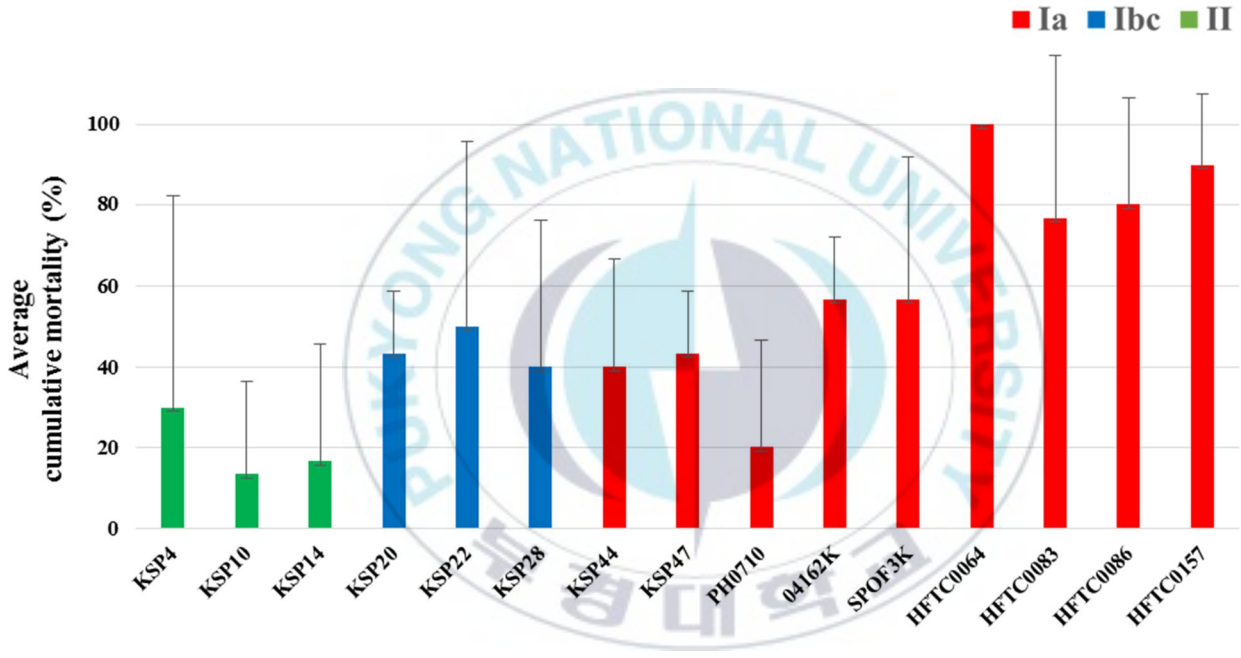

Figure 1-3. Average cumulative mortality of fifteen *S. parauberis* strains in olive flounder.

3.3. Bacterial survival in olive flounder serum

Five *S. parauberis* strains (SPOF3K, HFTC0064, KSP20, KSP10 and KSP14) used in this study were able to survive and grow in olive flounder serum for six hours (Figure 1-4). Strain SPOF3K in the serum multiplied more rapidly than other tested strains (Table 1-5).

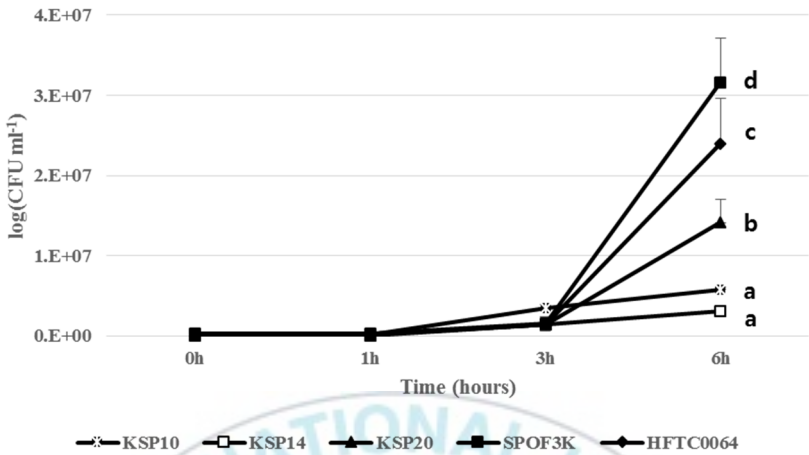

Figure 1-4. Survival of five *S. parauberis* strains cultured in the serum of olive flounder

Table 1-6. Relative growth of *S. parauberis* (CFU) in the serum of olive flounder at 6 hr compared to 0 hr

Strain	**6 hr / 0 hr**
KSP10	x 47.9[a]
KSP14	x 56.3[a]
KSP20	x 60.9[a]
SPOF3K	x 115.7[c]
HFTC0064	x 88.1[b]

3.4. Comparative genome analysis

3.4.1. General feature of genome sequences

The whole genome of five *S. parauberis* strains (SPOF3K, HFTC0064, KSP20, KSP10 and KSP14) were sequenced and *de novo*-assembled in complete level without any gaps or partial contigs. The general features of genome sequences are shown in Table 1-6. Briefly, five genome were 2.08 ~ 2.26 mega-base pairs (Mbp) in size with 35% average G+C contents, harboring 2020 ~ 2325 protein coding sequences (CDS), 68 ~ 70 tRNAs and 18 rRNAs. The sequencing depth of coverage was high enough (> 572.26 x) to ensure the reliable quality of genome sequences (Chun et al., 2018).

Of note, a 1.3 kilo-base pair sized plasmid harboring thirteen CDSs was predicted in the genome of SPOF3K and HFTC0064, serotype Ia strains. These genes were also identified in genome of KRS02083 and KCTC11980, serotype Ia strains, which indicates that they also harbor this identical plasmid in their genome (The assembly status of these two genomes is not complete, hence the plasmid was not

identified).

To clarify the correlation between the presence of the plasmid and serotypes, PCR verification was performed on three genes (*inlJ*, *mobA* and *tetS*) that are harbored in for 75 strains used in this study plasmid (Table 1-4 and Table 1-8). As a result, only serotype Ia strains (n = 33) were found to harbor these three genes, indicating the presence of a plasmid only in this serotype.

Predicted CDS of five *S. parauberis* genomes were assigned to COG functional categories based on the annotation (Table 1-8). The number of genes assigned in the functional categories did not show considerable differences within strains, except for the number in the category of replication, recombination and repair. Genes of SPOF3K (176) and HFTC0064 (180) assigned to this category substantially outnumbered those of KSP20 (125); KSP10 (136) KSP14 (135). Most of genes were involved in transposable elements including insertion sequences and transposons. As reported previously, CRISPR / Cas9 system was identified in only serotype II strains (KSP10 and KSP14) (Nho et al., 2013).

Table 1-7. General features of genome sequences performed in this study.

Strain	**SPOF3K**			**HFTC0064**			**KSP20**	**KSP10**	**KSP14**
Genome	Total	Chromosome	Plasmid	Total	Chromosome	Plasmid	Chromosome	Chromosome	Chromosome
Size (bp)	2,141,524	2,128,737	12,787	2,159,181	2,146,395	12,786	2,225,704	2,081,310	2,081,053
Average G+C contents (%)	35.66	35.67	34.39	35.64	35.65	34.31	35.6	35.61	35.61
Coding DNA sequences	2137	2124	13	2155	2142	13	2325	2020	2020
tRNA	69			69			70	68	68
rRNA	18			18			18	18	18
Sequencing depth of coverage	579.76 x			586.23 x			583.58 x	615.07 x	572.26 x
N50 (bp)	2,128,737			2,146,395			2,169,433	2,081,310	2,081,053
Median of CDS length (bp)	771			768			750	795	795
Mean length of intergenic region	119			119.2			114.7	123.6	123.6

Table 1-8. Annotation of 12 Kbp-sized plasmid in genome sequence of *S. parauberis* SPOF3K and HFTC006

Gene locus		Annotation
SPOF3K (SPSF3K_)	**HFTC0064 (SPHC0064_)**	
02212	02230	Internalin-J
02213	02231	Tetracycline resistance protein *TetS*
02214	02232	hypothetical protein
02215	02233	hypothetical protein
02216	02234	Replication initiator protein *RepB*
02217	02235	Replication initiator protein *RepB*
02218	02236	Adenosine monophosphate-protein transferase *NmFic*
02219	02237	Mobilization protein *mobB*
02220	02238	Mobilization protein *mobA*
02221	02239	Mobilization protein *mobC*
02222	02240	Transposase for insertion sequence element IS*257* in transposon Tn*4003*
02223	02241	Ribosomal-protein-alanine N-acetyltransferase
02224	02242	Transposon Tn*552* resolvase

Table 1-9. Functional eggNOG distribution of CDS in five *S. parauberis* genomes.

Gene category	Number of CDS				
	SPOF3K	**HFTC0064**	**KSP20**	**KSP10**	**KSP14**
Translation, ribosomal structure and biogenesis	154	155	150	154	154
Transcription	150	152	156	147	147
Replication, recombination and repair	176	180	125	136	135
Cell cycle control, cell division, chromosome partitioning	19	19	16	18	18
Defense mechanism	53	55	49	52	53
Signal transduction mechanism	50	50	46	43	43
Cell wall/membrane/envelope biogenesis	96	96	95	90	90
Cell motility	3	3	5	3	3
Intracellular trafficking, secretion, and vesicular transport	28	28	24	26	26
Posttranslational modification, protein turnover, chaperones	63	63	60	59	59
Energy production and conversion	73	73	72	69	69
Carbohydrate transport and metabolism	204	205	214	216	216
Amino acid transport and metabolism	137	137	144	143	143
Nucleotide transport and metabolism	75	75	69	70	70
Coenzyme transport and metabolism	42	42	42	42	42
Lipid transport and metabolism	49	48	50	50	50
Inorganic ion transport and metabolism	109	111	100	108	108
Secondary metabolites biosynthesis, transport and catabolism	13	13	13	14	14
Function unknown	529	533	570	506	506

3.4.2. Genome identification and phylogenomic analysis

Average nucleotide identity (ANI) between genomes of *S. parauberis* strains used in this study showed more than 98.43%, while ANI between *S. parauberis* and other closely related species strains (e.g., *S. uberis* and *S. iniae*) were lower than approximately 80% (Figure 1-5).

The phylogenetic tree constructed based on 1412 core genes from ten fish derived *S. parauberis* strains from Asia (Including our strains) showed clear clustering into three groups with correspondence to serotypes (Figure 1-7A).

Hierarchical clustering based on binary analysis using pan-genome of ten fish-derived strains originated from Asia also showed clustering into three distinct groups (Figure 1-6). Serotype Ia and Ibc strains showed close relationship in phylogenetic analysis, while serotype Ia strains were separated in the hierarchical clustering analysis.

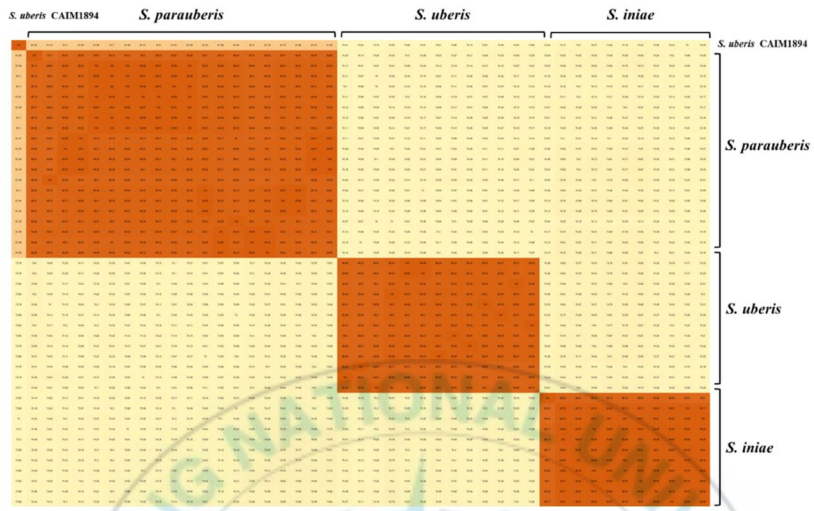

Figure 1-5. Average nucleotide identity (ANI) using genome sequences of *S. parauberis* strains and closely related species (*S. iniae* and *S. uberis*)

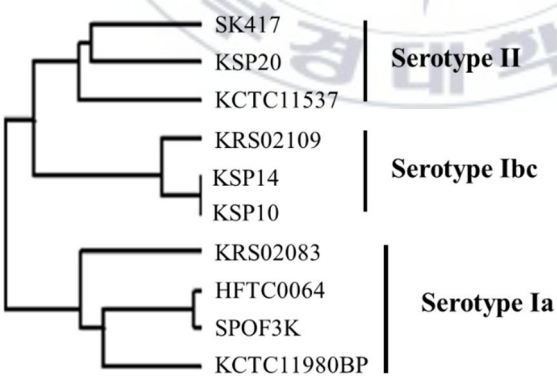

Figure 1-6. Hierarchical clustering based on binary analysis of pan-genome of ten fish derived strains.

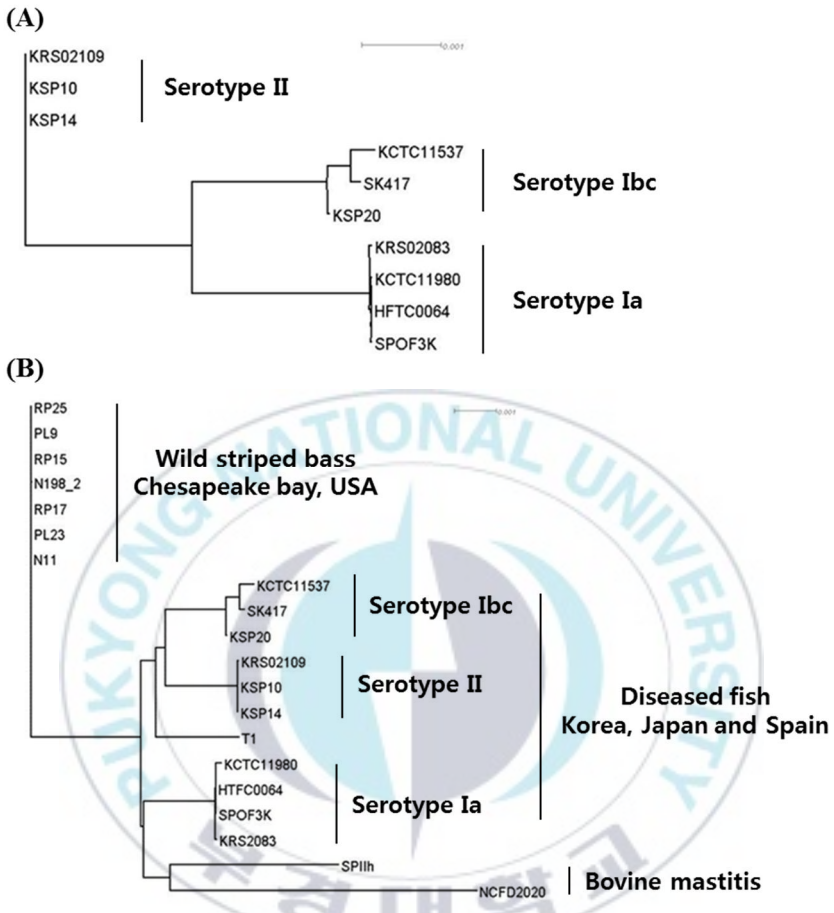

Figure 1-7. Phylogenetic tree based on (A) 1412 core genes of ten *S. parauberis* strains derived from fish in Asia, and (B) 1300 core genes of 20 *S. parauberis* strains.

3.4.3. Virulence factors

Five strains (HFTC0064, SPOF3K, KSP20, KSP10 and KSP14) were used to identify virulence-related genes. Based on virulence factor database (Chen et al., 2015), a total of 269 virulence-related genes were extracted from all the 5 strains, and 223 genes were present in common (Figure 1-8).Twelve, eight and nine genes were uniquely found in serotype Ia, Ibc and II strains, respectively. Five, Eight and four genes were shared between Serotype Ia / Ibc, Ibc / II and Ia / II, respectively. In particular, genes present only in high virulent strains (HFTC0064; SPOF3K) and intermediate strains (KSP20) were focused on to analyze correlation with its pathogenicity (Table 1-9).

PCR verification was performed on three genes (*inlJ*, *oatA* and BCS) that are only presence in serotype Ia genomes for 75 strains used in this study (Table 1-4 and Table 1-10). As a result, only serotype Ia strains (n = 33) were found to harbor these three genes clarifying that these genes are uniquely distributed only in serotype Ia *S. parauberis* strains.

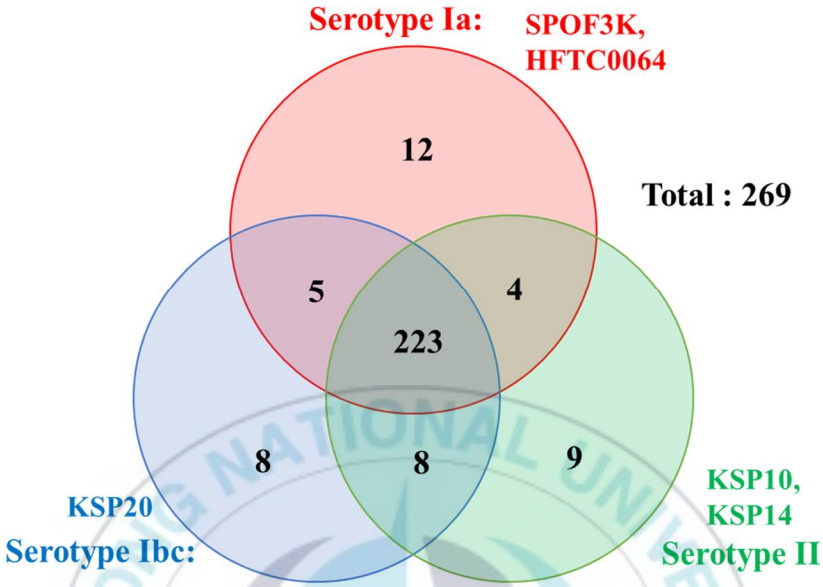

Figure 1-8. Distribution of identified virulent-related genes in genome of five strains.

Table 1-10. Predicted virulence related genes present only in serotype Ia and Ibc.

Locus		Annotation based on Virulence Factor DataBase	e-value
Serotype Ia	Serotype Ibc		
SPSF3K_01374	-	(*oatA*) peptidoglycan O-acetyltransferase	8E-25
SPSF3K_01483	-	(*lytA*) Autolysin (N-acetylmuramoyl-L-alanine amidase)	9E-65
SPSF3K_01565	-	(*cap8N*) capsular polysaccharide synthesis enzyme Cap8N	8E-51
SPSF3K_01699	-	(*hitC*) iron(III) ABC transporter, ATP-binding protein	2E-22
SPSF3K_01703	-	(*bfmR*) biofilm-controlling response regulator	2E-26
SPSF3K_01903	-	(*clbF*) colibactin biosynthesis dehydrogenase ClbF	3E-16
SPSF3K_01915	-	(*mgtB*) Mg2+ transport protein [MgtBC]	1E-14
SPSF3K_01917	-	(*cylA*) ABC (ATP-binding cassette) transporter CylA [Beta-hemolysin/cytolysin]	2E-27
SPSF3K_02074	-	N-acetylglucosaminyltransferase, involved in polysaccharide intercellular adhesion (PIA)	5E-11
SPSF3K_02077	-	synthesis [Intercellular adhesion proteins]	3E-50
SPSF3K_02147	-	(*cbpD*) choline binding protein D [CBPs]	E-21
SPSF3K_02212	-	Internalin-J	4E-31
SPSF3K_00488	SPK20_02030	(*mgtB*) Mg2+ transport protein [MgtBC]	2E-14
SPSF3K_01556	SPK20_00933	(*tviC*) Vi polysaccharide biosynthesis protein, epimerase TviC [Vi antigen]	7E-33
SPSF3K_01562	SPK20_00939	(*cps4H*) capsular polysaccharide biosynthesis protein Cps4H	3E-42
SPSF3K_01564	SPK20_00940	(*wbbO*) glycosyltransferase family 1 protein	3E-28
SPSF3K_01566	SPK20_00942	(*cps4E*) capsular polysaccharide biosynthesis protein Cps4E	1E-59
-	SPK20_00540	(*gtrB*) bactoprenol glucosyl transferase	2E-43
-	SPK20_00603	(*iroC*) ABC transporter [Sal]	4E-13
-	SPK20_00941	(*neuD*) neuD protein [Capsule]	9E-21
-	SPK20_00943	(*per*) perosamine synthetase [LPS]	3E-39
-	SPK20_01279	(*nagI*) hyaluronidase [mu-toxin]	2E-50
-	SPK20_01602	(*cylG*) 3-ketoacyl-ACP-reductase CylG [Beta-hemolysin/cytolysin]	1E-30
-	SPK20_01603	(*hitC*) iron(III) ABC transporter, ATP-binding protein [HitABC]	5E-25

3.4.4. Antibiotic resistance genes

Ten *S. parauberis* strains (HFTC0064, SPOF3K, KCTC19980BP, KRS02083, KSP20, KCTC11537, SK417, KSP10, KSP14, and KRS02109) were used to identify antibiotic resistance genes, and the results are shown in Figure 1-9. Serotype Ia strains (HFTC0064, SPOF3K, KCTC19980BP and KRS02083) harbored four to six antibiotic resistance genes. Tetracycline resistance gene, *tetS* (Roberts et al., 1996), was located in a plasmid of serotype Ia strains (Table 1-7). Also, Macrolide / Lincosamide / Streptogramin resistance gene, *ermB* (Min et al., 2008), and aminoglycoside resistance gene, ANT(6)-*Ia* (Gill et al., 2005) were located only in the chromosome of serotype Ia strains. Strain KCTC19980BP uniquely harbored three more antibiotic resistance genes (*isaE*, *inuB* and ANT(9)-*Ia*) in addition to *tetS*, *ermB*, ANT(9)-*Ia*. None of antibiotic resistance genes were detected in serotype Ibc strains (KSP20, KCTC11537 and SK417). Serotype II strains (KSP10, KSP14 and KRS02109) harbored one antibiotic resistance gene, *tetM* (Roberts et al., 1996) in their chromosome.

PCR verification was performed on four antibiotic resistance-related genes (*tetM*, *tetS*, *ermB* and ANT(6)-*Ia*) for 75 strains used in this study (Table 1-4 and Table 1-9) to clarify the relationship between the distribution of antibiotic resistance genes and serotypes. The result from PCR assay was corresponding to the result mentioned above (Figure 1-9).

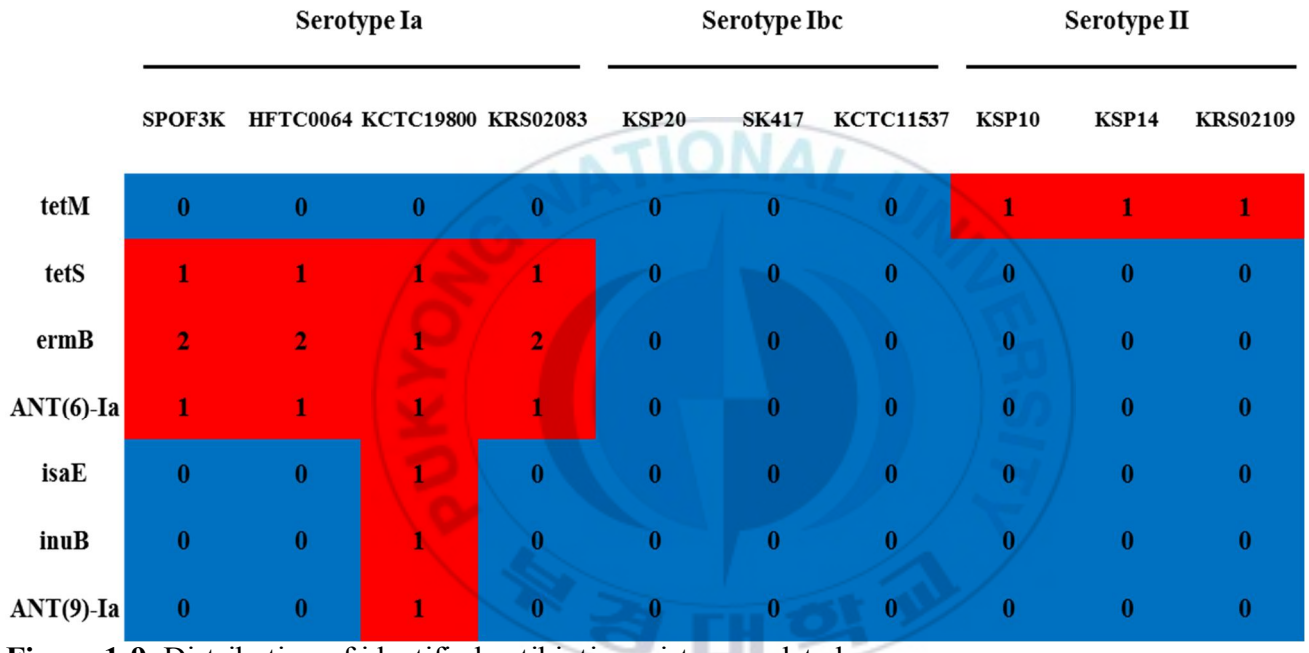

Figure 1-9. Distribution of identified antibiotic-resistance related genes

4. Discussion

Most of 75 *S. parauberis* strains used in this study were classified as serotype Ia regardless of its origin except for strains obtained in 2005 (Figure 1-3). It is important to note that serotype II strains has not been isolated since 2005. Monitoring of serotypes of *S. parauberis* will be needed to determine what type is dominating in recent years.

For pathogenicity, like former studies (Han et al., 2011 and Woo et al., 2013), serotype II strains showed relatively lower virulence compared to other strains. Of note, serotype Ia strains isolated from 2013 to 2015 were more pathogenic to olive flounder. Two strains (KSP44 and KSP47) isolated in 1999 showed intermediate degree of pathogenicity. These results indicate that recent strains of Ia type might have experienced genetic evolution to better adapt in olive flounder, resulting in higher pathogenicity (Diard et al., 2017). Another serotype Ia strain (PH0710) showed relatively lower degree of pathogenicity. A previous study (Woo et al., 2013) argued that this strain might have possible host-specific virulence as it was

higher pathogenic to starry flounder and lower pathogenic to olive flounder. Indeed, in this study, three strains (HFTC0083, HFTC0086 and HFTC0157) derived from starry flounder were highly pathogenic to olive flounder, and thus host specificity might not exist and/or cannot be concluded.

Serotype Ia strains (HFTC0064 and SPOF3K) showed remarkable ability to adapt and grow in olive flounder serum than that of other serotypes (Figure 1-7). It is important to note that all *S. parauberis* strains used in this study were able to resist and multiply in the serum, indicating that they all harbor factors to resist against immune responses of olive flounder. In addition to phenotypic characteristics of different serotypes of five *S. parauberis* strains obtained from *in vivo* and *ex vivo* model, analysis of differential genomic traits of the five strains will contribute to the understanding of virulence mechanism in olive flounder.

In this study, it was confirmed that only serotype Ia (SPOF3K and HFTC0064) harbored a 12 Kbp sized plasmid. This plasmid contains essential genes for plasmid transfer including mobilization

proteins (MobA, MobB and MobC), replication initiator protein RepB and replicator associated proteins (van Zyl et al., 2003). Any plasmids showing more than 41% of nucleotide sequence query cover were not detected within other species in public database at present (*Lactobacillus helveticus* strain FAM22155 plasmid pFAM22155; Nucleotide sequence identity = 92%; Accession: CP015499.1) (http://ftp.ncbi.nih.gov/), indicating that this plasmid might be genetically conserved in *S. parauberis* serotype Ia strains. This plasmid harbored Internalin J, which was previously recognized as a virulence factor in *Listeria monocytogenes,* encoding a protein mediating internalization into host tissue and cells (Sabet et al., 2005) (Figure 1-5 and Table 1-7).

Phylogenetic analysis at whole-genome level showed that ten fish derived *S. parauberis* strains isolated in Asian countries were clustered into three groups, which clearly corresponded with their serotypes (Figure 1-6). In line with this, Kanai et al. (2015) suggested the existence of three pulsotypes in *S. parauberis* based on PFGE corresponded with serotypes of *S. parauberis* (Pulsotype I, II and III corresponded with serotype Ibc, Ia and II respectively).

Therefore, it is proposed that *S. parauberis* strains derived from diseased fish can be divided into three genotypes: Genotype I (Serotype Ia strains, SPOF3K; HFTC0064; KRS02083 and KCTC11980), Genotype II (Serotype Ibc strains, KSP20; SK417 and KCTC11537) and Genotype III (Serotype II strains, KSP10; KSP14 and KRS02109).

Table 1-11. Three genotypes of *S. parauberis* proposed in this study

Genotype (Serotype)	Strain	Isolation origin		
		Host	Region	Year
Genotype I (Serotype Ia)	KRS02083	Olive flounder	Japan	2002
	KCTC11980	Olive flounder	Korea	2010
	SPOF3K	Olive flounder	Korea	2013
	HFTC0064	Olive flounder	Korea	2014
Genotype II (Serotype Ibc)	KSP20	Olive flounder	Korea	2005
	KCTC11537	Olive flounder	Korea	2006
	SK-417	Rock fish	Japan	2013
Genotype III (Serotype II)	KRS02083	Olive flounder	Japan	2002
	KSP10	Olive flounder	Korea	2003
	KSP14	Olive flounder	Korea	2004

However, the phylogenetic analysis including strains derived from udder mastitis or fish in USA and Spain showed more variant phylogenetic diversity, indicating the presence of additional genotypes. Further studies are needed to compare the difference between Asian strains analyzed in this study and others in phenotypic characteristics, along with genomic features.

In hierarchical clustering based on bianary data from pan-genome, genotype II and III showed closer relationship (Figure 1-6), whereas genotype I and II strains clustered closer in phylogenetic analysis (Figure 1-7A). Phylogenetic analysis provide understanding on evolutionary relationship, while hierarchical clustering better reflects differences in genomic contents (Presence/absence) mediated with major genetic rearrangement events in intra-species level (Douillard et al., 2013). Genotype I strains of *S. parauberis* harbored a conjugative plasmid and considerably higher number of genes in replication, recombination and repair category mainly composed of transposable elements (Table 1-8 and Table 1-9). Therefore, our result shows that genotype I is different from other genotypes in terms of genomic contents and arrangements, even

though genotype III (not genotype I) is evolutionary more distinct from other genotypes.

The horizontal gene transfer (HGT) is one of the most key issues in understanding microbial genetic diversity, evolution, ecological and pathogenic characters (Ochman et al., 2000). HGT occurs in three major pathways, conjugation (Lederber and Tatum, 1946), transformation (Avery et al., 1944) and transduction (Zinder and Lederberg, 1952). CRISPR / Cas9 system, a bacterial adaptive immunity against bacteriophage (Ran et al., 2013) was identified only in serotype II strains (KSP10 and KSP14). This system provides bacterial protection from bacteriolysis by viruses, while it is reported to limit HGT, specifically when the prophage decays through accumulation of mutants (Marraffini et al., 2008 and Garneau et al., 2010). Hence, the presence of CRISPR / Cas9 system in serotype II strains might have brought adverse effect in terms of genetic diversity and rapid evolution for better adaptation in hostile environment of the host.

Virulence factors in microorganisms are defined as ‘factors that mediate the relative capacity of microorganism to cause damage in a host’ (Casadevall et al., 2003). In this study, virulence factors of high and low pathogenic *S. parauberis* strains were identified and compared as shown in Table 1-10. Among 269 genes identified as virulence factors from the genome of five strains in this study, 223 were found to be core genes, the majority of which were well-known and -studied in various bacterial species. M-like protein and hyaluronic acid capsule encoding genes are major virulence factors in Group A Streptococci (GAS) (Moses et al., 2004), and they were found in five strains used in this study. All the five strains harbored sortase A, encoding cell wall anchoring protein (Paterson et al., 2004), as well as *lgt* and *lspA*, lipoprotein processing enzymes (Buddelmeijer et al., 2015). Also, several genes encoding as extracellular matrix binding proteins including laminin- (Tenenbaum et al., 2007) plasminogen- (D’Costa et al., 1997) and fibrinogen- binding proteins (Hasty et al., 1989) were found to be core. GAPDH (Boradia et al., 2014), enolase (Zhang et al., 2009), and elongation factors (Widjaja et al., 2007), which are

moonlighting proteins with multiple biological functions, were also found to be in all the strains. In addition, GroESL and DnaJ-DnaK-GrpE, bacterial major heat-shock chaperones (Roncarati et al., 2017) and Clp proteases (Bosl et al., 2006), major stress-related proteases were identified as a virulence factor as well.

Twelve and eight virulence related genes were found only in serotype Ia strains (SPOF3K and HFTC0064) and only in serotype Ibc strains (KSP20), respectively (Table 1-10). Both Ia and Ibc strains commonly possessed five virulence related genes. One of the major differences between Ia and Ibc type was capsular polysaccharide (CPS) operon, found only in Ia type of *S. parauberis*. Indeed, capsular polysaccharide have been reported as virulence factors with and serotype determinants various streptococcal species (Marques et al., 1992; Kim et al., 1998; Llull et al., 2001; Lowe et al., 2007 and Kanai et al., 2015). Capsule of *S. parauberis* is reported to have the ability to resist the bactericidal of the serum and resist phagocytic activities of macrophages in olive flounder (Hwang et al., 2008). In particular, Han et al (2011) showed that serotype I strains had thicker acidic polysaccharide capsule than that

of serotype II strains. Therefore, uniquely identified CPS related genes of *S. parauberis* would be crucial in virulence as well as antigenic properties.

Three ATP binding cassette (ABC) transporters (SPSF3K_01699; SPSF3K_01915 and SPSF3K_01917) were identified as a virulence factor uniquely in high virulent strains. As ABC transport system regulates physiological balance of microorganisms by importing / exporting essential biological substances, its importance for survival in hostile environment and bringing full virulence into host is emphasized in numerous studies (Davidson et al., 2004 and Chimalapati et al., 2012).

Peptidoglycan O-acetyltransferase encoding gene, *oatA* (SPSF3K_01374) which was found only in high virulent strains (Table 1-10) provides resistance to lysozyme, one of the most important and widespread compounds of the constitutive defense system in host (Bera et al., 2005). Many pathogenic bacterial species including *Staphylococcus aureus*, *listeria monocytogenes*, and *Neisseria gonorrhoeae* are resistant against lysozymes, and OatA is

regarded as the most critical lysozyme-resistance mediating factors (Bera et al., 2006; Moynihan et al., 2010 and Aubry et al., 2011). By modification of C6-OH group of muramic acid with O-acetylation, it serves as inhibitor for lysozyme as well as cell-wall associated antibiotics and autolysins, clearly attenuating the cell wall lysis (Bera et al., 2006). Studies using gene knockout mutants have not only proved its importance in resistance to cell wall lysis, but also in pathogenicity (Crisóstomo et al., 2006; Moynihan et al., 2010 and Aubry et al., 2011). Therefore, this gene would be one of powerful candidates for major virulence factors in *S. parauberis* strains.

Choline-binding protein encoding gene, *cbpD* (SPSF3K_02147) was uniquely found in virulent strains. Choline-binding proteins contain a number of short choline-binding-repeats which anchor them noncovalently to teichoic and lipoteichoic acid in pneumococcal cell wall (Swiatlo et al., 2004). Previous studies have demonstrated its contribution to virulence in adherence, colonization, immunogenicity, autolysis and DNA competences (Rosenow et al., 1997; Gosink et al., 2000 and Kausmally et al., 2005). Autolysin encoding gene, *lytA* (SPSF3K_01483) was also

uniquely found in virulent strains with several studies reporting that LytA acts together with CbpD to degrade the cell wall undergoing lysis during competence and hence release intracellular substances including its DNA to induce competence and toxins to induce inflammations in host (Gosink et al., 2000 and Kausmally et al., 2005). Based on our genomic finding of these two uniquely found genes in virulent strains, they would be one of the key factors in virulence of *S. parauberis*.

Bacterial biofilm organizes communities of aggregated cells, facilitating the bacteria to better survive better in hostile environments and to colonize in new niches by various dispersal mechanisms (Gupta et al., 2016). This microbial glue is formed with extracellular polymeric substances comprised of variety of high-weight polymers (Flemming et al., 2000 and Staudt et al., 2004). Based on BLAST search in VFDB, two genes (SPSF3K_02074 and SPSF3K_2077) that were uniquely found only in high virulent strains were predicted as Intercellular adhesion protein and N-acetylglucosaminyltransferase which involves in polysaccharide intercellular adhesion (PIA) synthesis. PIA or poly-N-

acetylglucosamine (PNAG) acts as adhesins and are required for microbial biofilm formation in pathogenic Staphylococcal species (Mack et al., 1996) as well as well-known Gram negative pathogens (Darby et al., 2002 and Wang et al., 2004). With the pathway analysis using KEGG and other functional databases, the former gene (SPSF3K_02074) was predicted as bacterial cellulose (BC) synthase. BC is one of the most major and well-known components of EPS (Ross et al., 1991 and Augimeri et al., 2015). Several studies have revealed loss of BC production leads to decreased adhesion ability, biofilm formation and multicellular behaviors (Da re and Ghigo, 2006 and Lapidot and Yaron, 2009). BC synthesis is positively regulated with the concentration of cyclic-di-GMP, which again is generated and degraded by Diguanylate synthase (Dgc) and Phosphodiesterase (PdeA) respectively (Figure 1-10B) (Gupta et al., 2016). In this study, a gene cluster composed of seven genes (SPSF3K_02074 ~ 02080) were identified to participate in bacterial BC synthesis (Figure 1-10A) gene cluster comprised with seven genes including BC synthase (BCS; SPSF3K_02074), PNAG synthase (*pga*; SPSF3K_02077) and Diguanylate synthase /

Phosphodiesterase (*dgc / pdeA*; SPSF3K_02080). This gene cluster differed from any of studied BC synthesis operons reviewed in Römling and Michael (2015) and three genes in this cluster did not show any significant sequence similarity in public databases. Hence, identification of this gene cluster might be important finding in terms of not only importance as key virulence factor of *S. parauberis*, but also a proposal of novel BC synthesis-related gene cluster type. However, further experimental studies are still needed to functionally characterize and comprehensively understand this finding.

As a result of antibiotic gene identification, genotype I strains harbored at least three antibiotic resistance genes targeting tetracycline (*tetS*), Macrolide / Lincosamide / Streptogramin (*ermB*), and Aminoglycoside (ANT(6)-*Ia*), while genotype II and III strains did not harbor any genes except for *tetM* in genotype III. (Table 1-7). Meng et al. (2009) reported that five out of 44 serotype I strains of *S. parauberis* harbored *ermB* and *tetS*, and 20 out of 20 serotype II strains possessed only *tetM* gene, which corresponded to the genomic findings in this study. It is considered that the five resistant

serotype I strains are genotype I strains, while non-resistant 39 serotype I strains are genotype II strains based on genomic evidences found in this study. Antimicrobial susceptibility test using *S. parauberis* strains showed that genotype I strains were usually multidrug resistant than others genotypes, indicating that more virulent strains are more resistance against antibiotics and thus there might be difficulties in treating the infection in the field.

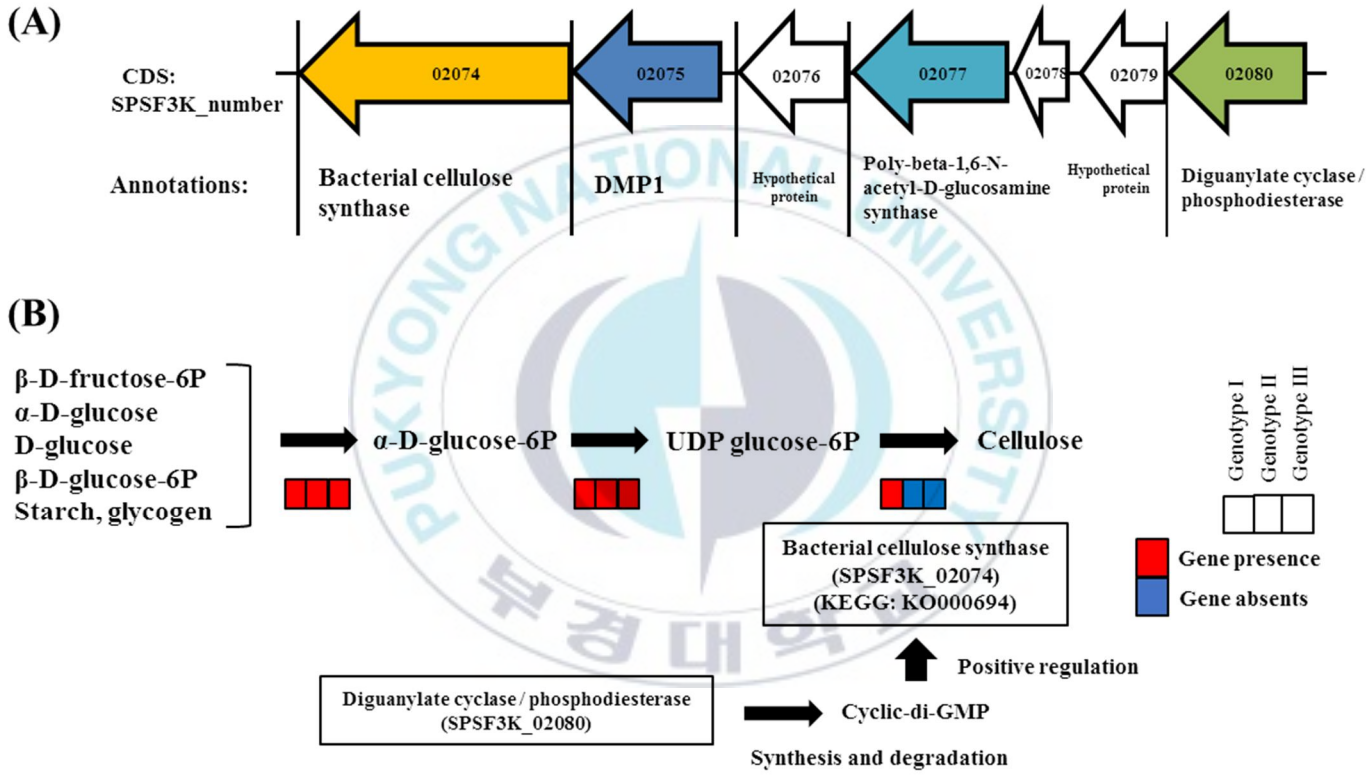

Figure 1-10. Bacterial cellulose synthesis (A) coding gene clusters (B) pathway

5. Conclusion

In this chapter, the genomic differences in *S. parauberis* high and low virulent strains were identified using comparative genomics. Serotype Ia strains showed relatively higher degree of pathogenesis in olive flounder and better resistance against olive flounder serum.

Phylogenetic analysis revealed three distinct genotypes in fish derived *S. parauberis* in Asia which corresponded with the serotypes. Importantly, genotype I strains harbored 12 Kbp plasmid containing tetracycline resistance gene and Internalin J, a virulence factor. Also, genotype I strains harbored at least three antibiotic resistance-related genes while II and III contain none and only one. Finally, key virulence factors which presence in virulent strains, but not in low strains were identified including bacterial cellulose synthase, peptidoglycan O-acetyltransferase and Internalin J.

In the following chapter, the important findings in this chapter are screened in mRNA level with bacterial transcriptome analysis in olive flounder serum environment, revealing the survival strategy of *S. parauberis*.

Chapter II. Understanding survival strategy of *Streptococcus parauberis* with global transcriptome profiling

1. Introduction

Gene expression technologies have been one of the most powerful tools in functional genomics, providing insights into normal cellular processes as well as disease pathogenesis for the last decades (Ritchie et al., 2015). In recent years, genome-wide transcriptome assays are increasingly being performed by high-throughput RNA sequencing methods not only in eukaryotic organisms, but also in various pathogenic microorganisms (McClure et al., 2013).

The ex-vivo model using bacteria in various environment such as heparinized blood, fresh serum, milk, and saliva has been widely used to evaluate global bacterial transcriptome, providing better understanding on bacterial survival and immune evasion mechanism (Graham et al., 2005; Camejo et al., 2009; Mereghetti et al., 2008; Malachowa et al., 2011; Li et al., 2011; Richards et al., 2013; Verhagen et al., 2014 and Williams et al., 2014). Specifically, bacteria in serum could face first hostile environment as nutrition and free iron concentration are extremely limited, and also various humoral immune factors, including complements, opsonins,

lysozymes, antitoxins, bacteriolysins, bacterial agglutinins, and antimicrobial peptides (Taylor et al., 1983 and Li et al., 2011). In recent years, several studies using important human pathogen, *Vibrio vulnificus, Staphylococcus aureus* and *Escherichia coli* have revealed global bacterial transcriptome profiles in relation with their survival/resistance in serum (Malachowa et al., 2011; Li et al., 2011 and Williams et al., 2014).

In this chapter, global gene expression profile of *S. parauberis* cultured in olive flounder serum was monitored to provide sufficient data for better understanding on survival and growth strategy of this bacterial pathogen. Together with virulence factors found by genomic approaches in the previous chapter, transcriptome analysis will provide insights into pathogenicity of *S. parauberis* in olive flounder.

2. Material and methods

2.1. Experimental design and culture conditions

S. parauberis strain SPOF3K was used for transcriptome analysis in this study. Bacteria were cultured in BN for 18 hours at 160 rpm at 26°C. Bacterial cells were harvested at early-stationary phase and washed with PBS by centrifugation for 10 min at 6000 rpm in room temperature. The bacterial pellet was re-suspended in PBS and adjusted to 2 x 10^9 CFU ml^{-1}. Two milliliters of bacterial suspension was mixed with the same amount of BN medium or olive flounder serum, and they were incubated for four hours at 160 rpm at 26°C in triplicate. Olive flounder blood was drawn from healthy one-year-old fish weighing approximately 100g with minimum anesthesia using MS-222 and serum was separated from the clot by centrifugation at 6500rpm at 4°C. At 0, 1, 2 and 4 hours post incubation (hpi), bacterial viable counts were measured by plate counting method: one hundred microliters of serially diluted cultures were plated onto BN at 26°C. The bacterial pellet was collected by centrifugation at 6000 rpm in room temperature.

2.2. RNA-sequencing

2.2.1. RNA isolation and cDNA library construction and sequencing

Total RNA was extracted from bacterial pellet using RiboPureTM Bacteria kit (Ambion Life technologies, Grand Island, NY, USA) according to the manufacturer's instruction with a modification; mechanical cell wall degradation time was doubled for the better yield of total RNA. Samples were treated with DNase I kit (Ambion Life technologies) for removal of traced-genomic DNA. RNA concentration and quality were determined using Qubit 3 Fluorometer and RNA High Sensitivity Assay kit (Invitrogen, Carlsbad, CA, USA), and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), respectively. Ribosomal RNA in samples were depleted using Ribo-zero-rRNA Removal kit (Epicentre, Madison, WI, USA). cDNA libraries were constructed using the TruSeq RNA Sample Prep Kit (Illumina, Sand Diego, CA) (Insertion size > 250 bp) and sequenced using the Illumina Hiseq 2500 (1 x 50 bp paired-end reads).

2.2.2. Sequence data processing and differential gene expression

Raw Illumina sequence reads were evaluated and trimmed using FastQC (Andrews et al., 2010) for removal of low-quality-reads. The remaining filtered reads were mapped onto reference SPOF3K genome using Bowtie2 (Langmead et al., 2012). Limma/Voom R package (Ritchie et al., 2015) was implemented for data normalization and differential gene expression (DGE) analysis by uploading the read counts for each gene into Degust (www.vicbioinformatics.com/degust). Statistical significance of DGE was calculated based on the false discovery rate (FDR, significance at < 0.005) of Benjamini and Hochberg (Benjamini et al., 1995).

Transcripts of broth-cultured samples were set as control for serum-cultured samples at every sampling time point. In addition, differentially expressed genes were assigned into functional categories based on Evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) database.

2.3. Quantitative-Reverse Transcriptase PCR

2.3.1. cDNA preparation

Quantitative reverse transcription-PCR (qRT-PCR) for four selected genes was conducted to validate the expression levels of RNA sequencing. Total RNA (1 ug for each) used for RNA-seq was reverse-transcribed using random hexamers (Bioneer, Daejeon, Korea) and moloney murine leukemia virus (MMLV) reverse transcriptase (Bioneer, Daejeon, Korea) according to manufacturer's instruction.

2.3.2. Primers

Primer sets used in qPCR were designed based on genome sequence of strain SPOF3K, using Primer3Plus (Untergasser et al., 2012) and in silico PCR at www.insilico.ehu.es/ (Bikandi et al., 2004). The primer pairs are listed in Table 2-1. The primer efficiency was evaluated with gel electrophoresis, Sanger sequencing analysis and standard curve assay as described by Schmittgen et al. (2000).

Table 2-1. Primers designed for real-time PCR

Target	Locus	Sequence information
ClpE	SPSF3K_01798	F- ATTTTGAGCCGGCTTCATCG
		R- AGCGCGTGGTATCAAAACAG
GroEL	SPSF3K_00142	F- AGAAGCCATTGCGCAAGTTG
		R- ACACCATCATTGCCAACACG
Cu(+) exporting ATPase	SPSF3K_02010	F- AGCGCGTGGTATCAAAACAG
		R- ATCCCAGCTGTTGTTGATGC
DnaK	SPSF3K_02051	F- TCAATTCCAACAGCGTCAGC
		R- TTGAATTGCAGCACCCATCG
Housekeeping gene		
50S ribosomal protein L17 *rplq*	SPSF3K_02101	F- AATCGCACCTCGTTATGCTG
		R- ATTGCCATTGGAGCAGCATC

2.3.3. Real-time PCR

Real-time PCR was performed with ExicyclerTM 96 Real-Time Quantitative Thermal Block (Bioneer). Each tube contained 25 μl of reaction mixture including 12.5 μl of AccuPower 2 x GreenStarTM qPCR MasterMix (Bioneer), 1 μl of each 10 μM primer set and 2 μl of cDNA template. PCR conditions were as follow: Pre-denaturation at 95°C for 10 min, followed by 40 cycles of 10 s of denaturation at 95°C, and 30 s of annealing/extension at 60°C. Expression levels were normalized by using the housekeeping gene 50 S ribosomal protein L17 (*rplq*) as control (Takle et al., 2007). Melting-curve analyses were performed after each reaction for validation of amplification specificity.

2.4. Statistical analysis

The experimental data of viable bacterial counts in broth and serum (Figure 2-1) were analyzed using SPSS (20.0), with significant differences among data being tested by ANOVA. Duncan method was implemented to compare statistical difference among the group ($P < 0.05$).

3. Result and Discussion

3.1. Bacterial adaptation in olive flounder serum

Strain SPOF3K showed growth both in a culture medium (BHIB supplemented with 1% NaCl) and olive flounder serum (Figure 2-1). The growth in the medium showed that bacterial cells proliferated from 1 hpi. Although the bacterial growth in serum lagged at 1 hpi, it began to increase from 2 hpi, indicating that this strain has an ability to resist and multiply in the serum environment.

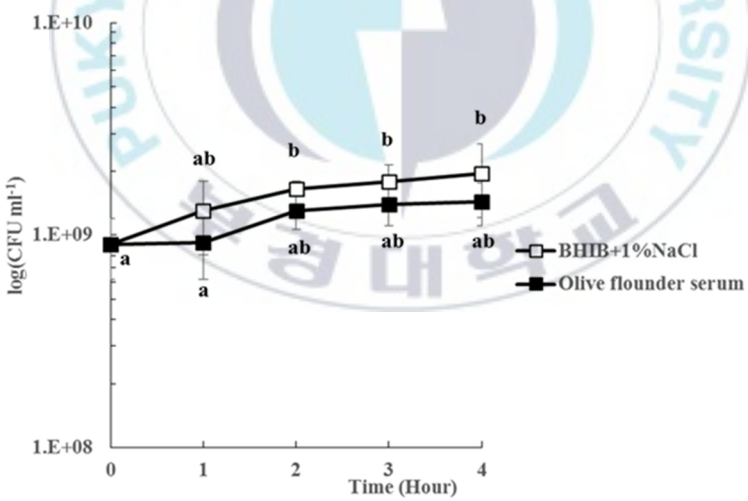

Figure 2-1. The number of viable bacterial cells in OF serum and BN medium during incubation: Viable bacterial counts were determined by plate counting.

3.2. Overview of transcriptome data

Remaining rRNA, intergenic reads and low-quality-reads through quality trimming were removed, and the final mapping rate of filtered transcript reads into the reference genome was 87.1-98.2% (Table 2-2). Principle component analysis (PCA) based on transcriptome data shows clear and extensive clustering among experimental groups; the triplicate samples clustered together according to the incubation conditions, indicating transcriptome data obtained in this study were highly reliable and reproducible. Samples taken at 2 and 4 hpi showed relatively large variance compared to those of other samples (Figure 2-2). In addition, samples at 1 hpi in serum were positioned closer to samples at 0 hpi in this plot. From DGE analysis, 737 (33%), 685 (30.7%) and 652 (29.2%), and 843 (37.8%), 609 (27.3%) and 666 (29.8%) genes showed significant up- and down-regulation in the serum at 1, 2, 4 hpi, respectively, compared with broth cultured bacteria at the same time points. Gene expression levels obtained from qPCR were consistent with RNA-seq results, indicating these data are reliable (Figure 2-3).

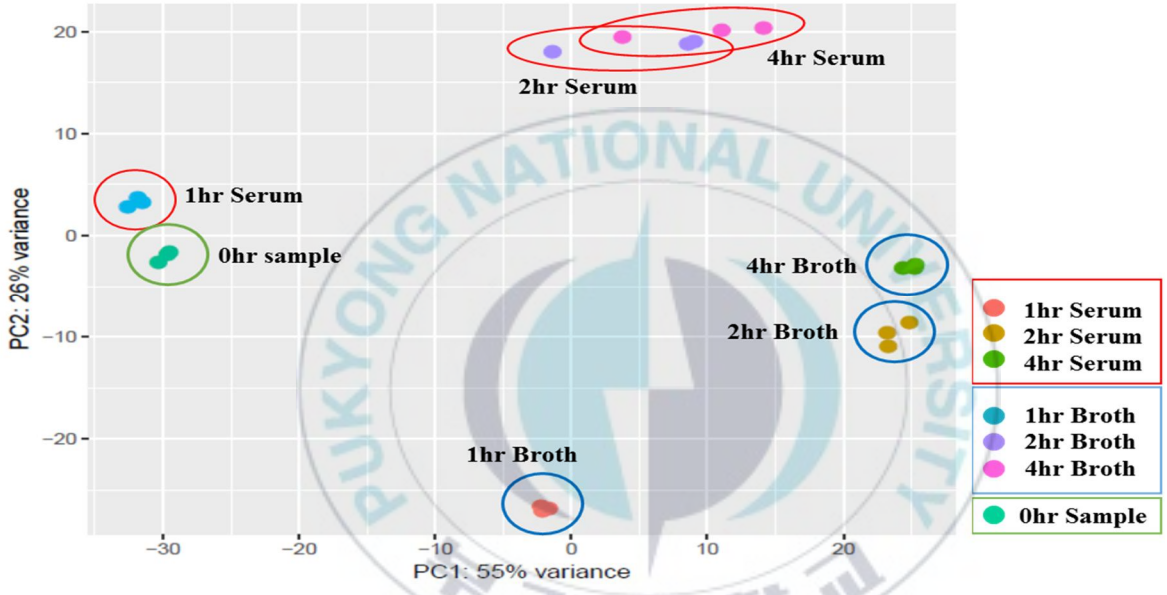

Figure 2-2. Principle component analysis (PCA) plot expression of RNA-sequencing data: This figure shows transcriptome differences between strain SPOF3K incubated in BN broth and Olive flounder serum. The PCA plot captures the variance in a dataset in terms of principal components and the percentages of the total variation are shown on the x- and y-axes.

Table 2-2. Summary RNA-Sequencing statistics.

Sample	Replication	Total reads	QC dropped reads	rRNA reads	Intergenic reads	Unmapped reads	mRNA reads	Mapping rate (%)
1hr Broth	Rep 1	26,770,092	32,867	82,679	3,946,011	509,151	22,199,384	98.1
	Rep 2	24,374,137	25,214	70,082	3,854,583	581,570	19,842,688	97.6
	Rep 3	23,179,668	24,232	59,893	3,457,539	348,692	19,289,312	98.5
1hr Serum	Rep 1	25,251,382	37,280	1,362,287	2,921,966	3,381,741	17,548,108	86.6
	Rep 2	24,401,998	53,251	125,402	2,958,888	3,488,787	17,775,670	85.7
	Rep 3	26,812,486	75,319	114,781	3,322,495	3,820,511	19,479,380	85.8
2hr Broth	Rep 1	32,704,855	72,757	185,582	4,868,214	586,550	26,991,752	98.2
	Rep 2	22,241,039	24,044	43,744	3,351,331	512,304	18,309,616	97.7
	Rep 3	23,988,867	22,323	98,400	3,590,872	464,968	19,812,304	98.1
2hr Serum	Rep 1	21,681,056	35,977	46,249	3,001,294	1,533,612	17,063,924	92.9
	Rep 2	25,192,992	31,933	114,574	3,388,237	2,189,204	19,469,044	91.3
	Rep 3	28,994,847	30,108	164,176	3,834,481	1,972,758	22,993,324	93.2
4hr Broth	Rep 1	25,914,973	35,043	70,172	3,882,064	693,122	21,234,572	97.3
	Rep 2	31,124,601	45,938	66,256	4,809,186	834,349	25,368,872	97.3
	Rep 3	25,662,496	27,079	49,250	3,808,651	676,916	21,100,600	97.4
4hr Serum	Rep 1	21,280,621	21,749	38,338	3,133,132	1,327,861	16,759,541	93.8
	Rep 2	19,780,737	27,379	134,334	2,918,480	1,621,407	15,079,137	91.8
	Rep 3	25,141,336	24,005	153,175	3,591,375	1,815,921	19,556,860	92.8
0hr Control	Rep 1	20,532,875	26,979	90,679	2,896,498	1,956,868	15,561,851	90.5
	Rep 2	30,441,566	56,832	820,058	4,122,062	3,609,746	21,832,868	88.1
	Rep 3	23,777,037	74,470	913,612	3,004,745	3,078,588	16,705,622	87.1

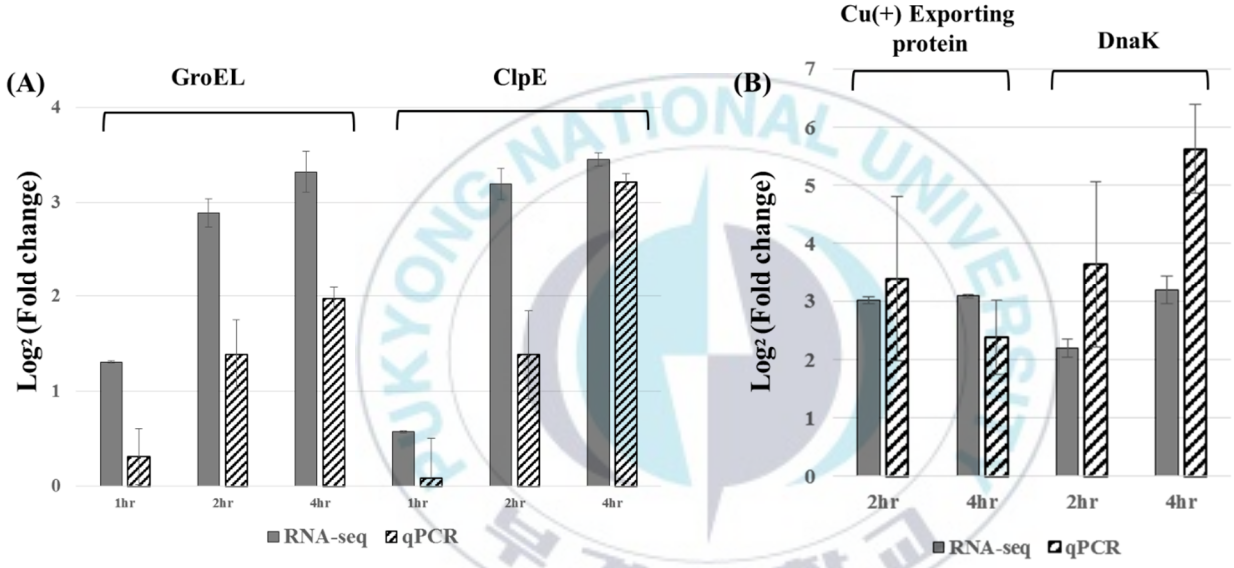

Figure 2-3. Comparison of gene regulation by RNA-seq and Real-time quantitative PCR. (A) RNA-seq expression of three genes were compared with qRT-PCR by setting broth-cultured transcripts as control for serum-cultured samples at every sampling time point. (B) RNA-seq expression of two genes were compared with qRT-PCR by setting 1 hpi transcript as control for serum-cultured samples at 2 hpi and 4 hpi.

3.3. Functional profiling of RNA-seq data

Transcripts obtained in this study were assigned into 18 functional categories based on eggNOG database, enabling global profiling of *S. parauberis* in serum compared to that in the medium (Figure 2-4). Carbohydrate transport and metabolisms category, amino acid transport and metabolism category, energy production and conversion category, translation, ribosomal structure and biogenesis category were under-represented. It means significantly down-regulated transcripts out-numbered significantly up-regulated transcripts. This might be due to comparatively low levels of nutrients in olive flounder serum than in the medium.

Expression on genes in the category of defense mechanism, inorganic ion transport and metabolism, and lipid transport and metabolism showed under-representation at 1 hpi, and over-representation at 2 and 4 hpi. Major components of defense mechanism category were transport-related proteins (Table 2-3). *msbA*, *cydCD*, *fbpC*, *bcrA*, *rbsA*, *mdlA* were significantly up-regulated, which would participate in lipid transport (Zhou et al.,

1998), iron import (Anderson et al., 2004), aerobic respiration (Truong et al., 2014) and drug resistance (Podlesek et al., 1995) in the serum. These genes would be essential for *S. parauberis* to better adapt in serum, which lacks inorganic materials, necessary for bacterial homeostasis and proliferation (Li et al., 2011). Of Note, acetylmuramoylpentapeptide -lysine N(6)-alanyltransferase *murM* (SPSF3K_00276) in defense mechanism category showed the highest degree of up-regulation. MurM is known to participate in streptococcal cell wall formation by adding L-serine/L-alanine into pentapeptide to form interpeptide bridge between the peptidoglycan precursor units (Filipe et al., 2001). It is also well-known for bringing resistance against cell wall associated antimicrobial substances by enriching the “mosaic-like” branched-structured muropeptides in the cell wall (Fiser et al., 2003). Two genomic loci (SPSF3K_00276 and SPSF3K_00910) with 39% identity in protein sequence level were predicted to function as MurM in SPOF3K genome and were significantly up-regulated. The fold change in expression of the former loci (SPSF3K_00276) was 7.89, 8.34, and 11.23 at 1, 2 and 4 hpi, while the latter (SPSF3K_00910) showed a

relatively lower degree of up-regulation only at 4 hpi with 1.34 fold change. These data indicate that the former loci (SPSF3K_00276) would function more crucially for bacterial survival in the serum environment with formation of compact form of peptidoglycan layer.

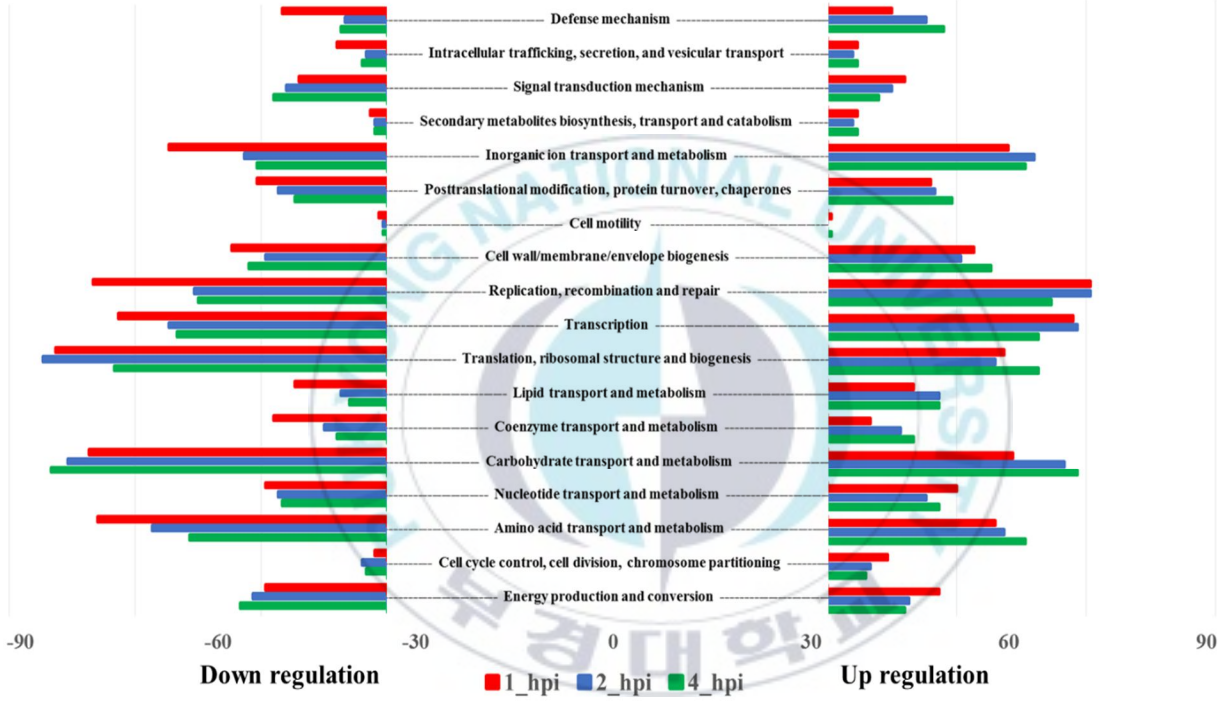

Figure 2-4. Differential regulation of transcript expression in SPOF3K during the incubation. Genes were classified into 18 functional categories. Bars indicate the number of differentially expressed genes at every sampling time point (1 hpi, 2 hpi and 4 hpi).

Table 2-3. Genes with significant up-regulation in Defense mechanism category.

Gene locus	Function of product	Fold change in expression		
		1 hpi	2 hpi	4 hpi
SPSF3K_00189	Probable multidrug resistance protein YpnP	0.57	-	1.33
SPSF3K_00195	Lipid A export ATP-binding/permease protein MsbA	1.49	2.61	3.60
SPSF3K_00196	Lipid A export ATP-binding/permease protein MsbA	-	2.32	3.56
SPSF3K_00218	ABC-2 type transporter	2.39	1.72	1.69
SPSF3K_00276	UDP-N-acetylmuramoylpentapeptide-lysine N(6)-alanyltransferase murM	7.89	8.34	11.23
SPSF3K_00321	Serine-type D-Ala-D-Ala carboxypeptidase	-	1.73	1.81
SPSF3K_00388	ATP-binding/permease protein CydC	-	1.66	1.46
SPSF3K_00389	ATP-binding/permease protein CydD	-	1.71	1.61
SPSF3K_00429	Type I site-specific deoxyribonuclease			
SPSF3K_00446	Fe(3+) ions import ATP-binding protein FbpC	-	1.58	1.70
SPSF3K_00449	ABC transporter G family member	-	2.15	1.84
SPSF3K_00473	Putative bacitracin ABC transporter, ATP-binding protein BcrA	2.18	2.34	-
SPSF3K_00476	Hypothetical protein	5.88	2.19	-
SPSF3K_00478	Ribose import ATP-binding protein RbsA	8.38	2.39	-
SPSF3K_00910	UDP-N-acetylmuramoylpentapeptide-lysine N(6)-alanyltransferase murM	-	-	1.34
SPSF3K_01011	Putative hemin transport system permease protein HrtB	0.51	1.47	1.71
SPSF3K_01012	Macrolide export ATP-binding/permease protein MacB	0.59	-	1.60
SPSF3K_01066	Macrolide export ATP-binding/permease protein MacB	0.72	-	1.41
SPSF3K_01138	Type I site-specific deoxyribonuclease			
SPSF3K_01363	N-acetylmuramoyl-L-alanyl-D-glutamyl-L-lysyl-(N(6)-glycyl)-D-alanyl-D-alanine-diphosphoundecaprenyl-N-acetylglucosamine:glycineglycyltransferase	-	-	1.72

(Continued)

Gene locus	Function of product	Fold change in expression		
		1 hpi	2 hpi	4 hpi
SPSF3K_01385	ABC transporter permease protein YxdM	1.29	1.61	1.27
SPSF3K_01386	Methionine import ATP-binding protein MetN	1.81	-	0.73
SPSF3K_01422	Probable ATP-dependent transporter ycf16			
SPSF3K_01446	Site-specific DNA-methyltransferase (adenine-specific)	1.51	-	-
SPSF3K_01447	Efflux ABC transporter, permease protein	0.47	1.71	1.99
SPSF3K_01448	ATP-binding cassette sub-family A member 8-A	0.46	-	1.52
SPSF3K_01755	D-alanyl-D-alanine carboxypeptidase	1.27	2.10	2.27
SPSF3K_01818	Putative ABC transporter ATP-binding protein exp8	-	-	1.58
SPSF3K_01819	Multidrug resistance-like ATP-binding protein MdlA	-	-	1.50
SPSF3K_01847	5-methylcytosine-specific restriction enzyme	4.54	-	-
SPSF3K_01880	Methionine import ATP-binding protein MetN	1.35	1.40	1.54
SPSF3K_01918	Inner membrane transport permease	1.88	2.12	2.55
SPSF3K_02131	Probable multidrug resistance protein NorM	-	1.80	2.17
SPSF3K_02201	Beta-lactamase	3.66	1.64	-

* -: No significance found.

3.4. Differentially gene expression (DGE)

3.4.1. ATP-Binding Cassettes transporters with DGE

ABC transport system is very essential for bacterial survival by regulating physiological balance in hostile environment and bringing full virulence into host (Davidson et al., 2004 and Chimalapati et al., 2012). Along with the over-representation of several substrate transporting categories, ABC transporter related genes with significantly up-regulation were identified based on KEGG pathway analysis (Table 2-4).

Biotin is a vital cofactor for numerous enzymes that are essential for microbial proliferation and survival, and many studies have also revealed its importance in virulence (Salaemae et al., 2016 and Satiaputra et al., 2016). In this study, Biotin transporter *bioY* (SPSF3K_00173-4), *ecfT* (SPSF3K_00021 and SPSF3K_00582) and *ecfA1* (SPSF3K_00583) were significantly up-regulated in olive flounder serum, showing their importance for survival and virulence of *S. parauberis* in host environment.

Spermidine/putrescine is one of predominant prokaryotic

polyamines that are necessary for broad range of biological functions including stress resistance (Igarashi et al., 2010). In RNA-seq analysis, *potABCD* operon, encoding spermidine/putrescine transporter (SPSF3K_01238-01241) was significantly up-regulated at 2 and 4 hpi. The importance of this operon in pathogenicity was well-demonstrated in several studies with *S. pneumonia*. Attenuated mutants of part of this operon lacked resistance to stress, virulence and choline concentration (Ware et al., 2006 and Martino et al., 2013). RNA-seq data in this study shows this operon would be also important in survival of *S. parauberis* in olive flounder.

Osmoprotectant *opuC* (SPSF3K_01282) and glycine betaine/proline transporter *proX*, *ProW* and *ProV* (SPSF3K_00166-167) were significantly up-regulated (Table 2-4). Osmotic tolerance is important for bacterial survival in hostile environment. Glycine betaine/proline is the major bacterial osmolytes, major substrates to counterbalance against osmotic stress (Graham et al., 1992 and Ko et al., 1994). The up-regulation of these transporters suggests that this bacteria might counter osmotic stress and take action to retain its homeostasis in serum.

Oligopeptide transporter encoding *opp* operon (SPSF3K_00631-00634) showed significant up-regulation (Table 2-4). Oligopeptide can be used for a good nutrition source for bacteria from external environment, and signal molecules for intercellular communication, which can further effect to the bacterial colonization, biofilm formation and quorum sensing (Alloing et al., 1994; Kerr et al., 2004 and Verhagen et al., 2014). Hence, the up-regulation of this operon in the serum may represent that this operon would serve a part in virulence of *S. parauberis* with better bacterial colonization and biofilm formation in olive flounder.

Also, several other ABC transporters including *cylAB* (SPSF3K_00217-8), multi-drug resistance / hemolysin transporters (Gottschalk et al., 2006), ABC.FEV.P/A (SPSF3K_00728-9 and SPSF3K_00008), iron complex transporters (Schneider et al., 1993) might be related to virulence mechanism of *S. parauberis*, as they were significantly up-regulated only in serum.

Table 2-4. ATP-binding cassette transporters with DGE

Gene	Locus	Fold change in expression			KO number
		1 hpi	2 hpi	4 hpi	
Biotin					
bioY	SPSF3K_00173	8.7	8.4	7.2	K03523
bioY	SPSF3K_00174	8.1	8.2	7.1	K03523
ecfT	SPSF3K_00021	-	1.8	1.4	K16785
ecfT	SPSF3K_00582	3.0	1.6	-	K16785
ecfA1	SPSF3K_00583	4.3	1.8	1.2	K16786
Multidrug resistance / Hemolysin					
cylB	SPSF3K_00218	2.4	1.7	1.7	K11051
cylA	SPSF3K_00217	2.0	1.6	1.3	K11050
ABCC Subfamily					
ABCC-BAC	SPSF3K_00478	8.3	2.3	-	K06148
cydC	SPSF3K_00389	-	1.6	1.6	K16012
cydD	SPSF3K_00388	-	1.6	1.4	K16013
CFTR, ABCC7	SPSF3K_01680	0.5	1.2	1.3	K05031
Iron complex					
ABC.FEV.P	SPSF3K_00728	-	1.6	2.1	K02015
ABC.FEV.P	SPSF3K_00729	-	1.3	1.8	K02015
ABC.FEV.A	SPSF3K_00008	4.3	1.9	-	K02013
Spermidine/putrescine					
potD	SPSF3K_01241	0.9	1.3	1.2	K11069
potC	SPSF3K_01240	0.9	1.3	1.2	K11070
potB	SPSF3K_01239	0.8	1.3	1.3	K11071
potA	SPSF3K_01238	0.9	1.3	1.2	K11072
glycine betaine/proline					
proX proW	SPSF3K_00167	2.2	1.7	1.5	K02001-2
proV	SPSF3K_00166	2.7	1.5	1.3	K02000
Osmoprotectant					
opuC	SPSF3K_01282	2.9	2.3	2.3	K05845
Oligopeptide					
oppA	SPSF3K_00630	3.1	0.9	-	K15580
oppB	SPSF3K_00631	3.2	1.2	-	K15581
oppC	SPSF3K_00632	2.5	1.4	1.2	K15582
oppD	SPSF3K_00633	2.4	1.4	-	K15583
oppF	SPSF3K_00634	2.3	1.5	1.2	K10823

* -: No significance found.

3.4.2. Stress responses and regulation

Induction of stress responses often represents bacterial responses to environmental changes, specifically with the adaptation into the host (Camejo et al., 2009). The heat-shock chaperones provide a cellular protection against stressed conditions by taking a key role in proper protein folding (Lindquist et al., 1988). Also, heat-shock proteins regulate transcription of various virulence-related factors in other streptococcal species (Woodbury & Haldenwang, 2003; Lemos et al., 2007; Ishibashi et al., 2010; Cui et al., 2011 and Tran et al., 2011). GroEL and DnaK are bacterial representatives of heat-shock chaperones together with co-chaperones (GroES and DnaJ-grpE) (Roncarati et al., 2017). Expression of *groESL* (SPSF3K_00141-142) and *dnaJ-dnaK-grpE* (SPSF3K_002050-2052) operons were increased as time went on, and significantly up-regulated at all the sampling time points (Table 2-5). The relative fold changes in these operon (7.12 ~ 28.69 in 2 and 4 hpi) was relatively higher than that of other virulence related genes, representing that bacteria is situated in highly stressful environment.

Furthermore, *clpP* multigene family, *clpP* family for ClpB (SPSF3K_00140), ClpE (SPSF3K_01798), ClpL (SPSF3K_01110) were significantly up-regulated. Those multigene family functions to efficiently disaggregate and refold a variety of protein aggregates (Schirmer et al., 1996; Goloubinoff et al., 1999 and Bosl et al., 2006). With significant expression of stress related genes shown here, *S. parauberis* would be able to better control and manipulate its pathogenicity in host.

Table 2-5. Stress-related transcripts with DGE

Gene name	Gene locus	Function of product	Fold change in expression		
			1 hpi	2 hpi	4 hpi
dnaJ	SPSF3K_02050	Chaperone protein DnaJ	-	18.29	22.20
dnaK	SPSF3K_02051	Chaperone protein DnaK	1.92	26.18	28.69
grpE	SPSF3K_02052	Heat shock protein grpE	2.73	27.52	26.73
groES	SPSF3K_00141	Chaperonin GroES	2.02	7.12	13.68
groEL	SPSF3K_00142	Chaperonin GroEL	2.33	7.61	10.11
clpL	SPSF3K_01110	ATP-dependent protease ClpL	11.48	13.31	12.67
clpB	SPSF3K_00140	ATP-dependent protease ClpB	3.11	3.73	4.22
clpE	SPSF3K_01798	ATP-dependent protease ClpE	1.40	9.41	11.09
MviM	SPSF3K_00109	Oxidoreductase	8.34	4.91	3.68

* -: No significance found.

3.4.3. Important virulence-related genes with DGE

In genome analysis, virulence-related genes of this strain were identified and the significantly differentially expressed virulence-related genes in serum were determined (Table 2-6). A comprehensive overview on their functions and possible pathogenesis in olive flounder are provided from here.

Table 2-6. Important virulence genes with DGE

Gene name	Gene locus	Function of product	Fold change in expression		
			1 hpi	2 hpi	4 hpi
hasA	SPSF3K_00187	Hyaluronan synthase	1.29	1.77	1.99
hasB	SPSF3K_00188	UDP-glucose 6-dehydrogenase	1.44	1.68	1.67
hasC	SPSF3K_00192	UTP-glucose-1-phosphate uridylyltransferase	1.40	-	-
	SPSF3K_00426	Antiphagocytic M protein	0.47	-	1.59
srtA	SPSF3K_01301	Sortase A	3.75	2.32	1.49
	SPSF3K_00379	C5a peptidase	1.94	1.92	2.19
lgt	SPSF3K_00871	Prolipoprotein diacylglyceryl transferase	2.19	1.55	1.16
GAPDH	SPSF3K_00249	Glyceraldehyde 3-phosphate dehydrogenase	1.77	1.52	1.51
EF-Tu	SPSF3K_00907	Elongation factor thermo unstable	1.52	1.96	1.57
MF2	SPSF3K_00129	Mitogenic factor 2	4.66	3.75	3.71
Genes present in only high virulent strains					
InlJ	SPSF3K_02222	Internalin J	1.61	1.50	1.25
	SPSF3K_01846	Lactocepin	3	1.94	2.74
	SPSF3K_02074	Bacterial cellulose synthase	0.45	1.93	1.93
oatA	SPSF3K_01374	Peptidoglycan O-acetyltransferase	1.84	1.53	1.23

* -: No significance found.

Hyaluronic acid capsule and M protein are considered to be the most prominent virulence factors in several streptococcal species due to their anti-phagocytosis, anti-opsonization and adhesion activity in host environment (Cunningham et al., 2000). Genes involved in biosynthesis of hyaluronic acid capsule were significantly up-regulated; *hasA* (SPSF3K_00187), a gene encoding hyaluronan synthase, and *hasB* (SPSF3K_00188), a gene encoding UDP-glucose 6-dehydrogenase, were up-regulated in all serum treated samples, and UTP-glcuose-1-phosphate uridylytransferase encoding gene, *hasC* (SPSF3K_00192) at 1 hpi was significantly up-regulated (Table 2-6). Alberti et al. (1998) suggested that expression of *hasA* and *hasB* was sufficient for encapsulation in GAS. Accordingly, results here show that hyaluronic capsule in *S. parauberis* would play an important role in resistance against serum. However, M-like protein (SPSF3K_00426) showed significant up-regulation only at 4 hpi (Table 2-6), which corresponds to the result of Moses et al. (1997). Therefore, hyaluronic acid capsule rather than M-like protein would be more crucial for bacterial survival in serum.

GAPDH (SPSF3K_00249) was significantly up-regulated samples in serum at all the sampling time points (Table 2-6). GAPDH is anchorless and cytoplasmic enzyme, playing a key role in glycolysis by catalyzing the oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphoshpoglycerate (Pancholi et al., 1992). However, its multifunctional ability to serve as virulence factors by binding into host structural proteins (fibronectin, laminin, myosin, actin, lysozyme) and to human fibrinolytic system, mediating impair phagolysosome, regulating host cell signaling, and maintaining iron homeostasis are reported in various bacterial species (Pancholi et al., 1997; Alvarez-Dominguez et al., 2008 and Boradia et al., 2014). Also, its potential as a vaccine candidate has been reported in different bacterial species (Rosinha et al., 2002; Jin et al., 2005; Liu et al., 2005 and Matta et al., 2010). Therefore, finding in this study shows that GAPDH of *S. parauberis* would be also important virulence factor, as well as one of vaccine candidates.

Elongation factor (Ef-Tu; SPSF3K_00907), a well-known housekeeping and multifunctional protein, in all serum-treated samples was significantly up-regulated (Table 2-6). Ef-Tu is as an

essential and universally conserved GTPase that adds the correct amino acid to the nascent polypeptide chain to ensure translational accuracy (Sprinzl et al., 1994). Differential expression of this gene in host has been widely reported in pathogenic bacterial species and its role in virulence is also known to be various: It serves as adhesins and contribute to bacterial dispersal by binding into multiple substrate of host and by converting plasminogen into plasmin which consequently lead into fibrinolysis respectively (Widijaja et al., 2017). The significant expression of *Ef-Tu* in olive flounder serum verifies that this gene would role in pathogenicity of *S. parauberis*.

Bacterial lipoproteins function in a variety of physiological functions such as nutrient acquisition, adaptation to environmental changes, protein maturation and adherence (Kovacs-Simon et al., 2012). The majority of bacterial lipoproteins are reported to account for substrate-binding proteins for ABC transporters (Chimalapati et al., 2012). Prolipoprotein diacylglyceryl transferase encoding gene, *lgt* (SPSF3K_00871), which functions the maturation and consequent anchoring of the lipoprotein to the cytoplasmic

membrane (Buddelmeijer et al., 2015). In this study, this gene was significantly up-regulated at all the sampling time points (Table 2-6), suggesting its vital role in virulence in *S. parauberis* infection.

Sortase A encoding gene, *srtA* (SPSF3K_01301), was up-regulated at all sampling time points (Table 2-6). Sortase A is an extracellular transpeptidase in Gram-positive bacteria, responsible for covalently sorting out and anchoring surface proteins to the peptidoglycan cell wall by recognizing and cleaving off the signal peptide containing LPXTG motif at C-terminal site (Paterson et al., 2004). The effect of this enzyme on pathogenicity was experimentally proven in many Gram-positive bacterial species including *Listeria monocytogenes* (Garandeau et al., 2002), *Staphylococcus aureus* (Mazmanian et al., 2000) and other Streptococcal species (Lee et al., 2003; Kharat et al., 2003 and Lalioui et al., 2005). The over-expression of *srtA* indicates that a variety of extracellular surface proteins with LPXTG motif would have been anchored to the cell wall, which might link to adaption process of this strain in the serum environment. Eight CDSs containing LPXTG motif in SPOF3K genome were predicted based on Hidden Markov Model (HMM) (Krogh et al., 2001) by

using using CW-PRED (Fimereli et al., 2012) (Table 2-7). They include M-like protein (SPSF3K_00426), C5a peptidase (SPSF3K_00379-00380) and Internalin J (SPSF3K_02222) which are reported as important bacterial virulence factors.

Table 2-7. CDSs containing LPXTG motif predicted in this study

Gene name	Gene locus	Function of product	Fold change in expression		
			1 hpi	2 hpi	4 hpi
	SPSF3K_00144	Hypothetical protein	2.92	4.77	4.72
KEX1	SPSF3K_00154	Pheromone-processing carboxypeptidase	-	0.78	-
	SPSF3K_00379-SPSF3K_00380	C5a peptidase	2.05	2.27	2.69
	SPSF3K_00426	Antiphagocytic M protein	0.47	-	1.59
	SPSF3K_00579	Uncharacterized protein	4.27	1.26	1.29
	SPSF3K_01115	Hypothetical protein	0.56	-	0.73
DAN4	SPSF3K_01126	Cell wall protein	0.42	-	-
in1J	SPSF3K_02222	Internalin J	1.61	1.50	1.25

* -: No significance found.

C5a peptidase (SPSF3K_00379-00380), a sortase-mediated cell wall-anchored with LPXTG motif (Lalioui et al., 2005, Severin et al., 2007), was significantly over-expressed in all samples in the serum. C5a peptidase specifically cleaves off complement component 5a (C5a), which is an important chemotactic agent in innate immunity as it induces increase of migration and adherence of leukocytes to vessel walls (Manthey et al., 2009). The over-expression of C5a peptidase shown in this study may provide a significant evidence that this gene bring *S. parauberis* better ability to resist against the host innate immunity.

Internalin J encoding gene, *inlJ* (SPSF3K_02222) was significantly up-regulated in all the samples cultured in serum (Table 2-6 and Table 2-7). InlJ is a sortase-mediated cell wall-anchored protein harboring LPXTG motif, and reported as one of the major virulence factors in *Listeria monocytogenes*, contributing to bacterial internalization into host cells (Sabet et al., 2005). In addition, our comparative genome analysis revealed that this gene is uniquely harbored in a plasmid of only genotype I strains, which showed relatively higher degree of pathogenicity.

Lactocepin (SPSF3K_01846), a cell wall-associated protease with a wide range of specificity is especially known for its action on caseins, but its hydrolysis on hemoglobin and insulin B chain has been previously reported (Visser et al., 1991). Recent studies have revealed its ability of anti-inflammatory effects by selectively degrading pro-inflammatory chemokines, inhibiting lymphocyte recruitment (von Schillde et al., 2012). This protein was significantly up-regulated in all the samples cultured in serum (Table 2-6), and found only in genotype I strains, indicating its possible importance in the virulence of *S. parauberis*.

Peptidoglycan O-acetyltransferase (*oatA*; SPSF3K_01374) was significantly expressed all the time points in serum. As mentioned in the previous chapter, OatA which provides resistance to lysozyme, and is presence only in genotype I strains, that showed high virulence in olive flounder (See discussion in Chapter I). The significant up-regulation of this gene in olive flounder serum indicates that this gene would participate in immune evasion of *S. parauberis* in the host.

In the comparative genome analysis, bacterial cellulose synthase (BCS; SPSF3K_02074) and its genetic cluster (SPSF3K_02075-02080) were uniquely found only in virulent strains which were predicted to participate crucially in adhesion, biofilm maturation and cell aggregations (See discussion in Chapter I). This gene cluster shared very similar gene expression pattern: Significant down-regulation was shown in 1 hpi, but they were uniquely up-regulated in 2 and 4 hpi when the bacterial cells proliferate in serum (Figure 2-5). The consensus expression pattern might show that this gene cluster can work as other reported BC synthesis operons (Römling and Michael, 2015), even though the further experimental studies are needed to comprehensively characterize the mode of this gene cluster. The uniqueness and differential expression of this gene cluster found in the comparative genome and transcriptome analysis provide crucial evidence on this BCS gene cluster as a major virulence factor of *S. parauberis*.

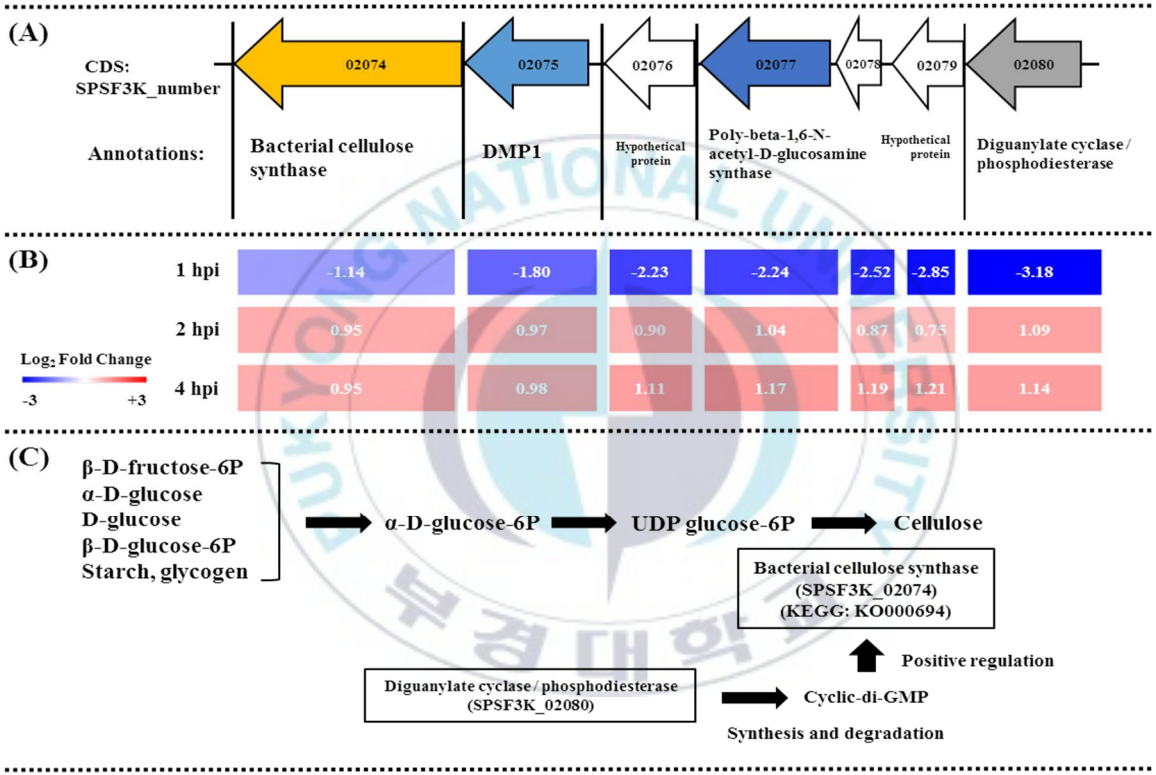

Figure 2-5. Bacterial cellulose synthesis (A) encoding gene cluster, (B) its gene expression in serum and (C) pathway

4. Conclusion

The aim of this chapter was to gain insights into survival strategy of *S. parauberis* using its culture in olive flounder serum with global transcriptome profiling. *S. parauberis* strain SPOF3K derived from diseased olive flounder was shown to have ability to survive and multiply in olive flounder serum. The result obtained from transcriptome analysis revealed differential up-regulation of various important ABC transporters, virulence and stress related factors, illustrating the mode of pathogenicity of *S. parauberis* host.

In particular, along with findings in the previous chapter, virulence factors which presence only in virulent strains, but not in low virulent strains including bacterial cellulose synthase, peptidoglycan O-acetyltransferase and Internalin J were significantly up-regulated in serum, providing strong evidence that those factors would be key in *S. parauberis* pathogenicity. The result obtained in here would provide an important background knowledge for further prevention and treatment strategies against *S. parauberis* infection in field.

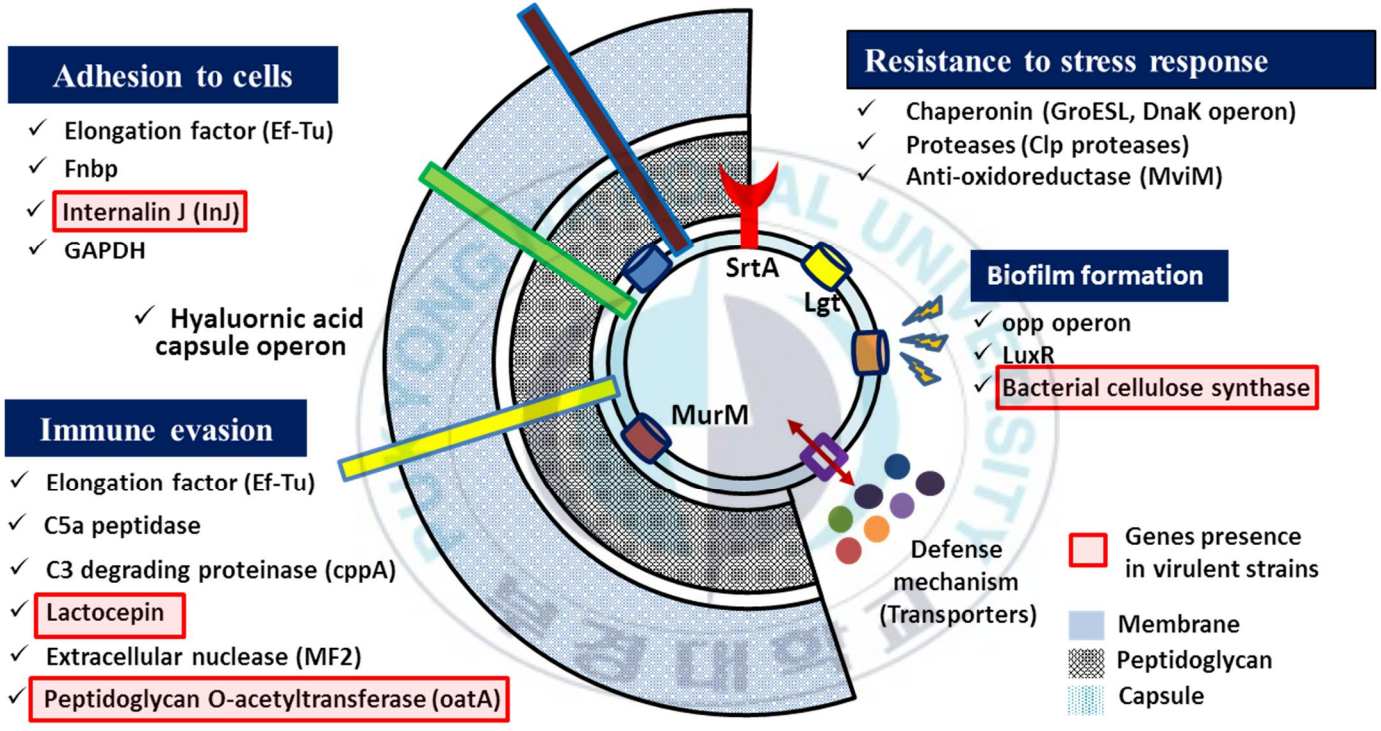

Figure 2-6 Summary overview of the important factors involved in pathogenesis of *S. parauberis* in olive flounder determined in this study

국문요약

넙치, *Paralichthys olivaceus*에 대한 *Streptococcus parauberis* 의 병원성 연구

이윤항

부경대학교 대학원 수산생명의학과

본 연구는 국내 어류양식의 대표 어종인 넙치, *Paralichthys olivaceus*의 주요 세균성 질병인 연쇄구균병의 원인체 *Streptococcus parauberis*의 전반적인 병원성을 이해하는 것을 목적으로 하였다. 본 연구는 *S. parauberis*의 감염기전 및 주요 병원성 인자를 탐색한 첫 보고이며, 추후 연쇄구균증에 대한 예방 및 관리에 기초 자료를 제공하고자 한다.

제 1장에서는 75개 균주에 대하여 혈청형 분석을 실시하였으며, 15개 *S. parauberis* 균주에 대한 병원성 시험을 통해 고 병원성 및 저 병원성 균주를 탐색하였다. 선정된 2개 고 병원성 균주와 1개 중간 병원성 균주, 2개 저 병원성 균주에 대하여 넙치 혈청에서의 생존능을 확인하

였으며, 이들의 전유전체를 비교 분석하여 유전적 차이를 확인하였다.

혈청형 분석의 결과, 국내에서 혈청형 Ia 가 가장 우점적으로 분리된 것을 확인할 수 있었으며, 병원성 시험의 결과 이들의 병원성이 전반적으로 다른 혈청형에 비하여 높게 나타났다. 넙치 혈청에 5개 균주 모두가 저항성을 나타내었지만, 2개 고 병원성 균주가 더 강한 저항성을 나타내었다. 비교 전유전체 분석의 결과로 세 개의 유전형을 확인할 수 있었으며, 이는 혈청형 분석의 결과와 일치하였다. 유전형 I (혈청형 Ia) 균주는 공통적으로 Tetracycline 저항성 유전자 *tetS*와 병원성 유전자 *inlJ*를 포함하는 12 Kbp 크기의 plasmid를 가지는 것으로 확인되었고, Macrolide / lincosamide / Streptogramin 저항성 유전자 *emrB*과 Aminoglycoside 저항성 유전자 ANT(6)-*Ia*를 가지므로 다른 유전형들에 비하여 강한 항생제 저항성을 나타낼 수 있음을 확인하였다. Bacterial cellulose synthase, Lactocepin, Internalin J 및 Peptidoglycan O-acetyltransferase 등의 병원성 인자가 고 병원성 균주에서만 확인되었으며, 이들이 다른 저 병원성 유전형에 존재하지 않는다는 점에서 넙치에 고 병원성 *S. parauberis* 의 중요한 병원성 인자로 생각된다.

제 2장에서는 *S. parauberis*를 넙치 혈청에서의 전사체를 분석하여 감염 기전 및 생존 전략을 확인하고자 하였다. 이에 대한 결과로, 다양한 병원체에서 주요 병원성 인자로 보고된 병원성 유전자들과 스트레스 저항 관련 유전자들의 유의적인 과 발현이 확인되었다. 뿐만 아니

라, 제 1장에서 확인된 고 병원성 균주에만 존재하는 병원성 인자들의 과발현이 확인되어, 이 유전자들이 *S. parauberis*의 주요 병원성 인자임을 증명하는 중요한 자료가 될 수 있었다.

감사의 글

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