16S rDNA-Based Analysis of Dominant Bacterial Populations Associated with Early Life Stages of Coho Salmon (*Oncorhynchus kisutch*)

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Abstract

In this study, we used a 16S rDNA–based approach to determine bacterial populations associated with coho salmon (*Oncorhynchus kisutch*) in its early life stages, highlighting dominant bacteria in the gastrointestinal tract during growth in freshwater. The present article is the first molecular analysis of bacterial communities of coho salmon. Cultivability of the salmon gastrointestinal microbiota was estimated by comparison of direct microscopic counts (using acridine orange) with colony counts (in tryptone soy agar). In general, a low fraction (about 1%) of the microbiota could be recovered as cultivable bacteria. Using DNA extracted directly from individuals belonging to the same lot, bacterial communities present in eggs and gastrointestinal tract of first-feeding fries and juveniles were monitored by polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE). The DGGE profiles revealed simple communities in all stages and exposed changes in bacterial community during growth. Sequencing and phylogenetic analysis of excised DGGE bands revealed the nature of the main bacteria found in each stage. In eggs, the dominant bacteria belonged to β-Proteobacteria (*Janthinobacterium* and *Rhodoferax*). During the first feeding stage, the most abundant bacteria in the gastrointestinal tract clustered with γ-Proteobacteria (*Shewanella* and *Aeromonas*). In juveniles ranging from 2 to 15 g, prevailing bacteria were *Pseudomonas* and *Aeromonas*. To determine the putative origin of dominant *Pseudomonas* and *Aeromonas* found in juvenile gastrointestinal tracts, primers for these groups were designed based on sequences retrieved from DGGE gel. Subsequently, samples of the water influent, pelletized feed, and eggs were analyzed by PCR amplification. Only those amplicons obtained from samples of eggs and the water influent presented identical sequences to the dominant bands of DGGE. Overall, our results suggest that a stable microbiota is established after the first feeding stages and its major components could be derived from water and egg epibiota.

Introduction

Commercial finfish aquaculture in Chile began in the middle of the 1980s and Chile is currently a leading producer worldwide. The main products are Atlantic salmon (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*). This production is hampered by unpredictable mortalities during the early life stages that may be due, at least in part, to negative interactions between salmon larvae and the bacteria they routinely encounter. Understanding the bacterial ecology of these farmed fish could help to improve both the management of hatcheries for higher productivity and the safety of salmon as food.

It is generally recognized that gastrointestinal microbiota of homoeothermic animals serves several functions, namely, digestion and development of the mucosal system, angiogenesis, and protection barrier against disease [17, 22, 36, 38, 45]. A recent report showed that microbiota in fish can regulate the expression of 212 genes in the digestive tract of zebrafish, some of them related to stimulation of epithelial proliferation and promotion of nutrient metabolism and innate immune response [28]. An important aspect of these results was the specificity of the host response, which depends on the bacterial species that colonize the digestive tract [28]. Hence, it is relevant to know the composition of this microbiota in fish. Several authors have investigated the composition of

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digestive tract microbiota [2, 18, 25, 30, 42]; however, these studies have been mostly carried out by culture-dependent methods [41], and identification of the fish microbiota has typically been based on phenotypic and biochemical characteristics [7, 40]. Furthermore, a striking characteristic of indigenous bacteria in many environments is the lack of cultivability of the majority of the living bacteria [1, 39]. Recently, several molecular techniques have been developed to study natural samples. These methods have allowed for the identification of microorganisms without isolation and for the determination of the phylogenetic affiliation of community members, revealing the enormous extent of microbial diversity. Methods based on the amplification of fragment coding for 16S rRNA have emerged as a powerful tool [16]. Specifically, denaturing gradient gel electrophoresis (DGGE) has been introduced into molecular microbial ecology to determine the genetic diversity of natural microbial communities. Recently, this technique was used to characterize the presumed dominating microorganisms in haddock [14] and Atlantic halibut larvae [20] as well as intestinal microbiota of rainbow trout intestine [19].

Little is known about the early steps of colonization of the gastrointestinal tract of fish, the establishment of normal microbiota and its stability, especially after dietary changes or treatment with antibiotics, which are routine practices in aquaculture. Understanding some aspects of microbial ecology in aquaculture systems, such as knowing the types, numbers, and sources of bacteria commonly associated with different developmental stages, could be useful for manipulating microbiota as a strategy to prevent pathogenic infection or to improve nutrition. In this study, we used a 16S rDNA-based approach to determine the bacterial community associated with the early life stages of coho salmon and to define the changes in dominant populations during growth in freshwater. The bacterial community present in eggs, first-feeding fries, and juveniles was determined using PCR–DGGE analysis from DNA extracted directly from the sample. The dominant bacterial populations were recognized as intense bands in each DGGE pattern. Furthermore, we identified dominant cultivable bacterial microbiota by sequencing 16S rDNA and a comparative sequence analysis. The retrieved sequences were used for the construction of a set of primers, which were subsequently applied to detect dominant bacterial groups by PCR amplification from samples of pelletized feed, water, and eggs.

Materials and Methods

Sample Collection and Homogenate Preparation. Eggs and gastrointestinal tract samples of first-feeding fries and juveniles were sampled from a hatchery (latitude 32° S, Chile). All salmon samples belonged to the same salmon lot, which was followed up during growth. The examination started in July and was terminated in October 2003. For each sample (eggs, first-feeding fries, and juveniles) 20 specimens with similar weight were collected in sterile plastic recipients and transported to the laboratory on ice. The weight of first-feeding fry specimens was about 0.5 g and those of juvenile samples ranged from 2 to 15 g. Simultaneously, freshwater samples were obtained directly from the aquifer access, the hatchery's water source (water influent). Pelletized feed currently consumed by sampled fish was also collected and transported on ice. Samples were processed immediately upon arrival in the laboratory.

The fishes were size graded and divided into eight groups, each composed of 10 specimens with an average weight of 2, 3.5, 4, 5, 7, 9, 13, and 15 ± 0.1 g. Gastrointestinal samples of first-feeding fries and juveniles were obtained by aseptically dissecting the fish and carefully extracting the entire gastrointestinal tract under stereomicroscope. In first-feeding fries, the gastrointestinal tract consisted mainly of a poorly developed tube from mouth to anus, with slight enlargement where the stomach will develop. The gastrointestinal tract of juveniles consisted of a U-shaped stomach, an early-developed pylorus region, and intestine. After dissection, the portion including stomach to anus was separated for analysis. Gastrointestinal tracts from fishes of the same size were pooled for analysis of bacterial communities. Individual gastrointestinal tracts were also obtained and analyzed to study the interindividual variation. Eggs and gastrointestinal tissues were shucked and weighed, then an equal amount of cold sterile 0.9% (w/v) NaCl was added; this mixture was subsequently homogenized in an ice bath with a vortex for 3 min. A similar process was used to prepare food homogenates.

Bacterial Counts and Cultivation. Total bacterial counts present in salmon samples (eggs and gastrointestinal tract of juveniles) and water were performed by epifluorescence microscopy using acridine orange, as previously described [32]. Serial dilutions of homogenates were plated in trypticase soy agar (TSA, Difco, Sparks, MD, USA) and the plates were incubated for 5–10 days at 17°C in aerobic conditions. Colonies were counted after 10 days and the colony-forming unit (CFU) per gram of eggs or gastrointestinal tract was calculated. The colonies from each sample were selected according to the most dominant colony morphology and then identified by molecular methods described below.

DNA Extraction and Purification. DNA from salmon samples was obtained from homogenates by lysis using sodium dodecyl sulfate and incubation at 70°C. The lysates were extracted with phenol/chloroform and

subsequently precipitated with ethanol as previously described [33]. A final purification was carried out using Wizard DNA Clean Up (Promega, Madison, WI, USA). DNA from water supply samples was obtained by filtering 5 L through a 0.2-μm filter, bacteria were resuspended in TE buffer (Tris 0.01 M, EDTA 0.001 M, NaCl 0.15 M, pH 7.8), and lyses were performed as described above. DNA from pelletized feed was obtained by homogenizing 15 g in TE buffer followed by PCR amplification.

PCR Amplification. To obtain fingerprints of the bacterial communities present in different samples, the extracted DNA was PCR-amplified using conserved 16S rDNA bacterial domain-specific primers 341F (5′-GCCTACGGGAGGCAGCAG-3′ with GC clamps in its 5′ end) and 907R (5′-CCGTCAATTCMTTTGAGTTT-3′) as previously described [24]. PCR reactions were performed as described [35] with a reaction mixture (30 μL) containing 0.2 mM of each deoxynucleoside triphosphate, 0.05 U/μL Platinum *Taq* DNA polymerase (Invitrogen, San Diego, CA, USA), 1× polymerase reaction buffer, 2 mM $MgCl_2$, and 0.25 pmol/μL of each primer. To identify bacterial isolates, amplification of 16S rRNA from positions 28 to 1492 was performed according to Espejo *et al.* [12] using primers previously described [10]. PCR products were analyzed by polyacrylamide electrophoresis and silver nitrate staining as previously described [11].

Restriction Fragment Length Polymorphism Analysis. Isolates obtained from each sample (egg, first-feeding, and juvenile) were analyzed and grouped according to their 16S rDNA restriction fragment length polymorphism (RFLP) profile. Products of the 16S rDNA PCR amplification were digested for 2 h at 37°C with 1.5 U of *Alu*I restriction endonuclease (Invitrogen). The resulting fragments were subsequently analyzed by polyacrylamide gel electrophoresis and silver nitrate staining as described [11].

DGGE Analysis. PCR products obtained from 341F and 907R primers were separated on a 6% (w/v) polyacrylamide gel in a TAE 1× running buffer (Tris–acetate 0.04 M, EDTA 0.002 M, pH 8.5) and a denaturing gradient from 30 to 60% or 40 to 60% of urea and formamide. Electrophoresis was run for 16 h at 85 V with a constant temperature of 60°C in a D-Code System (Bio-Rad, Hercules, CA, USA). After electrophoresis, gels were stained by 1 h of incubation with Sybr Green at room temperature.

Sequence Analysis. The dominant bands were recognized as intense bands in each DGGE pattern and were excised from the gel and eluted overnight in 50 μL of MilliQ water; 1 μL was used for reamplification. To test the presence of similar amplicons, bands showing the same migration in different lanes were digested with *Alu*I as described above. 16S rDNA from the reamplified bands or from the bacterial isolates were purified using Wizard PCR Preps (Promega) and then sequenced with an Applied Biosystems 310 automatic sequencer (Foster City, CA, USA). The ABI Prism dye terminator sequencing kits were used with primers 907R for the eluted bands as well as for bacterial isolated genes. Sequences were deposited in GenBank (accession numbers AY707641–AY707648, AY745885–AY745889, AY745891–AY745892) and aligned with reference sequences, using Sequence Match software from the Ribosomal Database Project II (RDP II) Web site [8]. Distance matrices were constructed from the aligned sequences and corrected for multiple base changes at single nucleotide positions by the method of Jukes and Cantor included in the TREECON program [43]. Using the same program, a phylogenetic tree was constructed by neighbor-joining method. Bootstrapping was performed using the bootstrap modus of the program, and values above 40% are reported.

Primer Design. Using the 16S rDNA sequence of dominant bacteria obtained from DGGE analysis, specific forward primers to detect *Pseudomonas* (PsF) and *Aeromonas* (AeF) were designed by using the Primer3 Software [34]. Primer selection was based on the primer's capability to hybridize *in silico* specifically with the corresponding sequence retrieved from the DGGE profile and simultaneously nonmatching with other *Pseudomonas*/*Aeromonas* sequences present in the RDP II database. The corresponding positions in *Escherichia coli* numbering of primers were as follows: PsF, 441–458; AeF, 578–595. Sequences of primers were as follows: PsF, 5′-AGT TGGGAGGAAGGGTTGT-3′; AeF, 5′-CACGCAGGCG GTTGGATA-3′. To check for specificity, the selected primer target sites were compared to all available 16S rDNA sequences by using the latest version of the probe match function at the RDP II (http://rdp.cme.msu.edu/html/). For *Pseudomonas* detection, thermal cycling was applied in 30 cycles of 1 min each at 95, 63, and 72°C, then a final extension for 5 min at 72°C. Annealing at 62°C was used for *Aeromonas* amplification. Since 907R was used as reverse primer, amplicons of about 480 bp for *Pseudomonas* and 350 bp for *Aeromonas* were expected. PCR products were sequenced as described above to confirm the identities of the amplicons. Simultaneously, bacterial isolates from these samples were tested with these primers to detect *Pseudomonas* and *Aeromonas*.

Results

Bacterial Enumeration. The average total bacterial density yielded 3×10^7 bacteria per egg and 2×10^5 bacteria per gram of juvenile gastrointestinal tract. The average

counts of cultivable bacteria were 10^5 CFU per egg and 6×10^3 CFU per gram of juvenile gastrointestinal tract. Total bacterial counts from the water supply were 1×10^6 bacteria/mL, whereas cultivable counts were 7×10^4 CFU/mL. In pelletized feed samples, 3×10^3 CFU/g was obtained. Considering all samples assessed, the cultivability was about 1%.

Analysis of PCR–DGGE Profiles. To study the diversity and the dynamics of the dominant bacterial communities during early growth of coho salmon, samples from eggs and gastrointestinal tract of first-feeding fries and juveniles were studied with PCR–DGGE. Figure 1 shows the DGGE profiles of PCR-amplified 16S rDNA obtained from DNA extracted directly from salmon tissues. These profiles revealed a small number of distinguishable bands per sample, reflecting a relatively simple and specific structure of bacterial community in each stage. The profile present in eggs (Fig. 1A, lanes Egg^1 and Egg^2) was remarkably different from that obtained from the gastrointestinal tract of fishes. Dominant amplicons in egg samples (band E1 and E2) disappeared in

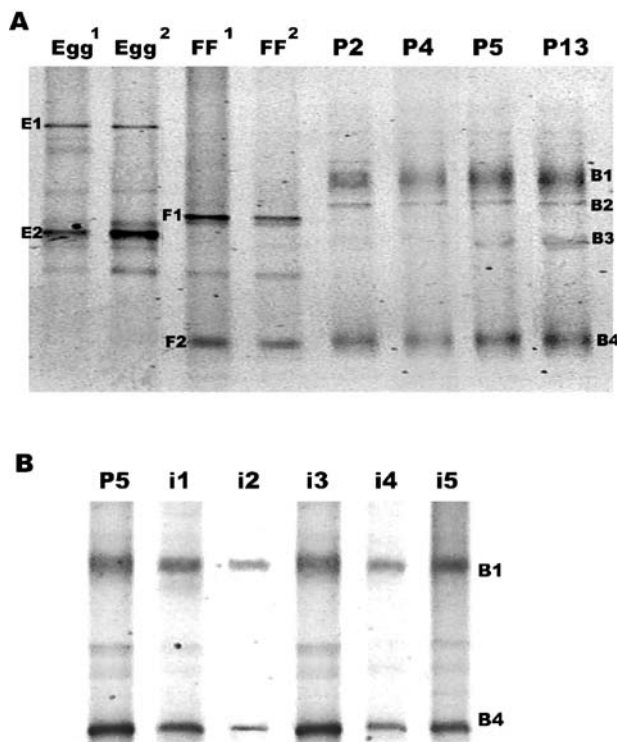

Figure 1. DGGE profiles of 16S rDNA amplification products from early stages of coho salmon (*O. kisutch*). (A) DGGE profiles of eggs (*lanes Egg^1* and *Egg^2*), first-feeding fries (*lanes FF^1* and *FF^2*), and juveniles of several sizes (pool of 2 g: *P2*; 4 g: *P4*; 5 g: *P5*; and 13 g: *P13*). (B) DGGE profiles of pooled samples (*lane P5*, pooled gastrointestinal tracts from 5 g fish) compared to individuals fish samples of 5 g (*lanes i1*–5).

both first-feeding fries (FF^1 and FF^2) and juveniles (P2, P4, P5, and P13). On the other hand, in juveniles ranging from 2 to 15 g, the DGGE patterns obtained were almost identical among them, suggesting that they maintain similar bacterial community throughout this stage. We also studied the DGGE profile of the gastrointestinal tract of individual juveniles. Figure 1B shows that the bacterial patterns of different individuals were similar to those observed in the pools. Bands with the same electrophoretic migration were eluted and reamplified with 341F and 907R primers. Amplicons showed identical restriction patterns when they were digested with *Alu*I (data not shown).

Identification of Dominant Band in DGGE by Sequence Analysis. The more intense bands detected in DGGE (band E1, E2, F1, F2, B1, B2, B3, and B4, Fig. 1A), which were indicative of the most abundant populations of each sample, were excised, reamplified, and sequenced. Partial 16S rDNA sequences of approximately 500 bases were directly obtained from reamplified bands. These sequences were compared with sequences available in RDP II (Table 1), and the phylogenetic affiliation of the sequences was further analyzed by Treecon version 1.3b (Fig. 2). Our results indicated that organisms represented by the main bands in egg samples belong to the class β-Proteobacteria. They were specifically related to the genera *Janthinobacterium* and *Rhodoferax*. In contrast, the main bands from the gastrointestinal tract in the first-feeding stage clustered with γ-Proteobacteria and corresponded to the genera *Shewanella* and *Aeromonas*. The principal bands obtained from gastrointestinal of juveniles also clustered with γ-Proteobacteria and belonged to *Aeromonas* and *Pseudomonas*. Two additional weak bands (B2 and B3) were also observed and were related to *Bacillus* and *Photobacterium*, respectively.

Identification of Bacterial Isolates by 16S rDNA Analysis. Cultivable bacterial populations were isolated in TSA from samples of eggs, first-feeding specimens, and juvenile gastrointestinal tracts. Twenty isolates per sample were typed by RFLP of PCR-amplified 16S rDNA using *Alu*I. Four RFLP groups were obtained from egg samples, each comprising five bacterial isolates showing identical RFLP profile. For first-feeding fries and juveniles, two RFLP groups were obtained, respectively, each comprising 10 bacterial isolates. One bacterial isolate from each RFLP group was selected and partial 16S rDNA sequences were obtained. Comparison with sequences available in the RDP database revealed values highly similar to those of described taxa (Table 1). Analyses revealed that isolates recovered in egg samples were closely related to the *Flavobacterium* and *Pseudomonas* genera. Isolates from the gastrointestinal tract of first-feeding fries were related to the genera *Exiguobacterium* and *Pseudomonas*,

Table 1. **Nearest-match identification of 16S rDNA sequences obtained with molecular and culture approaches from coho salmon (*O. kisutch*) to known sequences in the RDP II database**

Name	*Accession no.*	*% Identity*	*Affiliation phylum/class*	*Closest sequence*
Bands				
Egg band 1 (E1)	AY745891	97	Proteobacteria/ β-Proteobacteria	Unclasiffied oxalobacteraceae glaciar bacterium FXS9 (AY315179)
		96		*Janthinobacterium* sp. (AY212674)
Egg band 2 (E2)	AY745892	97	Proteobacteria/ β-Proteobacteria	*Rhodoferax ferrireducens* T118 (AF435948)
First-feed fries band 1 (F1)	AY745885	94	Proteobacteria/ γ-Proteobacteria *Shewanella decolorationis* S12 (AJ609571)	
First-feed fries band 2 (F2)	AY745884	96	Proteobacteria/ γ-Proteobacteria	*Aeromonas* sp. (AB076859)
		96		*Haemophilus piscium* NCIMB 1952 (AJ009860)
Juvenile pool band 1 (B1)	AY745886	99	Proteobacteria/ γ-Proteobacteria	*Pseudomonas* sp. (AY331379)
Juvenile pool band 2 (B2)	AY745887	93	Firmicutes/bacilli	*Bacillus thuringiensis* (Z84587)
Juvenile pool band 3 (B3)	AY745888	99	Proteobacteria/ γ-Proteobacteria	*Photobacterium phosphoreum* (AY292916)
		99		*Vibrio* sp. (AY542526)
Juvenile pool band 4 (B4)	AY745889	100	Proteobacteria/ γ-Proteobacteria	*Aeromonas* sp.
		100		*Haemophilus piscium* NCIMB 1952 (AJ009860)
Isolates				
Egg isolate 1 (Eis1)	AY707641	98	Bacteroidetes/ flavobacteria	*Flavobacterium* sp. (AF321008)
Egg isolate 2 (Eis2)	AY707642	98	Bacteroidetes/ flavobacteria	*Flavobacterium* sp. (AF493649)
Egg isolate 3 (Eis3)	AY707643	99	Proteobacteria/ γ-Proteobacteria	*Pseudomonas* sp. (AF456220)
Egg isolate 4 (Eis4)	AY707644	97	Bacteroidetes/ flavobacteria	*Flavobacterium* sp. (AF493646)
		97		Rainbow trout intestinal bacterium (AY374109)
First feed fries isolate 1 (Fis1)	AY707645	99	Proteobacteria/ γ-Proteobacteria	*Pseudomonas* sp. (AY121983)
First feed fries isolate 2 (Fis2)	AY707646	99	Firmicutes/bacilli	*Exiguobacterium* sp. 5138 (AY831656)
Juvenile pool isolate 1 (Pis2)	AY707647	98	Proteobacteria/ γ-Proteobacteria	*Pseudomonas* sp. (AY331345)
Juvenile pool isolate 2 (Pis3)	AY707648	99	Proteobacteria/ γ-Proteobacteria	*Pseudomonas* sp. (AJ291841)

whereas those from the gastrointestinal tract of juvenile samples were related to *Pseudomonas*.

Origin of Dominant Gastrointestinal Bacterial Populations. To determine the possible sources of the most abundant bacteria found in the gastrointestinal tract of juvenile salmon, DNA was extracted from several water and food samples and their PCR–DGGE profiles were obtained. Principal bands detected in gastrointestinal samples were not observed in the profiles from these samples (data not shown). Therefore, to detect the prevalent bacteria of the gastrointestinal tract of salmon and learn about their putative origin, samples of water influent, pelletized feed, and eggs were analyzed by specific 16S rDNA PCR amplification and subsequent sequencing of the amplicons. Primers to detect the *Pseudomonas* (PsF) and *Aeromonas* (AeF) groups were designed based on the 16S rDNA sequence of dominant bands obtained from DGGE analysis (*Pseudomonas*, B1; *Aeromonas*, B4). Primer specificity was assessed by comparison to all available 16S rDNA sequences deposited in the RDP II. Probe match analysis indicated that the PsF primer matched

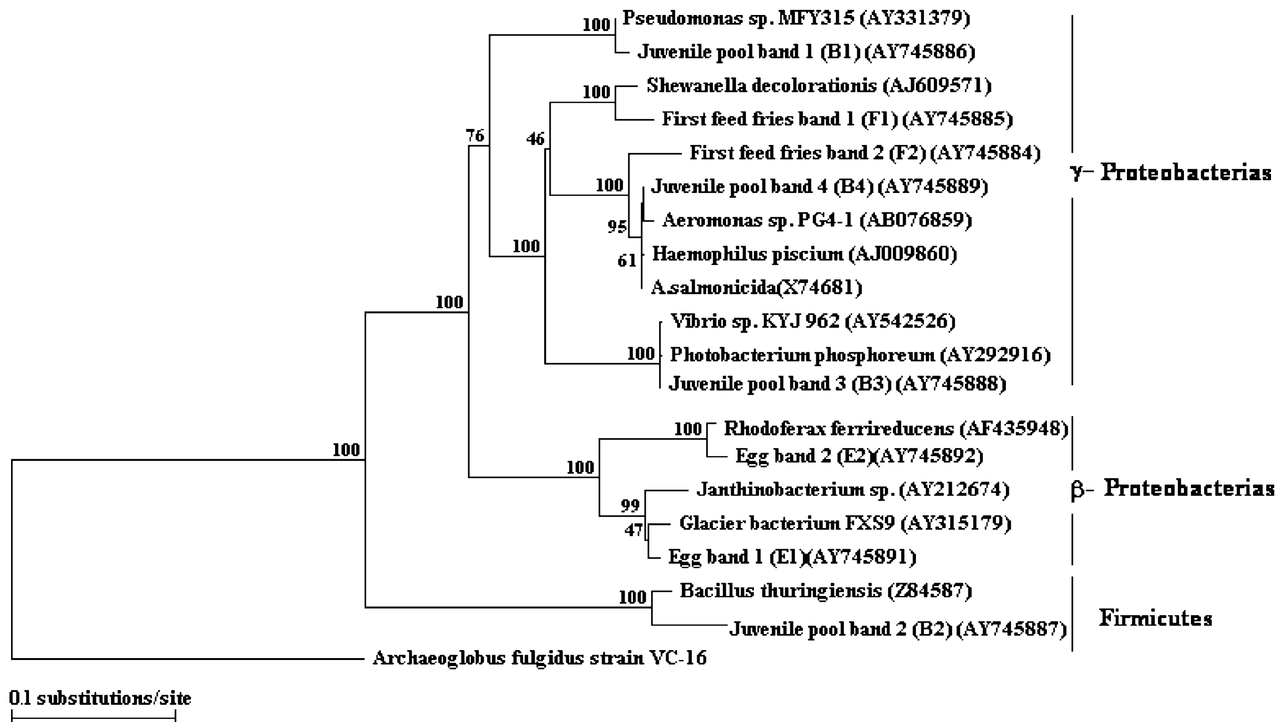

Figure 2. Phylogenetic tree of 16SrDNA sequences obtained from early stages of coho salmon. Neighbor-joining phylogenetic tree showing the relationship between sequences retrieved from the DGGE profiles and their closest relative sequences deposited in the RDP II database. The tree was constructed based on the 341–907 region of the 16S rDNA genes, using Treecon version 1.3b. A bootstrap analysis was performed with 100 repetitions, and values greater than 40% are shown.

with 438 of 4412 *Pseudomonas* sequences in the RDP II database. The AeF primer matched with 357 of 541 *Aeromonas* sequences in this database. The region amplified with this primer was highly conserved within the *Aeromonas* genus. The region amplified with PsF primer showed both conserved sequences and some polymorphic positions that behave as a signature for some clusters within the genus *Pseudomonas*. The quality of the DNA from water, pelletized feed, and eggs as a template was checked using the universal eubacterial primers (341F and 907R, data not shown). Simultaneously, bacterial isolates from these samples were tested with these primers to detect *Pseudomonas* and *Aeromonas*.

Table 2 shows that amplicons for *Pseudomonas* were obtained from water and food samples. The sequences obtained were not identical (98% of identity) to dominant band B1 of juvenile DGGE profiles. By culturing, however, several isolates of *Pseudomonas* were also recovered. Among them, only one sequence was identical to B1 (Eis3, 100% of identity, Table 2). The identical sequence corresponded to an isolate obtained from egg samples, which showed total coincidence in the variable nucleotide positions observed when RDP II deposited sequences closest to band 1 (B1) were aligned. These results suggest that dominant *Pseudomonas* found in juvenile gastrointestinal tract could be derived from bacteria present in eggs. Moreover, 16S rDNA sequences obtained from bacteria isolated from juveniles and first-feeding gastrointestinal tract (Fis1 and Pis3, Table 1) were identical to the *Pseudomonas* amplicons retrieved from the water sample. On the other hand, *Aeromonas* was only detected in water samples. The identity of this

Table 2. Detection of *Aeromonas* and *Pseudomonas* sequences by PCR amplification with specific primers AeF and 2 PsF, respectively, and 907R primer

	PCR amplification of DNA extracted from			
	Egg	*Water*	*Pelletized feed*	*Egg isolate Eis 3*
Pseudomonas	−	+	+	+
Identity with B1		98%	98%	100%
Aeromonas	−	+	−	−
Identity with B4		100%		

Samples analyzed consisted of DNA extracted directly from eggs, water, and pelletized feed, as well as from bacterial isolates of eggs.

amplicon was confirmed by sequencing and showed 100% identity with band B4. However, sequence identity has little resolution in this case, because 16S rDNA presents high conservation in this region within the *Aeromonas* genus. In spite of this, water could be a source of this bacterium since *Aeromonas* was only detected from water samples.

Discussion

To study the microbiota of the gastrointestinal tract of fishes, the general approach has been the use of conventional culture methods [3, 30, 41]. However, it has been found that these methods present several disadvantages since the number and species of bacteria detected are affected mainly by the conditions of the culture used [37]. The first molecular study on salmonid fish applied phylogenetic approaches to survey bacterial populations present in the distal intestine of fish [16]. The study was accomplished through PCR amplification, cloning, and sequencing of partial 16S rDNA extracted directly from fish intestines [16]. In the present study, we determined the composition of microbial communities during the early life stages of coho salmon by using a culture-independent approach based on PCR amplification and DGGE analysis. When we compared the number of cultured bacteria and direct counts through microscopy, we found low cultivability rates ranging from 1 to 10%, suggesting that the largest part of microbiota failed to grow under the conditions used for isolation of salmon bacteria. Therefore, a molecular approach based on analysis of DNA extract direct from the sample seems to be a more adequate strategy to determine the principal components in the microbial community of coho salmon.

The analysis of DGGE profiles showed very simple bacterial communities in the early stages of coho salmon (eggs, gastrointestinal tract of first-feeding fries and juveniles), with dominance of two or four bands per profile. Important shifts in the bacterial community structure were also observed during these developing stages. The DGGE pattern obtained from eggs showed that dominant species belonged to β-Proteobacteria. This bacterial class has been previously identified in aquatic habitats [9, 13, 29] and associated with some fish: *Janthinobacterium* spp. have been detected in Atlantic halibut larvae by molecular methods [20], and *Oxalobacter*-related bacteria were reported as part of cultivable intestinal microbiota of rainbow trout [19]. In first-feeding fries and juvenile DGGE profiles, the dominant bands corresponded mainly to γ-Proteobacteria; the *Pseudomonas* and *Aeromonas* genera have been previously observed in the gastrointestinal tract of fishes and suggested as components of the normal gastrointestinal microbiota of fishes reared in freshwater [15, 21]. The observed shift from predominantly nonfermentative organisms associated with the eggs to fermentative species present in juvenile stages could be explained by the presence of some food components or by mucus secreted by the gastrointestinal tract, which could provide nutrients that favor the establishment of these species [31]. Similar transitions in the gastrointestinal-associated bacteria have been reported by different authors in early stages of Atlantic halibut development [20, 44].

One important attribute of gastrointestinal microbiota is that bacterial components must be present in the majority of healthy individuals [6] and represent populations that are readily stable over time [4, 23]. In this study, we analyzed microbiota of juvenile individuals from the same lot collected during a 3-month period. All individuals tested showed identical DGGE profiles with common bands corresponding to *Pseudomonas* and *Aeromonas*. These data suggest that these bacteria may be part of the gastrointestinal microbiota of coho salmon and seem to be stable during the stages analyzed (2–15 g). However, further studies are required to determine the stability of this microbiota, for example, after antibiotic treatment or dietary change. The data also suggest that this stable gastrointestinal microbiota of coho salmon could be acquired in the juvenile stage. Other authors also propose that a persistent microbiota could be established at this phase [15]. It should be noted, however, that the individuals analyzed in the current study were all from the same cohort and were cultivated on identical feed and under identical conditions. This may explain why these individuals exhibit less variation in intestinal microbiota composition than was previously observed when other salmonid fish (Atlantic salmon) were compared across geographic locations in the North Atlantic Ocean [16]. Therefore, it should be considered that gastrointestinal microbiota determined in this study may not be representative of all coho salmon, especially those in the open environment.

Some investigations have reported that bacteria present in the hatchery environment may influence the composition of gastrointestinal microbiota [7, 31]. Using a culture-based approach, these results suggest that bacteria present in the gastrointestinal tract generally seem to be those from water or the diet, and which can survive and multiply [27, 45]. Furthermore, larvae may ingest substantial amounts of bacteria by grazing on suspended particles and egg debris [5]. Hence, it is tempting to suppose that egg microbiota would also affect the primary colonization of the fish larvae. To explore the origin of dominant bands observed in juvenile gastrointestinal tracts (*Aeromonas* and *Pseudomonas*), samples of water, pelletized feed, and eggs, were analyzed by PCR–DGGE. Bands corresponding to these taxa were not observed in DGGE profiles, probably because they were present in a very small proportion, less than 1% [26]. Therefore, these samples were analyzed by PCR using

primers designed to detect these groups. Sequences identical to the dominant *Aeromonas* were only retrieved from amplicons of water samples. Although 16S rDNA in the amplified region is highly conserved within the *Aeromonas* genus, water from the aquifer could be regarded as a source of this bacterium since *Aeromonas* was only detected in those samples. *Pseudomonas* amplicons were obtained from water and pelletized food.

However, the sequences retrieved were not identical to the dominant band found in the DGGE profile from gastrointestinal tract of juvenile salmon. Nevertheless, one isolate obtained from egg samples possessed 16S rDNA that was identical to the dominant band B1. Both sequences shared the same signature in the variable nucleotide positions observed when sequences from RDP II database closest to B1 were aligned. This was noticeable since it allowed for clearly distinguishing these sequences from those obtained from other putative sources. These data suggest that some minor component of the egg epibiota could persist during growth and contribute to the microbiota of the gastrointestinal tract. On the other hand, bacterial isolates obtained from juveniles and first-feeding fry gastrointestinal tract presented 16S rDNA identical to the *Pseudomonas* amplicons retrieved from water samples. These data showed the occurrence of several *Pseudomonas* strains that were in contact with coho salmon in this aquatic environment. In this study, however, the sequence retrieved from B1 (*Pseudomonas*) was obtained directly from the reamplified band. This procedure could not reveal others phylotypes present in a minor proportion, for example, those corresponding to *Pseudomonas* sequences found in the environment. Altogether, molecular and cultured-based analyses suggest that the gastrointestinal microbial community could be acquired in the juvenile stage of salmon and could be derived from the bacterial populations present in water and egg epibiota. In conclusion, the molecular approach provides a more complete picture about bacterial community composition than do cultured-based methods. However, comprehensive overview of coho salmon microbiota can be better achieved when both approaches are combined.

Acknowledgments

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