RESEARCH ARTICLE

Molecular analysis of intestinal microbiota of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The aim of this study was to evaluate different molecular tools based on the 16S rRNA gene, internal transcribed spacer, and the *rpo*B gene to examine the bacterial populations present in juvenile rainbow trout intestines. DNA was extracted from both pooled intestinal samples and bacterial strains. Genes were PCR-amplified and analysed using both temporal temperature gradient gel electrophoresis (TTGE) and restriction fragment length polymorphism methods. Because of the high cultivability of the samples, representative bacterial strains were retrieved and we compared the profiles obtained from isolated bacteria with the profile of total bacteria from intestinal contents. Direct analysis based on *rpo*B-TTGE revealed a simple bacterial composition with two to four bands per sample, while the 16S rRNA gene-TTGE showed multiple bands and comigration for a few species. Sequencing of the 16S rRNA gene- and *rpo*B-TTGE bands revealed that the intestinal microbiota was dominated by *Lactococcus lactis*, *Citrobacter gillenii*, *Kluyvera intermedia*, *Obesumbacterium proteus*, and *Shewanella marinus*. In contrast to 16S rRNA gene-TTGE, *rpo*B-TTGE profiles derived from bacterial strains produced one band per species. Because the single-copy state of *rpo*B leads to a single band in TTGE, the *rpo*B gene is a promising molecular marker for investigating the bacterial community of the rainbow trout intestinal microbiota.

Introduction

Chile has become the world's second largest producer of farmed salmonids. Over the last 15 years, the total salmon and trout production has increased 10-fold, reaching a total of 498 360 tonnes worth US$2.326 million in 2007. The main species produced are Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and coho salmon (*Oncorhynchus kisutch*). However, during the last year, production was negatively impacted due to the increase of several diseases, which may be due, at least in part, to negative interactions between salmonids and pathogens.

The importance of gastrointestinal microbiota for the health of fishes has recently been revealed by several studies on the gnotobiotic zebrafish (*Danio rerio*), demonstrating the involvement of the microbiota in stimulating epithelial proliferation and promoting nutrient metabolism and innate immune responses (Rawls *et al.*, 2004; Bates *et al.*, 2006, 2007). Some bacterial components of the gut microbiota can produce inhibitory compounds and control the colonization of potential pathogens in fish (Spanggaard *et al.*, 2001; Irianto & Austin, 2002a, b). Recently, it has been proposed that the digestive tract could represent an entry point for invading bacteria (Ringø *et al.*, 2004; Jutfelt *et al.*, 2006). Thus, a normal gut microbiota is beneficial, contributes to the nutritional processes of fish, and helps evade colonization by pathogens.

The bacterial composition of the fish gastrointestinal tract has been studied previously using culture techniques (Cahill, 1990; Hansen & Olafsen, 1999); however, these methods are time consuming, and only part of the total bacterial community can be recovered using traditional agar substrates (Romero & Navarrete, 2006; Navarrete *et al.*, 2009). On the other hand, alternative molecular methods based on PCR amplification of DNA extracted from frozen samples have been proven to be efficient in studying the

gastrointestinal bacterial community of fishes (Griffiths *et al.*, 2001; Jensen *et al.*, 2004; Romero & Navarrete, 2006; Hovda *et al.*, 2007; Kim *et al.*, 2007).

Examination of bacterial communities has commonly been performed by analysis of temporal temperature gradient gel electrophoresis (TTGE) and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified DNA fragments that produce a profile of all dominant bacterial species in environmental samples (Muyzer *et al.*, 1993; Muyzer & Ramsing, 1995) or of a particular group, for example, the genus level (Salles *et al.*, 2002; Garbeva *et al.*, 2003; Mangin *et al.*, 2006). Because of its ubiquity and the growing database, the gene encoding the 16S rRNA is usually used for the analysis. In addition, these gene sequences contain highly conserved primer-binding sites and hypervariable regions that can provide species-specific signature sequences that are useful for bacterial identification. However, the disadvantages of this method include the heterogeneity of the different 16S rRNA genes and the poor discrimination between closely related bacteria (Magne *et al.*, 2006). This point has led us to propose using other genes, such as either the spacer region between the 16S and 23S rRNA genes [internal transcribed space (ITS)] or the *rpo*B gene.

The spacer region between 16S and 23S rRNA genes (ITS) is often used to study microbial communities because of its great discriminatory power (Gonzalez *et al.*, 2003; Brown & Fuhrman, 2005; Oliveira *et al.*, 2006). As the length of the ITS varies between microorganisms, mainly due to the presence of several tRNAs in the sequence (Garcia-Martinez *et al.*, 2001), it is possible to distinguish between species based on their ITS PCR profiles.

The *rpo*B gene, encoding the RNA polymerase β subunit, exhibits greater differences between species than the 16S rRNA gene (Renouf *et al.*, 2006). This gene has been described as being suitable for PCR-DGGE or TTGE (Dahllof *et al.*, 2000). In contrast to the 16S rRNA gene, only one band was observed per species, and the banding pattern from a mixture of bacteria could clearly be related to single strains. Moreover, multiple copies of *rpo*B have never been reported in bacteria (Mollet *et al.*, 1997).

In aquaculture, fish can be exposed to feed changes or antibiotic treatment. One of the primary and most interesting questions is how the bacterial population of a fish's microbiota can be altered due to these practices. Therefore, the application of easy and rapid fingerprinting methods could help to evaluate these changes. The overall objective of the present study is to evaluate a combination of molecular methods [TTGE and restriction fragment length polymorphism (RFLP)] based on different markers, such as the 16S rRNA gene, ITS, and the *rpo*B gene, to accurately study the composition of juvenile rainbow trout (*O. mykiss*) intestinal microbiota.

Materials and methods

Trout samples

Thirty rainbow trout, weighing 15.2 ± 0.6 g, were collected from a freshwater fish farm in Chile. Intestinal contents were obtained by squeezing the abdomens of anaesthetized fishes. Equal quantities of intestinal contents from three rainbow trout were pooled, and a total of 10 pooled samples of the intestinal contents were analysed. The trout were sampled in the winter when the water temperature was $15 \pm 2\,^{\circ}$C. The fish were fed conventional pelleted feed (from EWOS Aquaculture International) without probiotic, prebiotic, immunomodulatory, or inhibitory agents and were randomly collected from different tanks.

Bacterial counts and cultivation

The total bacterial counts of pooled samples were assessed by epifluorescence microscopy using acridine orange, as described previously (Romero & Espejo, 2001). For cultivation, serial dilutions of the homogenates were generated in sterile phosphate-buffered saline and 100 μL of the appropriate dilutions were spread onto trypticase soy agar (TSA) plates in triplicate (Difco, Sparks, MD). The plates were incubated for 10 days at 17 °C under aerobic conditions. Bacterial colonies were counted, and the number of CFUs g^{-1} of intestinal content was calculated. Twenty colonies per sample were randomly selected and analysed using the molecular methods described below.

DNA extraction and purification

The pooled intestinal homogenates were weighed and an equal weight of cold sterile TE buffer (Tris 0.1 M, EDTA 0.01 M, NaCl 0.15 M, pH 7.8) was added. Overnight bacterial cultures were centrifuged at top spin and resuspended in TE buffer. DNA from pooled intestinal homogenates and bacterial strains was obtained by incubating for 30 min at 37 °C with lysozyme (1 μg μL^{-1}), 30 min at 37 °C with proteinase K (0.1 mg mL^{-1}), and then at 70 °C with sodium dodecyl sulphate (10%). The samples were frequently vortexed during the incubation. DNA was extracted with phenol/chloroform, and subsequently precipitated with ethanol as described previously (Romero *et al.*, 2002). In order to eliminate salts and nucleases, a final purification was carried out using the Wizard DNA Clean Up System (Promega, Madison, WI).

PCR amplification

To obtain fingerprints of the bacterial communities present in intestinal samples and to compare them with those of the bacterial strains, the extracted DNA from intestinal samples

and strains was PCR-amplified using 16S rRNA gene, *rpo*B, and ITS primers (Table 1).

Amplification of the 16S rRNA gene from positions 27 to 1492 (*Escherichia coli* numbering) for RFLP analysis and sequencing was performed as described previously (Romero *et al.*, 2002). Amplification of the V3–V5 region of the 16S rRNA gene for TTGE was performed as optimized previously (Romero & Navarrete, 2006). Amplification of the 16S–23S intergenic regions (ITS) was performed as described previously (Gonzalez *et al.*, 2003). Amplification of *rpo*B was carried out as described previously (Rantsiou *et al.*, 2004); however, the annealing temperature of the first 10 cycles was reduced from 58 to 52 °C, in order to obtain amplicons for *Lactococcus lactis*.

All PCR reactions were performed in a reaction mixture of 30 μL containing 0.2 mM of each dNTP, 0.05 U mL^{-1} of Platinum Taq DNA polymerase (Invitrogen, San Diego, CA), 1 × polymerase reaction buffer, 2 mM $MgCl_2$, and 0.25 pmol mL^{-1} of each primer. For *rpo*B amplification, the $MgCl_2$ concentration was increased to 2.5 mM (Rantsiou *et al.*, 2004). PCR products were analysed by polyacrylamide gel electrophoresis (PAGE) and silver nitrate staining as described previously (Romero *et al.*, 2002).

RFLP analysis

Intestinal contents and bacterial strains obtained from each sample were analysed according to their 16S rRNA gene RFLP profile. Products of the 27F-1492R 16S rRNA gene PCR amplification were digested for 2 h at 37 °C with 1.5 U of AluI restriction endonuclease (Invitrogen). The digested fragments were subsequently analysed using PAGE and silver nitrate staining as described previously (Romero *et al.*, 2002).

TTGE analysis

PCR products obtained using 16S rRNA gene (341F-GC and 907R), *rpo*B, and ITS primers were separated by TTGE as described previously (Magne *et al.*, 2006). Electrophoresis was performed for approximately 20 h at 65 V in a D-Code System (Bio-Rad, Hercules, CA). After electrophoresis, gels were stained for 1 h with SYBR Green, and the bands were reamplified for sequencing.

Sequencing analysis

Bacterial strains corresponding to different patterns derived from 16S rRNA gene PCR-RFLP and dominant 16S rRNA gene- and *rpo*B-TTGE bands were selected for sequencing (Macrogen USA sequencing service). Dominant bands from the TTGE gels were excised and eluted overnight in 50 μL of sterile MilliQ water, and 1 μL was used for reamplification. The 16S rRNA gene sequences were compared with those available in the public Ribosomal Database Project II (Cole *et al.*, 2007) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) in order to ascertain their closest relatives. The *rpo*B sequences were compared with those available in GenBank using the BLASTN software (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequences obtained from the bacterial culture and from the intestinal contents were aligned to check their identity using BIOEDIT software (Hall, 1999).

Results

Bacterial counts and identification using the culture method

The average number of total bacteria was 8.9×10^8 bacteria g^{-1} of intestinal content. The average count of cultivable bacteria was 1.3×10^8 CFU g^{-1} of intestinal content; furthermore, in all samples, the cultivability was approximately 18% (ranging between 9% and 27%). A total of 200 strains recovered on TSA medium were analysed by RFLP of PCR-amplified 16S rRNA gene digested with AluI. This enzyme yielded four different, easily distinguishable PCR-RFLP patterns, with at least three bands per profile.

Table 1. Specific primers and PCR conditions for the amplification of 16S rRNA gene, ITS, and *rpo*B genes

Primer	Target	Molecular method	5′–3′ sequence	References	PCR conditions*
27-F	16S rRNA gene	PCR-RFLP	AGA GTT TGA TCM TGG CTC AG	Hicks *et al.* (1992)	95 °C, 60 s; 55 °C, 60 s; 72 °C,
1492-R	16S rRNA gene	PCR-RFLP	GGT TAC CTT GTT ACG ACTT	Kane *et al.* (1993)	60 s; 35 cycles
341F-GC	16S rRNA gene	PCR-TTGE	CCT ACG GGA GGC AGC AG	Muyzer *et al.* (1993)	94 °C, 60 s; 65–55 °C
907-R	16S rRNA gene	PCR-TTGE	CCG TCA ATT CMT TTG AGT TT	Muyzer *et al.* (1998)	(−0.5 °C each cycle); 72 °C, 3 min; and 94 °C, 60 s; 55 °C, 72 °C, 3 min, 16 cycles
1698-F	*rpo*B	PCR-TTGE	AAC ATC GGT TTG ATC AAC	Dahllof *et al.* (2000)	95 °C, 60 s; 52 °C, 60 s; 72 °C,
2041-R	*rpo*B	PCR-TTGE	CGT TGC ATG TTG GTA CCC AT	Dahllof *et al.* (2000)	90 s; 30 cycles
L1	ITS	PCR and PCR-TTGE	GAA GTC GTA ACA AGG	Jensen *et al.* (1993)	95 °C, 60 s; 55 °C, 60 s; 72 °C,
G1	ITS	PCR and PCR-TTGE	CAA GGC ATC CAC CGT	Jensen *et al.* (1993)	60 s; 35 cycles

*Denaturation, annealing, and elongation temperatures and times, respectively.

At least one representative of each RFLP pattern was characterized by partial sequencing of their 16S rRNA gene and *rpo*B (Supporting Information, Table S1). Bacterial strains were identified as *Citrobacter gillenii*, *Obesumbacterium proteus*, *Kluyvera intermedia*, *Shewanella* sp., and *L. lactis*, and were recovered at different abundances among the samples (Fig. 1). It is important to notice that *K. intermedia* and *C. gillenii* exhibited indistinguishable RFLP types with AluI in this study (Fig. S1a); only colony morphology or partial 16S rRNA gene sequencing could discriminate between them.

Analysis of intestinal bacterial strains and bacterial populations based on the 16S rRNA gene

16S rRNA gene PCR-RFLP

The PCR-RFLP profiles of the total bacteria from intestinal samples were compared with the profiles of isolated strains (Fig. S1a). In the intestinal samples, it was possible to identify some dominant profiles that had the same electrophoretic migration as those corresponding to bacterial strains. This suggests that these bacterial strains could be present in the intestinal samples. For example, *L. lactis* could dominate in sample 1, while *O. proteus* could dominate in samples 2, 3, 7, and 9. However, due to the indistinguishable RFLP pattern of *K. intermedia* and *C. gillenii*, these two bacteria could not be differentiated in the profiles of intestinal samples, although they may have been present in samples 2, 4, 5, 6, 8, 9, and 10 (Fig. S1a).

16S rRNA gene PCR-TTGE

PCR-TTGE analysis of the 16S rRNA gene V3–V5 region was performed on bacterial strains and intestinal samples. Most bacterial strains resulted in one band on the gel; however, multiple bands were detected for *K. intermedia*, possibly indicating intragenomic heterogeneity or the presence of a heteroduplex (Fig. S1b). Enterobacteria (*Kluyvera*, *Obesumbacterium*, and *Citrobacter*) bands migrated at the bottom of the gel, whereas *L. lactis* bands, with a low GC content, remained in the upper part. The TTGE patterns of the 10 intestinal samples revealed at least four bands that could be easily detected and showed identical electrophoretic migration as those corresponding to single species, suggesting the presence of these strains in the intestinal contents. However, bands corresponding to *C. gillenii* and *K. intermedia* comigrated and could not be separated under the TTGE conditions used. A comparison of the variable regions V3–V5 (TTGE bands C1, C2, K1, and K2) of these bacteria revealed 98% identity due to six mismatches.

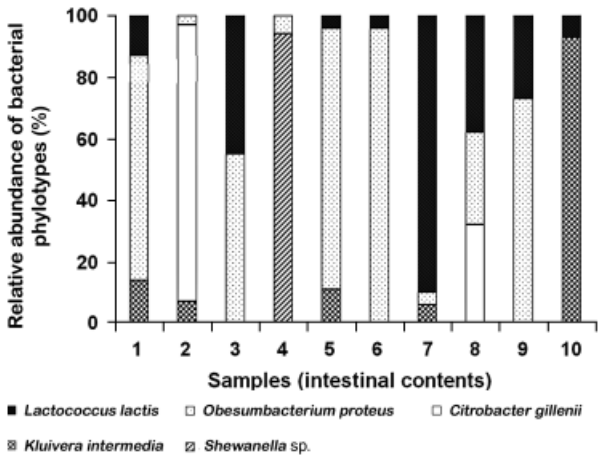

Fig. 1. Relative abundance (%) of the bacterial phylotypes obtained by 16S rRNA gene RFLP analysis and sequencing of bacterial strains obtained from the intestinal content of juvenile trout.

Analysis of intestinal bacterial strains and bacterial populations by the internal spacer regions (ITS)

ITS-PCR

Each bacterial isolate showed a particular ITS pattern characterized by one to three distinct bands (Fig. S1c). In some bacteria, weak and variable amplification products were also generated. These products were observed in *C. gillenii*, *K. intermedia*, and *Shewanella* sp., and could represent heteroduplex formations. Because of the inconsistent nature of these fragments, they were not included in the bacterial profiles. The profile of each isolate could be observed within the complex ITS pattern derived from the intestinal content. All bands in the direct ITS pattern seemed to be derived from an identified bacterial profile. However, many of the bands in this product overlapped; therefore, it was difficult to determine the bacterial composition of the intestinal samples. Particularly, the overlapping of ITS products from *C. gillenii* and *K. intermedia* made it difficult to differentiate between these two bacteria.

ITS PCR-TTGE

Because of separation based on GC content and length, the TTGE profiles of amplified ITS achieved better resolution than common electrophoresis. The profiles of bacterial strains displayed two to five bands per strain. These bands were frequently associated with the bands observed in the intestinal sample profiles (Fig. S1d). *Kluyvera intermedia* and *C. gillenii* exhibited three overlapping bands; however, an additional intense band in the *K. intermedia* profile facilitated its discrimination from the direct intestinal sample profiles.

Analysis of intestinal bacterial strains and bacterial populations by *rpo*B-TTGE

When the five bacterial strains were analysed by PCR-TTGE of *rpo*B, only one band per species was observed (Fig. 2), indicating either a single copy of the gene or multiple identical copies. TTGE profiles from intestinal samples showed sufficient separation between the different bands; moreover, these bands could be easily associated with the individual strains (Fig. 2).

Closely related bacteria that demonstrated comigration in the 16S rRNA gene-TTGE profile were clearly separated in the *rpo*B-TTGE profile (*Kluyvera* and *Citrobacter*). However, it was necessary to reduce the annealing temperature from 58 to 52 °C, in order to amplify *L. lactis* from the intestinal samples. Analysis of the *rpo*B primers showed one mismatch for each of them when their sequences were compared with the *L. lactis* genome sequences. The amplification efficiencies for *L. lactis* and Enterobacteria were not affected when the annealing temperature was reduced to 52 °C (data not shown).

Discussion

In the present investigation, the dominant bacteria isolated from the intestine of juvenile trout using culture methods and identified using 16S rRNA and *rpo*B gene sequencing belonged to five bacterial species: *C. gillenii*, *O. proteus*, *K. intermedia, Shewanella* spp., and *L. lactis*. The identification of these bacteria is shown in Table S1. Bacteria of the γ subclass of *Proteobacteria* and the *Firmicutes* phylum have been frequently found in the intestinal content of fishes (Huber *et al.*, 2004; Rawls *et al.*, 2004; Pond *et al.*, 2006; Romero & Navarrete, 2006). Particularly, *Enterobacteriaceae* have been described previously as common components of the intestinal content of trout (Spanggaard *et al.*, 2000; Skrodenyte-Arbaciauskiene *et al.*, 2006; Kim *et al.*, 2007). *Kluyvera intermedia* has been identified in both the intestinal content and the mucosal layer of the rainbow trout *O. mykiss* (Walbaum) from a commercial farm in Scotland (Kim *et al.*, 2007). It is worth noticing that the authors referred to this bacterium as *Enterobacter intermedius*; however, this species was recently reclassified in the genus *Kluyvera* (Pavan *et al.*, 2005). It has also been reported that lactic acid bacteria (LAB) are present in fish intestines (Cai *et al.*, 1998; Ringø & Gatesoupe, 1998; Gonzalez *et al.*, 2000; Ringø *et al.*, 2000), in particular *L. lactis*, which was isolated as the dominant LAB from the intestine of silver carp, common carp, channel catfish, and deep-bodied crucian carpin in the summer (Hagi *et al.*, 2004). Using molecular methods based on 16S rRNA gene analysis, Hovda *et al.* (2007) showed that populations of LAB such as *Lactococcus* and *Lactobacillus* are important in the Atlantic salmon intestine. Furthermore, some LAB isolated from rainbow trout intestines have been reported to possess probiotic properties (Balcazar *et al.*, 2008). Other bacterial populations, such as *Shewanella*, have been described in rainbow trout from Denmark (Huber *et al.*, 2004), but were not observed in trout from Scotland.

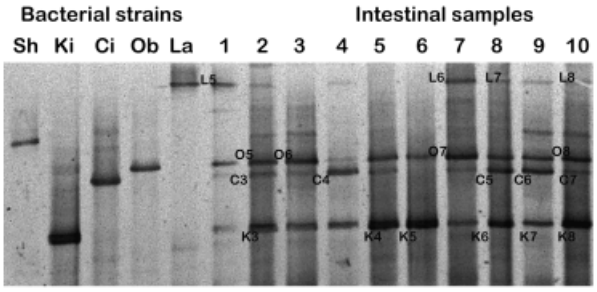

Fig. 2. TTGE profiles of *rpo*B amplification products from intestinal samples and bacteria isolated from trout intestinal contents. Ki/Ci/Ob/La/Sh, bacterial strains; Ki, *Kluyvera intermedia*; Ci, *Citrobacter gillenii*; Ob, *Obesumbacterium proteus*; La, *Lactococcus lactis*; Sh, *Shewanella* sp.; lanes 1–10, direct profile of intestinal samples. Each intestinal sample corresponds to a homogenate of the intestinal content from three 15-g trout. Marked bands correspond to sequenced bands shown in Table S1.

The bacterial counts determined in this study were similar to those reported previously in the gastrointestinal tract of trout (Huber *et al.*, 2004). A high proportion of bacteria could be recovered using a TSA culture method, and the cultivable bacterial counts were similar to those observed previously in trout (Huber *et al.*, 2004). The cultivability (18% average) was higher in comparison with the results of other studies on salmonids. For example, in the *S. salar* or coho salmon, cultivable bacteria represented < 1% of the dominant bacteria (Romero & Navarrete, 2006; Navarrete *et al.*, 2009). TSA medium, which has been extensively used to study the intestinal microbiota of fishes (Austin & Alzahrani, 1988; Hansen *et al.*, 1992; Spanggaard *et al.*, 2000), was found to be an adequate substrate to recover the dominant intestinal bacteria of juvenile trout. Sequencing of the bands excised from 16S rRNA gene- and *rpo*B-TTGE revealed that all dominant bacteria in these samples could be cultured on TSA plates. In comparison with molecular methods, the culture methods were time consuming and required immediate plating of the collected samples. On the other hand, the ability to culture dominant bacteria provided a good opportunity to compare the total bacterial DNA profiles from intestinal samples with the profiles of isolated bacterial strains. We compared these profiles using different molecular methods.

First, we studied the intestinal bacterial composition using 16S rRNA gene PCR-RFLP. This method revealed that many bands could be obtained from a single bacterial species. Moreover, several bands could comigrate in the gel, making the interpretation of the profile obtained for the

intestinal sample difficult. On the other hand, 16S rRNA gene-TTGE or -DGGE has been successfully used to characterize the bacterial populations associated with juvenile haddock (Griffiths *et al.*, 2001), intensively reared cod (Brunvold *et al.*, 2007), Atlantic halibut larvae (Jensen *et al.*, 2004), adult salmon (Hovda *et al.*, 2007), juvenile trout (Kim *et al.*, 2007), and salmon (Romero & Navarrete, 2006; Navarrete *et al.*, 2009). The banding pattern obtained using this method has been recognized previously to reflect the composition of the dominant bacterial community. However, the heterogeneity between 16S rRNA gene copies and the possibility of heteroduplex formation (Moreno *et al.*, 2002) led us to observe multiple bands for a single strain, such as those observed for *K. intermedia* in this study. Additionally, amplicons having the same melting behaviour, but different sequences, can comigrate to the same position in a gel (Muyzer *et al.*, 1993; Magne *et al.*, 2006). In our study, the TTGE conditions were unable to separate the bands corresponding to *K. intermedia* and *C. gillenii*, which comigrated in the gel, confirming that the 16S rRNA gene may be ineffective in distinguishing closely related bacteria.

The 16S–23S internal spacer regions (ITS) are very useful in characterizing microorganisms because they offer a higher resolution and have been used to study bacterial ecosystems (Gonzalez *et al.*, 2003), as this region exhibits sequence variability and length variation (Jensen *et al.*, 1993). Moreover, the amplification of this region in microorganisms can be achieved with a single pair of universal primers, and amplicons can be separated in polyacrylamide gels. Species with a high number of ribosomal operons (7–9 *rrn* operons) have been shown to generate complex ITS profiles, whereas those containing a low number of ribosomal operons produced simpler profiles (Perez-Luz *et al.*, 2004). In our study, direct amplification of the ITS for each species revealed a characteristic pattern that could be considered species specific. However, multiple bands were observed for a single species and the comigration of some bands was observed; consequently, the analysis of direct profiles from more diverse bacterial communities might be difficult. ITS-TTGE was more definitive than ITS-PCR, followed by acrylamide gel electrophoresis, as TTGE separates amplicons according to their length and GC content. As a result, bacteria with similar electrophoretic migration profiles in ITS-PCR, such as *L. lactis* and *Shewanella* spp., could be efficiently identified using TTGE due to their different GC contents. To summarize, the main drawback of this method is that individual bands in the ITS-TTGE profiles should not be taken as exact measures of bacterial diversity in these communities, as the number of bands cannot be strictly correlated to the number of distinct bacterial types.

Using both the 16S rRNA gene and the ITS markers, the intraspecies heterogeneity can severely hamper the community pattern analysis. To avoid this bias, the use of the *rpo*B gene (RNA polymerase β subunit-encoding gene) has been proposed as an alternative marker to study bacterial communities (Dahllof *et al.*, 2000), as this gene is present as a single copy and it is ubiquitously distributed among bacterial species (Mollet *et al.*, 1997). In the present study, the *rpo*B-TTGE profiles of bacterial strains exhibited one band per species. Based on the gel migration and the sequences retrieved from the profiles of intestinal samples, each band in the *rpo*B-TTGE was associated with one specific species. Furthermore, the main strains present in the bacterial community (as assessed using TSA culture) could be revealed using this *rpo*B PCR-TTGE method. The primers used in this study were designed based on the *rpo*B sequences of only four bacteria: *E. coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Helicobacter pylori* (accession numbers AE000472, 2632267, 677848, and AE000625) (Dahllof *et al.*, 2000). Furthermore, the primer regions for these species were not completely identical. As degenerate primers coupled with TTGE are not recommended (because they give rise to multiple products), it was necessary to reduce the annealing temperature in order to amplify all bacterial DNA samples, especially those from intestinal samples. Specifically, in the present study, *L. lactis* was identified only when the annealing temperature was lowered from 58 °C (suggested by Rantsiou *et al.*, 2004) to 52 °C (its sequence contains one mismatch with each *rpo*B primer). Future studies should be conducted to obtain complete *rpo*B sequences from fish bacteria to design more accurate *rpo*B primers.

To summarize, the fingerprinting methods used in this study were based on the amplification of gene markers using PCR of extracted DNA; consequently, only dominant bacterial populations could be identified. PCR-TTGE and PCR-DGGE methods are not quantitative, although many studies have reported that the band intensity in the TTGE profile may correspond to the bacterial abundance in the ecosystem, because all bacteria could be amplified with the same efficiency (Magne *et al.*, 2006). Bands within a profile represent the dominant bacteria within the ecosystem, and it can be assumed that the band intensity is proportional to the relative abundance of a particular species in the population (Calvo-Bado *et al.*, 2003). In the present study, within the same sample, bacterial abundance based on band intensity differed among the TTGE profiles obtained using different gene markers. This may be explained by the specificity of the primers used in the different PCRs.

Overall, this study suggests that *rpo*B-TTGE analysis is a promising approach for investigating bacterial communities, especially the composition of the intestinal microbiota of juvenile rainbow trout. The *rpo*B gene can be a powerful molecular marker, as its single copy state leads to single band profiles in TTGE.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Direct molecular methods to study the intestinal bacterial community of juvenile trout (*Oncorhynchus mykiss*).
Table S1. Nearest-match identification of partial 16S rRNA gene and *rpo*B sequences obtained from representative strains of each RFLP patterns retrieved from juvenile trout to known sequences in the RDP II database.

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