Research Article

Characterization of culturable bacterial flora in yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus* L.) with "gaping jaws" syndrome

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ABSTRACT. One of the main problems facing Atlantic halibut hatcheries is the high mortality in the early stages of larval development. Several factors could be involved, for example: water quality, diseases or abnormalities, such as deformities occurring in the yolk sac larvae prior to exogenous feeding. The aim of this study was to identify differences in bacterial flora associated with yolk sac larvae with oral deformity. We also aimed to establish whether there is any relationship between bacterial strains and the "gaping jaws" syndrome. During our study, 74 bacterial isolates were obtained using three different nutrient media: Marine Agar, R2A and TCBS. Some of these bacteria were characterized using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and 16S rRNA sequencing. The immune response in larvae exhibiting the "gaping jaws" condition was measured by real time PCR. Our results showed significant differences in bacterial flora between normal and gaping larvae. The gaping yolk sac larvae were predominantly colonized by members of the families *Vibrionaceae* and *Flavobacteriaceae*. Bacteria belonging to the *Bacillus* and *Pseudoalteromonas* genera were also present but less frequent. It was not possible to associate a type or group of bacteria directly related to "gaping". Strikingly, larvae with gaping jaws had an increase in the expression of two immune related genes, like hepcidin and chemokine (MIP-1ß). These results indicate activation of the immune response in larvae with "gaping jaws" syndrome and this response could be related to bacteria isolated from gaping condition.

Keywords: Atlantic halibut, *Hippoglossus hippoglossus*, bacterial flora, "gaping jaws", immune genes.

Caracterización de la flora cultivable y la respuesta inmune en larvas con saco vitelino del halibut del Atlántico (*Hippoglossus hippoglossus* L.) con el síndrome de "gaping jaws"

RESUMEN. Uno de los mayores problemas que enfrentan los criaderos de halibut del Atlántico es la alta mortalidad en estados tempranos del desarrollo larval. Distintos factores pueden estar involucrados, *e.g.*, calidad del agua, enfermedades o anormalidades tales como deformidades que ocurren en las larvas en estado de saco vitelino antes de la alimentación exógena. El objetivo de este estudio fue identificar diferencias en la flora bacteriana asociada a larvas con saco vitelino con deformidad oral. Además se buscó establecer si existió relación entre alguna cepa bacteriana y el síndrome de "gaping jaws". Durante el estudio, 74 cepas bacterianas fueron aisladas usando diferentes medios de cultivo: agar marino, R2A y agar TCBS. Algunas de las bacterias fueron caracterizadas usando reacción en cadena de la polimerasa con análisis del polimorfismo de los fragmentos de restricción (PCR-RFLP) y secuenciación del gen ribosomal 16S. La respuesta inmune de larvas con el síndrome de "gaping jaws" fue medida por PCR en tiempo real. Los resultados evidenciaron diferencias significativas en la flora entre larvas normales y "gaping jaws". Las larvas con "gaping" fueron principalmente colonizadas por miembros de las familias *Vibrionaceae* y *Flavobacteriaceae*, mientras que bacterias pertenecientes a los géneros *Bacillus* y *Pseudoalteromonas* se observaron en baja frecuencia. No fue posible asociar directamente a un grupo bacteriano con el desarrollo del "gaping", sin embargo fue llamativa la respuesta inmune de larvas con "gaping", ya que mostraron un aumento en la expresión de los genes de la hepcidina y la citoquina (MIP-1ß). Estos resultados indican la activación de la respuesta inmune de las larvas con el síndrome de "gaping" y podría estar relacionada con las bacterias aisladas para la condición de "gaping".

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INTRODUCTION

The aquaculture of Atlantic halibut (*Hippoglossus hippoglossus*) is well developed in Canada, Iceland, Scotland and Norway (Mangor-Jensen *et al*., 1998). It is in the early stages of development in South America and still on an experimental scale in the Magellan Strait, Chile. (Alvial & Manríquez, 1999). At present, all the rearing phases are in development, including broodstock management, incubation, yolk-sac larval, first feeding, weaning, nursery, ongrowing and harvest (Gallardo, 2008).

Although Atlantic halibut has been cultured in captivity for almost 40 years, juvenile production remains one of the most important bottlenecks (Olsen *et al*., 1999). During the early stages of development, the larvae are very sensitive to perturbations in environmental conditions which can affect development and survival (Pittman *et al*., 1989; Lein *et al*., 1997a, 1997b; Solbakken & Pittman, 2004). The proliferation of opportunistic bacteria present as part of the normal seawater microbiota in marine hatcheries may be an important reason for high mortality of fish larvae (Olsen *et al*., 1999; Bergh *et al*., 2001; Olafsen, 2001). Although the normal bacterial flora associated with fish larvae can have a probiotic effect, some studies have shown negative effects caused by the presence of opportunistic bacteria, such as, *Flexibacter ovolyticus*, *Vibrio salmonicida* and *Vibrio anguilarum* (Hansen *et al*., 1992; Bergh *et al*., 1994). Diseases caused by viruses have also produced high mortality and economic losses (Grotmol *et al.,* 1995; Dannevig *et al*., 2000; Johansen *et al*., 2002).

The "gaping jaws" syndrome is characterized by the breakdown of the oral membrane, premature opening of the jaw and subsequent locking of the jaw cartilage during the yolk sac stage. This pathology prevents effective feeding at the completion of yolk sac absorption and finally leads to death by starvation (Pittman *et al*., 1990; Morrison & MacDonald, 1995). Although, the exact cause of the "gaping jaws" syndrome remains unresolved, the development of gaping appears to be related to the injury of the skin through contact with the rearing container and subsequent infection by opportunistic bacteria (Bergh *et al*., 2001; Bjornsdottir *et al*., 2009).

The presence of pathogenic bacteria leads to the activation of the immune response by the expression of genes involved in pathogen recognition and effectors**.** However, the adaptive immune response of halibut is not mature at the first larval stage of feeding (Øvergård *et al*., 2010). Therefore, the innate (nonspecific) response of the immune system of halibut has to be used for protection against pathogens at this stage (Lange *et al*., 2006; Magnadottir, 2006). For this reason, we have used the innate immune response associated with the expression of certain effectors obtained after genomic approaches. Much of the genetic information obtained for halibut, corresponds to expressed sequence tag (EST) programs that have been made on various tissues (Park *et al*., 2005; Douglas *et al*., 2007). The expression of immune genes involved in adaptive immunity indicates that some of these genes are expressed in the thymus during the larval stage (Øvergård *et al*., 2010). However, there are no studies that measure the expression of immune genes associated with the "gaping jaws"syndrome.

The aim of this study was to characterize the culturable bacteria in yolk sac larvae exhibiting "gaping jaws", based on morphological characteristics and genotyping by PCR-RFLP, and to evaluate the expression of genes involved in innate immune response of the larvae by real time PCR (qPCR).

MATERIALS AND METHODS

Sampling of larval yolk sac stage

The samples were obtained, between October and November 2009, from the University of Magallanes Marine Research Center, located in Laredo Bay (52°58'S, 70°49'W) Punta Arenas, Chile. A total of 5 normal yolk sac larvae and 5 with "gaping jaws" syndrome were taken to each incubator (silo). In total, four silos were sampled reaching a sample size of 40 larvae aged 25-30 days post-hatch (dph).

Bacterial isolation and counting

Larvae was placed in a 15 mL tube and washed three times with sterile seawater in order to avoid any external contamination. Then larvae were rinsed twice in sterile sea water before being homogenized in 1 mL of sterile 1X PBS using a glass homogenizer. Subsequently, the sample was centrifuged for 10 min at 1460 g and the supernatant was removed and the

pellet was resuspended in 1 mL of 1X PBS. Then, 50 µL of the homogenized sample was plated on solid nutrient media, R2A (Merck), Marine Agar (MA, BD) and TCBS agar (Thiosulfate citrate bile sucrose, Merck-selective medium for isolation and culture of *Vibrio* bacteria). The plates were incubated in duplicate, at room temperature, for several days until growth was observed.

DNA extraction and PCR amplification of 16S rRNA genes

Genomic DNA of each bacterial colony was extracted and purified according to Sambrook & Russell (2001). Universal primers were used to amplify 16S rRNA genes (8-27F, AGAGTTTGATCCTGGCTCAG; 1422R, GGTTACCTTGTTACGACTT). PCR amplifications were performed in an Eppendorf Master cycler gradient PCR system in 25 µL reaction mixtures containing approximately 50 ng of DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 µM (each) deoxynucleotides, 3 mM MgCl2, 2.5 U of *Taq* DNA polymerase (Invitrogen), and 0.2 µM (each) primer and approximately 50 ng of DNA. Amplification conditions for the primer pair were: 94°C for 10 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 7 min. PCR products (~1.5 kb) were visualized under UV light after electrophoresis at 100 mV for 30 min on 0.8% agarose gels prepared in Tris-acetate- EDTA (TAE) 0.5X buffer and stained with ethidium bromide.

Bacterial characterization

Representative colonies of each morphology bacteria were isolated from culture plates containing solid bacterial medium. Morphological characterization of all the isolates was done according to Freeman (1986) and using Gram staining. Molecular characterization of all the isolates was done using RFLP. Five microliters of PCR product were digested with 10 U of each restriction enzyme: Rsa I, Alu I and Bsur I (Fermentas; sequence: GT^AC, AG^CT and GG^CC, respectively) and the associated Tango or R restriction buffer (Fermentas) for 2 h at temperatures specified by the supplier. The restriction fragments were resolved on a 2% agarose/TAE gel and visualized using ethidium bromide under UV illumination. A molecular weight ladder was included in each run (100-bp ladder, Invitrogen) (Jensen *et al.*, 2002). The partial sequences of 16S ribosomal RNA gene from 33 isolates are available in the GenBank with the following access numbers: JQ861982 to JQ862014.

Analysis of immune gene expression in larvae by real-time PCR

We evaluated the expression of three immune genes in control and gaping larvae groups obtained from three different incubators. For RNA extraction, larvae were homogenized with TRIZOL (Invitrogen TM) according to the manufacturer's instructions. For the reverse transcription of RNA, we used 1 µg of total RNA plus 50 ng µL^{-1} of oligo-(dT) 12-18 in a volume of 12 µL, containing 1 mM dNTPs, 1 unit µL^{-1} of RnaseOUT (Invitrogen TM) and 200 units µL^{-1} of Moloney murine leukemia virus (M-MLV) reverse transcriptase in buffer (Promega). The cDNAs were amplified using primers derived from conserved regions of genes involved in immunity (Table 4). The amplification program consisted of 10 min at 95°C, followed by 30 cycles of 95°C for 60 s, and 55°C for 60 s, 72°C for 60 s and finally an elongation stage of 72°C for 7 min. The amplified products were analyzed on agarose gels 1.5%, cloned into a Topo TA vector (Invitrogen) and subsequently sequenced (Macrogen, Korea).

We selected the β-actin gene as an internal control and three pairs of primers corresponding to CC chemokine (MIP-1ß), hepcidin and tissue inhibitors metalloproteinases (TIMP) were designed based on the homology between known mammalian and fish sequences, in the translated regions (Table 1). The real-time PCR were performed using dilutions 1/10 of each sample of control and gaping larvae. These reactions were performed in a final volume of 25 µL by adding the same primers used above and adding a solution ready to use SYBR Green PCR Master Mix (Applied Biosystems). The amplification conditions of the Applied Biosystems 7500 thermocycler, consisted of 40 cycles at 94ºC for 10 min, 94°C for 1 min, 55°C for 1 min, 72°C for 1 min. Results were finally analyzed by using the 7.500 program version 2.0.1. The specificity of the PCR reaction was verified in agarose gel 1.5% and the analysis of melting curves. The ratio of the number of copies of each cDNA was determined as the average of three independent replicates. The relative expression of different genes was calculated based where extracted from gaping larvae *versus* normal larvae and expressed in comparison with the reference gene (actin). The relative expression of the genes was calculated using the delta CP method for relative comparison of the results defined as: Ratio = 2 - [ΔCt sample-ΔCt control] = 2-ΔΔCt (Livak & Schmittgen, 2001).

Statistical analysis

Two-way ANOVA was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software,

Table 1. Specific primers used to measure the expression of immune-related genes by qPCR. MIP-1ß and TIMP.

Gene	Primer	Nucleotide sequence (5'→3´)	Product size (bp)
MIP-1β	MIP-1β-F	GAG GAG TGA GCT TCA CAG CA	220
	MIP-1β-R	AGA CAT CCA GAG CCC ACT TG	
B-actin	B-actin-F	ATC GTG GGG CGC CCC AGG	550
	B-actin-R	CAC C	
Hepcidin	Hep-F	CTC CTT AAT GTC ACG CAC GAT	179
	Hep´-R	TTC	
TIMP	TIMP-F	GCC ACC TTT CCT GAG GTA CA	368
	TIMP-R	CTG CAG CAA AGT CCA CAG AC	
		CAA CAG GCT TTT TGC AAC G	
		CAC GAT CTT GCA TTC ACA CC	

San Diego California, USA. Results obtained by restriction fragment plus morphological data were analyzed with the multivariate statistical software Primer 6 (Primer-E Ltd, Plymouth, UK). A similarity matrix was calculated using the simple matching (SM) of presence/absence. Two-way crossed analysis of similarity (ANOSIM), a multivariate randomization procedure analogous to ANOVA, was carried out to examine the statistical significance of grouping (Clarke, 1993). The output statistics, a global R and R values for pair-wise comparisons among groups, take a value of 0 if there is no separation of the community structure due to the factor analyzed, and 1 if total separation takes place (Clarke, 1993). Values higher than 0.5 account for high differences, whereas values between 0.25 and 0.5 differences are considered as moderate.

RESULTS

Comparison of bacterial CFUs between normal and gaping larvae

To evaluate the differences in bacterial load between gaping and normal larvae, we compared the number of bacteria in each, expressed as the total number of colony forming units (CFU) per larvae. The mean of the total number of CFU analyzed in two culture media, MA and R2A, showed a higher number in MA ($4,229x10^3 \pm 1,311x10^3$; n = 38) but not significantly different ($P = 0.164$) to the R2A medium ($2,035x10^3 \pm 0,876x10^3$; n = 38). Significant differences were not found between the two types of larvae (normal or gaping). The average number of CFU larvae^{-1} in MA for normal larvae was $3,226x10^3 \pm 1,601x10^3$ (n = 19) and $5,232x10^3 \pm 2,095x10^3$ (n = 19) for gaping ($P = 0.451$). The R2A media showed an average of $2,136x10^3 \pm 1,039x10^3$ (n = 19) for normal larvae and

$1,935x10^3 \pm 1,441x10^3$ (n = 19) for gaping ($P = 0.910$).

**Number of CFUs according to the morphology presented in different culture media**

The colonies obtained from both media, were characterized by their external morphology, pigmentation, edge, shape and consistency according to the criteria proposed by Freeman (1986). It was possible to recognize nine color types, four edge morphologies and three types of consistency. These colonies were classified into 13 different morphologies (Table 2), 8 obtained from the R2A medium and 5 from the MA medium (Fig. 1). All morphologies identified in the R2A medium were found in both normal and gaping larvae, but significant differences were not found between them (Table 2). In the MA medium, only three morphologies were found in normal larvae and five morphologies in gaping larvae and there were significant differences in abundance of morphologies (Table 3). These differences were showed in terms of bacterial abundance and presence of unique morphologies 12 and 13 in marine agar medium that correspond mainly to *Photobacterium* sp. and *Arthrobacter* sp. (Table 3, Fig. 1). Additionally, the use of TCBS culture medium also presented unique morphological colonies for gaping condition that correspond to *Vibrio* sp. (Table 3).

Genotypes obtained by RFLP and analysis of similarity of bacterial isolates

The use of three restriction enzymes (Rsa I, Alu I and Bsur I) revealed the heterogeneity of the isolates obtained from different media. A total of 43 isolates (21 isolates in MA, 21 in R2A and 1 in TCBS) were obtained from 66 colonies. The similarity analysis, based on RFLP data of bacterial isolates present in the different culture media, (MA, R2A and TCBS)

Table 2. Classification of bacterial morphology present in different culture media. R2A and MA media.

Culture medium	Morphology	Pigmentation	Edge	Form	Consistency
R2A	1	orange	irregular	round	viscose
	2	yellow	smooth	round	viscose
	3	cream	smooth	round	creamy
	4	pink	smooth	round	creamy
	5	transparent	irregular	round	creamy
	6	white	smooth	round	dry
	7	light yellow	irregular	irregular	viscose
	8	light yellow	sawing	round	creamy
MA	9	white	smooth	rhizoid	dry
	10	orange	smooth	round	creamy
	11	damask	irregular	irregular	creamy
	12	brown	lobed	punctate	dry
	13	yellow	sawing	round	viscose
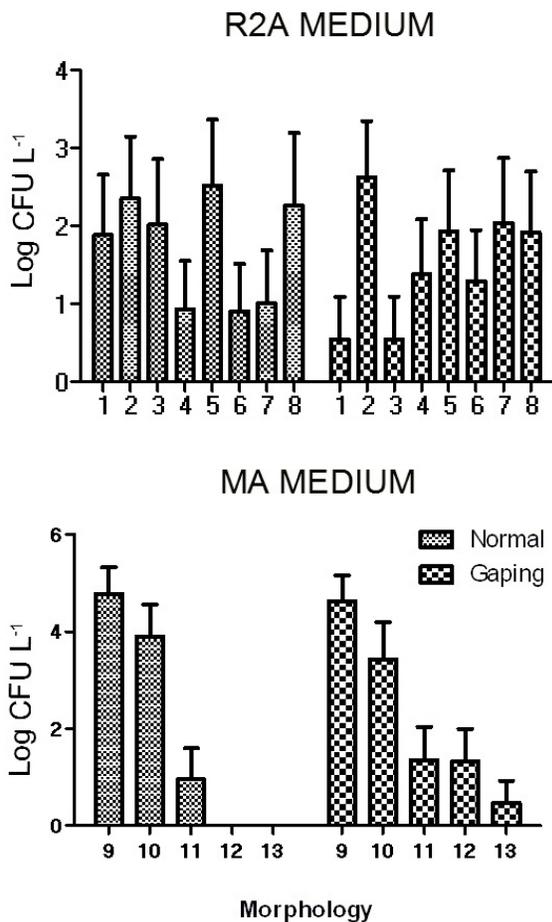
Figure 1. Comparison of the log average number of colony forming units (CFU) per liter based on morphologies recovered from each larval condition in distinct culture medium (numbers correlate to morphology presented in Table 1). R2A and MA media.

showed differences between isolates as a function of culture media (two-way crossed ANOSIM, r = 0.502, P = 0.001), but not in terms of gaping or normal larvae (two-way crossed ANOSIM, r = -0.035, P = 0.868).

Identification of culturable bacteria

The types of bacteria found colonizing the yolk sac larvae, both normal and gaping, are shown in Table 4. Some representative colonies were selected on the basis of the RFLP grouping and phylogenetic identification, based on 16 rRNA gene amplification. Partial sequencing revealed that the flora present was dominated by γ-Proteobacteria in both larvae types, but few bacteria belonging to the Bacteroidetes, Firmicutes and Actinobacteria phyla were also found (Table 4). The culturable bacterial community was highly diverse and complex, but some genera were more dominant than others. Several genera like *Pseudomonas*, *Pseudoalteromonas*, *Arthrobacter* were present in both larvae types (Table 4). However, *Vibrio*, *Photobacterium*, *Flavobacterium* and *Bacillus* genera only occurred in gaping larvae and these may be correlated to jaw deformity.

Immune gene expression

The relative expression of three immune genes was first evaluated by reverse transcription -PCR. The expression of all immune genes was detected in normal and gaping larvae. Quantitative PCR was used to determine whether the level of expression of these immune genes was changing. Using actin as an endogenous control, the gaping larvae showed the strongest relative increase in hepcidin mRNA level in comparison to normal larvae. Hepcidin expression was

Table 3. Analysis of differences in bacterial morphology composition present in normal and gaping larval condition (Two-way analysis of variance). R2A and MA media.

Source of variation		df	Sum-of-squares	Mean square	F	*P*
R2A	Interaction	7	27.92	3.989	0.734	0.644
	Condition	1	1.626	1.626	0.299	0.585
	Morphology	7	42.36	6.052	1.113	0.358
	Residual	144	782.9	5.437		
MA	Interaction	4	9.343	2.336	0.745	0.564
	Condition	1	2.428	2.428	0.775	0.381
	Morphology	4	314	78.490	25.040	$P < 0.0001$
	Residual	90	282.1	3.134		

elevated by a factor of 1196 times. The expression level of MIP1ß in gaping larvae was also significantly increased by the factor of 3.2 compared to normal larvae. However, the TIMP expression in gaping larvae did not show significant difference to normal larvae (Fig. 2).

DISCUSSION

The normal microbial flora of fish larvae may play an important role in disease control and nutrition of marine animals (Cahill, 1990; Jensen *et al*., 2004). The profile of normally occurring, culturable, heterotrophic bacteria may serve as an indicator of the health status of halibut larvae and a stable community may give protection against pathogens such as *Vibrio* species (Bolinches & Egidius, 1987; Bergh *et al*., 1994; Bergh, 1995). The bacterial community of halibut larvae has also been monitored using a combination of molecular techniques like PCR-RFLP of 16S rRNA genes and partial 16S rDNA gene analysis (Jensen *et al*., 2002; Verner-Jeffreys *et al*., 2003).

In order to compare the endogenous flora present in larvae with jaw gaping syndrome, the culture method was employed, and representative colonies differentiated by morphological criteria were characterized using the RFPL-PCR and sequencing of the 16S rRNA genes. The number of culturable bacteria in early yolk sac larvae ranged from $2x10^1$ to $3.6x10^4$ CFU larvae^{-1} which is in a similar range to those obtained in related studies (Verner-Jeffreys *et al.,* 2003; Jensen *et al*., 2004; Bjornsdottir *et al*., 2009). However, the numbers of CFU larvae^{-1} from individual production units were variable, as reported by Bjornsdottir *et al*. (2009). Although the highest number of CFU was found in gaping yolk sac larvae,
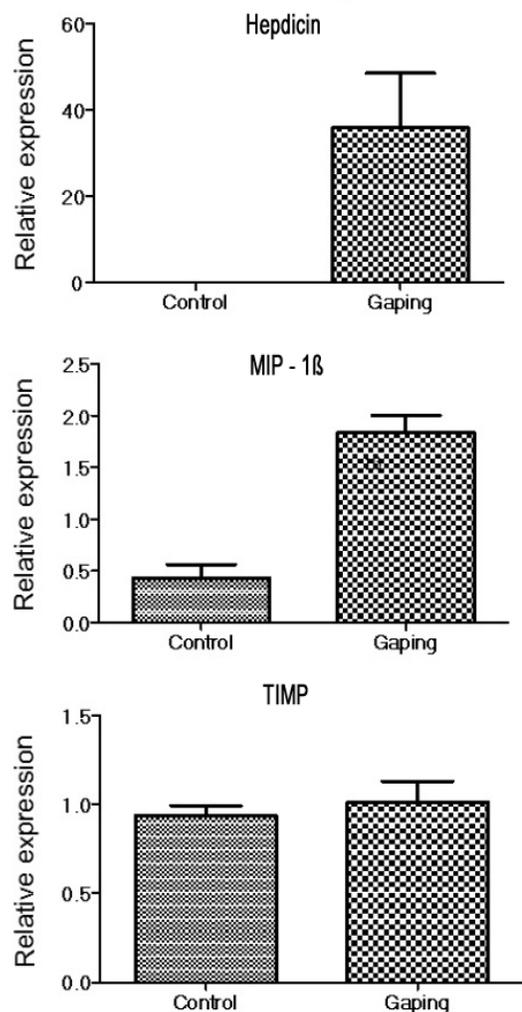
Figure 2. Relative expression in yolk sac larvae of *Hippoglossus hippoglossus* .Pool of five larvae gaping and control were collected from each incubator. The gene expression of Hepcidin, MIP-1ß and TIMP was evaluated by qPCR. The results are means ± SD of three independent incubators.

Table 4. Members of the bacterial community associated with yolk sac larvae in normal (N) and gaping (G) condition. The products identified are represented by 16S rDNA sequences covering 382~966 pb.

Bacterial isolate	Larvae condition	Culture medium	Most related sequences (GenBank accession number)	Morphology	16S rRNA gene sequence similarity in %	Taxonomical position of isolate	Notes/references of most related sequences
BACT-D (JQ861988)	G						
BACT-E (JQ861989)	G	MA	*Shewanella livingstonensis* (HM142581)	11	Identities = 590/590 (100%), Gaps = 0/590 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Shewanellaceae; Shewanella	Isolates from sandy sediment of Nella Fjord, Prydz Bay, Eastern Antarctic (Yu, unpublished)
BACT-F (JQ862008)	G	MA	*Photobacterium* sp. (EU153251)	12	Identities = 383/400 (96%), Gaps = 4/400 (1%)	Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Photobacterium	Isolated from East China Sea seawater (Liu, unpublished)
BACT-G (JQ862000)	G	MA	*Photobacterium* sp. (JF691588)	12	Identities = 681/688 (99%), Gaps = 1/688 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Photobacterium	Isolated from sea water sample from the coast (Agdour, unpublished)
BACT-H (JQ861990)	G	MA	*Photobacterium* sp. (JF691588)	12	Identities = 596/600 (99%), Gaps = 0/600 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Photobacterium.	Isolated from coastal seawater from the coast (Agdour, unpublished)
BACT-I (JQ862007)	G	MA	*Arthrobacter* sp. (AJ551167)	13	Identities = 464/467 (99%), Gaps = 0/467 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter	Isolated from deep sea sediment (Wang, unpublished)
BACT-O (JQ862004)	N	MA	*Lacinutrix* sp. (EU052711)	10	Identities = 382/399 (96%), Gaps = 9/399 (2%)	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Lacinutrix	Isolated from algal culture of Thalassiosira sp. (Hart, unpublished)
BACT-P (JQ862003)	G	MA	*Formosa* sp. (FJ596364)	10	Identities = 390/391 (99%), Gaps = 0/391 (0%)	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Formosa	Associated with marine sponges (Lee *et al.*, 2009)
BACT-Q (JQ862002)	G	MA	*Winogradskyella eximia* (NR_025804)	10	Identities = 395/396 (99%), Gaps = 0/396 (0%)	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Winogradskyella	Marine bacteria (Nedashkovskaya *et al.*, 2005)
BACT-X (JQ862001)	G	MA	*Pseudoalteromonas prydzensis* (HM583997)	9	Identities = 826/829 (99%), Gaps = 0/829 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Pseudoalteromonadaceae; Pseudoalteromonas	Isolated from diseased shrimp broodstock; female. (Gomez-Gil, unpublished)
BACT-Z (JQ861991)	N	MA	*Arthrobacter* sp. (GU733470)	9	Identities = 601/602 (99%), Gaps = 0/602 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter.	Isolated from lake sediment from Antarctica (Shivaji *et al.*, 2011. Res. Microbiol., 162 (2): 191-203)

Continuation

Bacterial isolate	Larvae condition	Culture medium	Most related sequences (GenBank accession number)	Morphology	16S rRNA gene sequence similarity in %	Taxonomical position of isolate	Notes/references of most related sequences
BACT-A1 (JQ862006)	N	MA	Colwellia sp. (GQ452867)	9	Identities = 423/423 (100%), Gaps = 0/423 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Colwelliaceae; Colwellia	Isolated from Kongsfjorden seawater (Zeng, unpublished)
BACT-*2 (JQ862010)	G	MA	Nautella sp. (FJ161342)	9	Identities = 396/400 (99%), Gaps = 1/400 (0%)	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Nautella.	Isolated from culturable bacterial communities in Shandong coast, China (Du, et al., unpublished)
BACT-*6 (JQ862005)	N	MA	Shewanella sp. (FJ196028)	11	Identities = 400/400 (100%), Gaps = 0/400 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Shewanellaceae; Shewanella	Isolated from Antarctic Ocean (Yu et al., 2010)
+BACT-V1 (JQ861993)	G	TCBS	Vibrio sp. (FM957480)	-	Identities = 368/419 (88%), Gaps = 15/419 (4%)	Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;Vibrionaceae; Vibrio	Isolated from Indian Ocean water of shrimp pond (Yuhana, unpublished)
BACT-V2 (JQ861994)	G	TCBS	Vibrio alginolyticus (EU834002)	-	Identities = 457/461 (99%), Gaps = 2/461 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;Vibrionaceae; Vibrio	Isolated from coastal waters in Fish Farm, Long Harbor (Ki et al., 2009)
BACT-8 (JQ861983)	G	R2A	Bacillus sp. (JF343174)	5	Identities = 502/502 (100%), Gaps = 0/502 (0%)	Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus	Isolated from Chilka Lake water (Saxena et al., unpublished)
BACT-10 (JQ861986)	G	R2A	Flectobacillus lacus (HM032866)	2	Identities = 719/721 (99%), Gaps = 1/721 (0%)	Bacteria; Bacteroidetes; Cytophagia; Cytophagales; Cytophagaceae; Flectobacillus	Isolated from water natural environments (Chun, unpublished)
BACT-14 (JQ861982)	N	R2A	Kocuria rhizophila (HQ641339)	2	Identities = 499/500 (99%), Gaps = 0/500 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Kocuria	Isolated from larviculture system in UMS hatchery (Ibrahim & Al-Azad, unpublished)
BACT-17 (JQ861995)	G	R2A	Pseudomonas migulae (EU111700)	7	Identities = 388/391 (99%), Gaps = 2/391 (1%)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	Isolated from rhizosphere antagonistic bacteria (Chen et al., unpublished)
BACT-18 (JQ861996)	G	R2A	Pseudomonas sp. (JF799932)	7	Identities = 353/359 (98%), Gaps = 3/359 (1%)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	Isolated from active-layer in permafrost the Qinghai-Tibet plateau highway (Li et al., unpublished)
BACT-19 (JQ862011)	G	R2A	Pseudomona sp. (JF523583)	7	Identities = 550/550 (100%), Gaps = 0/550 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	Isolated from nitrate-contaminated groundwater (Huang et al., unpublished)
BACT-21 (JQ862009)	N	R2A	Pseudomonas sp. (JF767410)	8	Identities = 580/580 (100%), Gaps = 0/580 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.	Isolated from intestinal microbiota of Atlantic salmon (Godoy & Wittwer, unpublished)

Continuation

Bacterial isolate	Larvae condition	Culture medium	Most related sequences (GenBank accession number)	Morphology	16S rRNA gene sequence similarity in %	Taxonomical position of isolate	Notes/references of most related sequences
BACT-23 (JQ861997)	G	R2A	Pseudomonas sp. (JF767410)	8	Identities = 630/630 (100%), Gaps = 0/630 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.	Isolated from intestinal microbiota of Atlantic salmon (Godoy & Wittwer, unpublished)
BACT-24 (JQ861998)	G	R2A	Pseudomonas sp. (GU784935)	8	Identities = 342/362 (94%), Gaps = 8/362 (2%)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	Isolated from soil (Bazhanov & Yatsevich, 2011. Microbiol., 80 (1): 89-95)
BACT-25 (JQ861999)	N	R2A	Brevundimonas sp. (GU377106)	1	Identities = 966/970 (99%), Gaps = 2/970 (0%)	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Brevundimonas.	Isolated from bacteria from the Shapotou region, China (Zhang, unpublished)
BACT-27 (JQ861984)	G	R2A	Flavobacterium sp. (FJ517631)	1	Identities = 500/500 (100%), Gaps = 0/500 (0%)	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Flavobacterium	Isolated from Antarctica: King George Island soil (Tam & Wong, unpublished)
BACT-28 (JQ862013)	G	R2A	Flavobacterium degerlachei (EU000232)	3	Identities = 504/504 (100%), Gaps = 0/504 (0%)	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Flavobacterium	Arctic marine bacteria (Lee, unpublished)
BACT-29 (JQ861985)	N	R2A	Pseudomonas fluorescens (HQ647251)	3	Identities = 501/501 (100%), Gaps = 0/501 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	Bacteria from Dongxiang wild rice (Fei, unpublished)
BACT-31 (JQ862012)	N	R2A	Pseudomonas sp. (JF523583)	3	Identities = 501/501 (100%), Gaps = 0/501 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	Isolated from nitrate-contaminated groundwater (Huang et al., unpublished)
BACT-33 (JQ861992)	G	R2A	Bacillus sp. (HQ173714)	4	Identities = 464/469 (99%), Gaps = 3/469 (1%)	Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus	Microorganisms in soil (Situmorang et al., unpublished)
BACT-40 (JQ862014)	G	R2A	Flavobacterium aquatile (AB517711)	2	Identities = 747/751 (99%), Gaps = 2/751 (0%)	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Flavobacterium	Unpublished
BACT-41 (JQ861987)	G	R2A	Moraxellaceae bacterium (GU731671)	5	Identities = 536/541 (99%), Gaps = 2/541 (0%)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae.	Isolated from seawater (Oh et al., 2011. Int. J. Syst. Evol. Microbiol., 61 (PT 6): 1382-1385.)

there was not a significant difference compared to normal larvae.

The *Vibrio*, *Photobacterium* and *Shewanella* are the three closely related genera of the Vibrionaceae family that have several pathogenic species. The *Vibrio* spp. has been reported as a common bacteria isolated from halibut (Bolinches & Egidius, 1987; Bergh *et al*., 2001; Verner-Jeffreys *et al*., 2003). However, in our work that is based on a culture-dependent approach, this genus was found only in larvae with gaping condition when a specific medium for vibrio was used. One of these isolates has high similarity with *Vibrio alginolyticus* (morphology V2, see Table 4) which is considered a marine pathogen (Colwell & Grimes, 1984; Toranzo *et al*., 2005). In the same way, some *Shewanella* and *Photobacterium* species are also considered opportunistic pathogens of aquatic animals. *Shewanella algae*, *S. putrefaciens*, *S. marisflavi* and *Photobacterium damselae* have been isolated from oyster, sea cucumber and water (Richards *et al*., 2008; Li *et al*., 2010).

Most species of *Flavobacterium* are harmless, but some are opportunistic or true pathogens. Many representatives of the family Flavobacteriaceae are involved in gill disease. *Flavobacterium aquatile*, *F. johnsoniae*, *F. hydatis*, and *F. succinicans* have been associated with fish disease and have also been detected in surrounding water during disease outbreaks (Bernardet *et al*., 1996; Bernardet & Bowman, 2006). However, little information about *Bacillus* has been reported as a pathogen in fish. As an example, *Bacillus mycoides,* isolated from moribund catfish (*Ictalurus punctatus*) is responsible for necrotic muscle lesions after a subcutaneous injection (Goodwin *et al.*, 1994). Despite the fact that culturable *Bacillus* strains were only detected in larvae with gapping condition, we cannot yet make a direct association with the gapping condition. Several probiotic studies include strains of spore forming *Bacillus* that possess adhesion abilities, capable of producing bacteriocins (antimicrobial peptides) (Cherif *et al*., 2001; Cladera-Olivera *et al*., 2004; Duc *et al*., 2004) and their presence could be associated to modular response linked to the probiotic functions of the *Bacillus* genera. In the same way, *Pseudoaltero-monas* is usually reported as belonging to the normal flora and not related to pathogenic events in fish due to their capability of producing biologically active compounds with antibacterial, algicidal, antifungal, agarolytic, cytotoxic and antiviral activities (Holmstrom & Kjelleberg, 1999; Kalinovskaya *et al*., 2004). However, some species like *P. undina,* have also been reported as a pathogen for marine fish (Pujalte *et al*., 2007). *Pseudomonas* is also present in normal flora and is capable of producing active compounds. This bacterial genus is present in normal probiotic bacterial flora due to their ability to adhere and colonize the surface of tissues, forming a barrier against pathogenic bacteria (Kesarcodi-Watson *et al*., 2008; Korkea-aho *et al*., 2011).

Members of the genera *Moraxella* were found only on halibut eggs by Hansen & Olafsen (1989). Infections by *Moraxella* sp. are becoming common in Mediterranean aquaculture plants causing metabolic alterations in fish and their bacterial colonization of internal organs can affect fish wellness and decrease growth rate, stress resistance, and immune response (Baya *et al.*, 1990; Addis *et al*., 2010). Further study is needed for a better metabolic characterization of these bacteria isolates and in order to confirm their phylogenetic relationships. Physiological-biochemical characteristics, optimal growth temperature, different pH or salt concentration could help us to identify the bacterial strains and complement the 16S rRNA information.

During the early life stages of larvae fish, the defense mechanism is based on an innate immune response (Magnadóttir, 2006). Many fish larvae have poorly developed immune response and are exposed to pathogens before their lymphoid organs have matured and adaptive immunity has developed (Seppola *et al*., 2009), even though these larvae may be protected by maternally transferred components, like lectins and antibodies (Bly *et al*., 1986; Avtalion & Mor, 1992; Seppola *et al*., 2009). The halibut has been characterized by several defense gene components like immunoglobulin light chain, MHC class I and II, antimicrobial peptide, complement system, CC chemokine and B-cell factor (Park *et al*., 2005). Little information exists about the innate effectors, such as, the antimicrobial peptide in halibut yolk sac larvae. Maternal immunity transferred as RNA could not explain the high levels of hepcidin or MIP-1 expression in gaping condition; therefore it could be associated with induction of the larvae itself.

The antimicrobial peptides like hepcidin play a key role in the natural defense against invading microorganisms and iron metabolism (Ganz, 2011). The hepcidin gene is widely expressed in various fish, suggesting that this antimicrobial peptide is a very important component in the innate immune system. During metamorphosis of the Atlantic cod, hepcidin showed a high level of transcriptional up-regulation at 1295 hpf (Seppola *et al.*, 2009). The expression in other fish of this AMP is up regulated in several tissues and organs in response to a bacterial challenge (Shike *et al*., 2002; Park *et al*., 2005; Kim *et al*., 2008). Constitutive expression of hepcidin was seen in

the normal yolk sac larvae but their expression increased dramatically in the gaping condition. The elevated expression in this condition could indicate an increase in pathogen exposure, but further studies are necessary to establish whether this response was produced by a specific pathogen similar to bacteria present in the gaping condition, like *Photobacterium* and *Vibrio* genera. Our findings seem to be in agreement with bacterial challenge experiments performed in the sea bass (*Dicentratchus labrax*), indicating that hepcidin expression increases after injection with *Photobacterium damselae* (Rodrigues *et al*., 2006).

The expression in yolk sac larvae of Macrophage inflammatory protein (MIP)-1 was evaluated for the first time in yolk sac larvae. The MIP-1 is part of CC chemokine subfamily and these chemokines are produced by many cells, particularly macrophages, dendritic cells, and lymphocytes. The mature halibut MIP-1 was identified by Park *et al*. (2005), and their expression was evaluated in three different tissues which showed an induction after vaccination against *Vibrio* and *Aeromonas*. In Japanese flounder and rainbow trout, the CC chemokine increased their expression by DNA injection and LPS, respectively (Kono *et al*., 2003; MacKenzie *et al*., 2004). The MIP-1 gene in halibut larvae could be implicated in the migration of phagocytic cells and activation of macrophages.

Matrix metalloproteinases (MMPs) are responsible for proteolytic degradation of specific extracellular matrix (ECM) components. These MMPs could be involved in the embryonic development, morphogenesis and tissue remodeling (Kubota *et al*., 2003). On the other hand, the tissue inhibitors of metalloproteinases (TIMPs) are involved in the down-regulation of MMPs activities; therefore, TIMPs play a critical role in the regulation of ECM degradation. The bacterial proteinases may play an important role in tissue destruction and disintegration of extracellular matrix at the site of infections. In addition, several fish and mollusk proteinase produced by bacteria could be activated by the expression of TIMPs. In this context, TIMPs could increase their expression against degradation processes of the larva´s ECM, produced for example, by bacteria present in the gapping condition. Given the importance of these proteins, little information exists on the presence of TIMPs in teleost fish and in the Atlantic halibut, the sequence of TIMP has not been reported (Kubota *et al*., 2003; Lodemel *et al*., 2004). In halibut, TIMP was constitutively expressed in normal larvae; the lack of change in the expression of TIMP in gaping larvae may indicate that there is not an activation process of

degradation of the ECM by bacterial proteinase. The TIMP expression and their protein function in the larvae stage needs to be resolved.

Several studies on the gaping phenomenon have been made, which have tried to relate the temperature, salinity, density of larvae with this condition. However, the factor (biotic or abiotic) that causes this malformation is still not clear. Further studies could use bacteria isolated in the present study to address the extent to which biotic or abiotic can induce the gaping condition in larvae.

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