Ontogenetic Development of the Gastrointestinal Microbiota in the Marine Herbivorous Fish *Kyphosus sydneyanus*

D. Moran, S.J. Turner and K.D. Clements

School of Biological Sciences, The University of Auckland, New Zealand

Abstract

Molecular techniques were used to investigate the composition and ontogenetic development of the intestinal bacterial community in the marine herbivorous fish *Kyphosus sydneyanus* from the north eastern coast of New Zealand. Previous work showed that *K. sydneyanus* maintains an exclusively algivorous diet throughout post-settlement life and passes through an ontogenetic diet shift from a juvenile diet which is readily digestible to an adult diet high in refractory algal metabolites. Terminal restriction fragment length polymorphism (T-RFLP) analysis was used to investigate the relationship between bacterial community structure and fish size. Bacterial diversity was higher in posterior gut sections than anterior gut sections, and in larger fish than in smaller fish. Partial sequencing of bacterial 16S rDNA genes PCR amplified and cloned from intestine content samples was used to identify the phylogenetic affiliation of dominant gastrointestinal bacteria. Phylogenetic analysis of clones showed that most formed a clade within the genus *Clostridium*, with one clone associated with the parasitic mycoplasmas. No bacteria were specific to a particular intestinal section or size class of host, though some appeared more dominant than others and were established in smaller fishes. Clones closely related to *C. lituseburense* were particularly dominant in most intestine content samples. All bacteria identified in the intestinal samples were phylogenetically related to those possessing fermentative type metabolism. Short-chain fatty acids in intestinal fluid samples increased from 15.6 ± 2.1 mM in fish <100 mm to 51.6 ± 5.5 mM in fish >300 mm. The findings of this study support the hypothesis that the ontogenetic diet shift of *K. sydneyanus* is accompanied by an increase in the diversity of intestinal microbial symbionts capable of degrading refractory algal metabolites into short-chain fatty acids, which can then be assimilated by the host.

Introduction

Gastrointestinal bacteria play an important role in digestion in a wide range of amphibian, reptilian, avian and mammalian herbivores [28]. Despite the great differences between these groups of vertebrate herbivores in terms of phylogenetic relationship, digestive physiology, and anatomy of the alimentary tract, their bacterial gastrointestinal communities display strong similarities in composition. Common bacterial symbionts include members of the genera *Streptococcus*, *Bacteroides*, and *Clostridium* in reptiles and amphibians [3], *Bacteroides*, *Clostridium* and *Eubacterium* in birds [11], and *Ruminococcus*, *Clostridium*, *Prevotella*, and *Fibrobacter* in ruminants [27].

Several lines of evidence indicate that the intestinal symbionts of some marine herbivorous fish are also an important component of the digestive process: (1) diverse and abundant intestinal symbiont communities are found in the hindgut of many species [5, 6, 26]; (2) nutritionally significant levels of microbial fermentation end products, notably short-chain fatty acids (SCFAs), occur in the hind gut [8–10, 22]; and (3) capacity for uptake [31–33] and assimilation [22] of SCFA has been demonstrated in herbivorous fishes. Despite this, our understanding of community composition and ontogenetic development in these fish gastrointestinal communities is poor.

The present study uses molecular methods to investigate the ontogenetic development of bacterial gastrointestinal symbionts in *Kyphosus sydneyanus* (family Kyphosidae) from northeastern New Zealand. Like many kyphosids, *K. sydneyanus* has a relatively long gut with an enlarged hindgut chamber separated from the proximal

Correspondence to: D. Moran; E-mail: d.moran@auckland.ac.nz

intestine by a sphincter [26]. This species maintains an exclusively herbivorous diet throughout postsettlement life, unlike some other marine herbivorous fish species that have a period of omnivory or carnivory during juvenile development [7]. *Kyphosus sydneyanus* exhibits an ontogenetic diet shift, with juvenile fish (<100 mm standard length, SL) consuming a diet dominated by rhodophytes (red algae) and sometimes chlorophytes (green algae), and adults (>300 mm SL) a diet dominated by phaeophytes (brown algae). This diet shift corresponds to a decrease in activity of endogenous carbohydrases, leading to the hypothesis that as the fish grow they rely increasingly on microbial degradation of algal carbohydrates [20]. Microbial fermentation is likely to be an important component of the digestive process in adult *K. sydneyanus* as fermentation rates, SCFA pool size, and acetate uptake in the hindgut of this species are relatively high [22].

Apart from some relatively simple morphological descriptions, little is known about the nature and characteristics of the microbial community in the intestine of *K. sydneyanus*. Rimmer and Wiebe [26] found that microbial diversity and abundance increased posteriorly along the intestine, with the highest density of microbes in the hindgut chamber (99.7×10^9 cells g^{-1} dry wt intestine fluid). Despite the high density of microbes and high SCFA concentrations in this chamber, fermentation rates are as high or higher in the intestine immediately proximal to it [22].

Although nothing is known about the ontogenetic development of the gastrointestinal microbiota of *K. sydneyanus*, Rimmer [25] studied the closely related fish *K. cornelii* and found that a switch from omnivory in juveniles to herbivory in adults was correlated with an increase in microbial abundance in the hindgut. The apparent diet shift in *K. sydneyanus* from endogenous digestion of rhodophytes to predominantly exogenous digestion of phaeophytes raises the question of whether this diet shift is accompanied by related changes in microbial diversity and abundance. The present study uses terminal restriction fragment length polymorphism (T-RFLP) analysis and 16S rRNA gene clone library sequencing techniques to describe qualitatively the ontogenetic development of the bacterial community in the mid and posterior intestine of *K. sydneyanus*. Hindgut SCFA concentrations were also quantified for different size classes of *K. sydneyanus* to provide a qualitative measure of bacterial fermentation.

Methods

Sample Collection and Sites. Specimens of *K. sydneyanus* were collected from a variety of locations around the greater Hauraki Gulf area (174°48'E, 36°20'S), on the northeastern coast of New Zealand. All specimens were collected from shallow reefs on snorkel using spearguns. Once shot, specimens were promptly taken to the RV *Proteus* and samples processed for transportation to the laboratory. Samples of intestinal fluid were collected from four size classes over four seasons, from July 1999 to May 2000. Where possible, three fish were taken per size class per season. Samples were taken from different seasons to minimize any effects that seasonal variation may have on the gastrointestinal microbiota. The size classes were designated as follows: <100 mm Standard Length (SL), 100–199 mm SL, 200–299 mm SL, and >300 mm SL. Otolith annual increment rings of *K. sydneyanus* collected from the study area suggests that the smallest size class are newly settled fish <1 year old; 100–199 mm SL fish are 1–2 years old, 200–299 mm SL fish are 2–4 years old, and fish >300 mm SL are >4 years old (J. H. Choat, pers. comm.). Upon removal from the water the fish were immediately killed by pithing and the gastrointestinal tract excised. The gastrointestinal tract was divided into five sections following Clements and Choat [9], with the stomach designated as section I, the hindgut chamber designated as section V, and the intestine proximal to the hindgut chamber divided into three equal parts designated sections II–IV. Microbial samples were collected from intestinal sections III, IV, and V by taking ∼1.0 mL of intestinal content and preserving it in isopropanol [24]. Samples from specimens in which intestinal sections were damaged by the spear were not used for analysis.

DNA Extraction and PCR Amplification for T-RFLP. Once in the lab, gut solids were separated from isopropanol by centrifuging for 20 min (10,000*g*, 4°C, Sorvall RC-5B Ultrafuge), after which the supernatant was discarded. The pellet was resuspended in 5 mL of 0.2 M sodium phosphate buffer (pH 7.2) and centrifuged for 10 min. The resulting pellet was resuspended in 1 mL of sodium phosphate buffer and transferred to a 1.5 mL microfuge tube. Samples were spun for 1 min at 9,500*g* in a benchtop centrifuge, the supernatant removed, and the samples stored frozen at −4°C until required for DNA extraction.

DNA extraction was performed using a modification of the method by Moré et al. [21] to lyse the bacterial cells. Frozen gut content samples were thawed at room temperature and 100 µL aliquots were transferred to sterile 15 mL Falcon tubes containing 150 µL TNE buffer (40 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA, pH 8.0) and 150 µL TNS solution (0.5 M Tris-HCl, pH 8.0; 100 mM NaCl; 4% SDS). The samples were placed into a 65°C water bath for 5 min, then immediately transferred to a liquid nitrogen bath to rapidly freeze the sample (2 min). A series of four freeze–thaw cycles were carried out. After freeze–thawing the samples were transferred to Multimix2 Matrix tubes (Bio101) and 600

µL of TNE buffer was added. A series of four cycles of vigorous shaking at speed 5.5 m/s for 45 s on the Fast-Prep Instrument (Bio101) was performed. Between cycles the samples were chilled on ice for 2 min to prevent overheating. From this point on the extraction procedure followed the protocol specified for the Bio101 FastDNA SPIN Kit for soil. Isolated genomic DNA was concentrated by ethanol precipitation.

A semiconserved region of the bacterial 16S rRNA gene was amplified using the bacterial consensus primers PB38 (5′-GKTACCTTGTTACGACTT) and PB36 FAM (5′-AGRGTTTGATCMTGGCTCAG). These primers amplified an ∼1500 bp product spanning positions 8 to 1509 of the *Escherichia coli* 16S RNA gene [4]. PB36 FAM was labeled on the 5′ end with 6-carboxyfluorescein (Applied Biosystems [ABI], CA, USA). Reaction mixtures for PCR contained buffer (10 mM Tris HCl pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$, 100 µM each deoxynucleotide, 0.2 µM of each primer, and 1.25 U of AmpliTaq DNA polymerase (PerkinElmer [PE], CT, USA) in a final volume of 50 µL. DNA amplification was performed using a program of 3 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 30 s at 72°C. Positive and negative (no template) controls were run with all sets of PCR reactions.

T-RFLP Analysis. A 10 µL aliquot of amplicon was digested with 1 U of *Hae*III (Life Technologies, MD, USA) at 37°C for 12 h. The terminal fragments were resolved and sized by denaturing gel electrophoresis using a model 373A automated sequencer in GeneScan mode (PE Applied Biosystems, Weiterstadt, Germany). Samples were prepared for electrophoresis at the Sequencing Facility at the School of Biological Sciences, The University of Auckland, by mixing a 2.5 µL portion of each digest with 2.0 µL formamide and 0.5 µL internal length standard (GeneScan 2500 TAMRA, PE Applied Biosystems). The samples were denatured at 94°C for 5 min and then immediately placed on ice until loaded onto the gel. A 1 µL portion of each sample of the samples were loaded onto a 36 cm 6% (w/v) denaturing polyacrylamide gel which was run for up to 9 h with limits of 2400 V, 40 mA, and 27 W. Following electrophoresis, the size of fluorescently labeled terminal fragments was determined by comparison with internal standards using GeneScan and Genotyper software (PE Applied Biosystems). The GeneScan default setting of 50 fluorescence units was set as the baseline noise threshold. Restriction fragments of similar sizes were designated as operational taxonomic units (OTUs) and were assumed to represent distinct taxa of bacteria. A bin size of 5 bp was employed to define an OTU.

Terminal restriction fragments of 70 bp were found in all samples, including negative controls. These fragments were therefore excluded from analysis as this fragment length was an artifact of the methodology, possibly due to the labeled primer dimers. The data were qualitatively interpreted by graphing total OTU abundance and mean OTUs per individual against size class and intestinal section, and statistically analyzed using the Kruskall-Wallis test for comparing medians and Dunn's test for nonparametric multiple comparisons with unequal sample sizes [35].

Cloning and DNA Sequence Analysis of PCR Products. Intestinal content samples were selected for bacterial 16S cloning that represented a variety of size classes and intestinal sections, and which demonstrated good PCR amplification in the T-RFLP analysis. DNA was extracted from intestinal content samples and the 1500 bp fragment of the 16S rRNA gene was amplified by PCR as described above. In this case an unlabeled PB 36 primer was used. PCR products were purified using a High Pure PCR Purification Kit (Roche, Basel, Switzerland) following supplier's directions. Purified products were ligated into a T-tailed PCR cloning vector (pGEM-T; Promega Madison WI) as described in the manufacturer's protocols. Ligation reactions were prepared in 15 µL volumes and incubated overnight at 4°C. The resulting products were transformed into *E. coli* DH5α competent cells in accordance with the manufacturer's protocol with the exception that Luria broth was used instead of SOC medium. Transformants containing inserts were identified by blue-white selection on L-agar plates. A clone library of 96 randomly selected white colonies was prepared and used for further analysis. Individual clones were subcultured in 150 µL of L-broth in a microtiter-tray format and incubated at 37°C overnight. Crude cell lysates were prepared for PCR analysis by transferring 50 µL of the broth culture to a new tube containing 100 µL of sterile H_2O and incubating at 94°C for 20 min. Lysates were centrifuged at 3000g for 5 min to sediment cell debris and were stored at −20°C prior to analysis. The remaining portions of the cultures were supplemented with 15% glycerol and stored at −80°C. Inserts were recovered from cell lysates by PCR amplification using vector-specific primers pGEM-F 5′-(GGCGGTCGCGGGAATTCGATT) and pGEM-R 5′-(GCCGCGAATTCACTAGTGATT) at a concentration of 100 µM and under the same conditions as above. The PCR products were digested using *Hae*III (Life Technologies) and separated on an acrylamide gel to obtain a RFLP profile for each clone. Twelve clones were selected for sequencing representing dominant restriction fragment profiles or those which were most likely to yield the 290–295 and 220–225 bp terminal restriction fragments (T-RFs) found commonly in the T-RFLP analysis. These clones were PCR amplified using pGEM-F and pGEM-R primers. PCR products were purified using a High Pure

PCR Product Purification Kit (Roche) and the yield estimated by acrylamide gel comparison. Sequencing PCR reactions were performed using the BigDye vs3 Dye Kit (PE Applied Biosystems) and one of each of the primers PB36, PB38, and 16S.5 following manufacturer's instructions.

Phylogenetic Analysis of DNA Sequences. Sequences were assembled and ambiguities resolved using AutoAssembler 2.0 software (PE Applied Biosystems), and the sequences compared to known sequences using the BLAST function provided through the NCBI website (http://www.ncbi.nlm.nih/BLAST). Sequences from the BLAST search with insufficient nucleotide length to allow for meaningful alignment with clones were discarded, while sequences of bacteria closely related to those found in other studies of gastrointestinal bacteria in marine herbivorous fish [1, IBY Pasch (2002) unpublished MSc thesis, The University of Auckland, New Zealand] were incorporated into the analysis. Comparative sequence data and clone sequence data were aligned using ClustalX1.08 [30], and sequences trimmed to 640 bp using BioEdit version 5.0.9 [15] to remove the effect of varying terminal sequence lengths. Following realignment, phylogenetic relationships were inferred using PAUP version 4.0b4a (Sinauer Associates, http://www.sinauer.com). Two phylogenetic trees were generated using neighbor-joining distances with the Kimura correction and consensus trees were constructed with 1000 bootstrap replicates. *Micrococcus luteus* and *Clostridium coccoides* were chosen as outgroups to allow comparison with a previous study on the phylogenetic relationships of bacteria found in the intestine of a herbivorous surgeonfish [1]. Additionally, *in silico* digests of rDNA clone sequences with *Hae*III were performed using GCG software (version 10, Wisconsin Computer Genetics Group Software, Wisconsin, USA), taking into account the extra sequence length added by the pGEM F primer. The T-RFLP Analysis Programme (TAP) from the Ribosomal Database Project II [12] was used to compare the T-RFLPs found in the present study to those in the database. Finally, the sequence data for the 12 clones were submitted to the NCBI Web site for incorporation into GenBank (accession numbers shown in Figs. 3 and 4).

Short-Chain Fatty Acid Analysis. The levels of the SCFAs acetate, propionate, and butyrate were quantified from intestinal section V of two size classes of *K. sydneyanus*. Three individuals <100 mm SL and six 100–199 mm SL were sampled between June and August 2000. Approximately 1 mL of intestinal content from section V was taken from captured specimens, frozen in liquid nitrogen for transport, and then stored at −4°C until required for analysis. SCFA analysis was performed on a Hewlett Packard 5890 gas chromatograph using the methods of Mountfort et al. [22].

Results

Specimen Capture and PCR Amplification. Samples of intestinal content were taken from four size classes of *K. sydneyanus* for four seasons with the exception of the <100 mm SL size class. Only one complete sample of this size class was taken (in winter) as juveniles settle onto coastal reefs in late summer/autumn. Generally, DNA extraction yields were higher in samples taken from larger fish and more posterior intestinal sections (data not shown). Of the 117 intestinal fluid samples taken, nine failed to yield PCR products. These included all three samples of intestinal section IV from the <100 mm SL size class, which was probably due to lower genomic DNA yield and PCR inhibition. PCR reactions performed with sample and control DNA indicated the presence of significant PCR inhibitors indicating the latter.

T-RFLP Analysis. Generally, total OTU diversity increased posteriorly along the intestine for all size classes (Fig. 1a). Total OTU diversity was highest in the >300 mm SL size class for all intestine sections, while the 100–199 and 200–299 mm SL size classes had similar OTU diversity for all intestinal sections. The <100 mm SL size class had only one OTU in each of intestinal sections III and V, although there was only an $n = 2$ for each intestinal section in this size class (Fig. 1a). Mean OTU number per individual per intestinal section was approximately the same for the >300 mm SL size class (7–8 OTUs/ind/section), but was lower in section III (2 OTUs/ind/section) than in section IV and V (5 OTUs/ind/section each) for the 100–199 and 200–299 mm SL size classes (Fig. 1b). A Kruskal-Wallis test found no significant difference in the medians of any of the intestinal sections for these latter size classes, though the data were greatly skewed by a single sample in each size class. The small number of samples analyzed for the <100 mm SL size class showed that only one OTU was found per sample in both the intestinal sections tested (Fig. 1b). Some OTUs were more common than others, with a distinct clustering of OTUs with terminal restriction fragment sizes in the 200–300 bp region (Fig. 2). The OTUs with 220–225 and 290–295 bp restriction fragments were common to most intestinal sections except for the <100 mm SL size class, where the 220–225 bp restriction fragment was the sole OTU in section III and the 290–295 bp restriction fragment was the sole OTU in section V (Fig. 2).

Phylogenetic Analysis of rDNA Clones. The phylogenetic analysis of the 12 clones resulted in four distinct clades. Eleven of the clones formed three clades derived
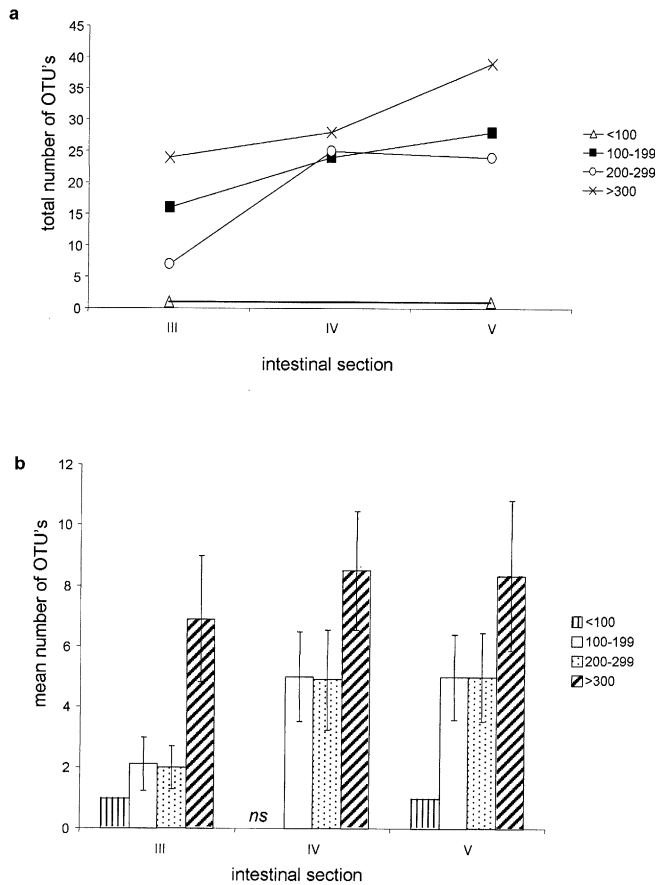
Figure 1. OTU diversity per intestinal section per size class for *K. sydneyanus* expressed as (a) total OTU diversity summed from all samples for each intestinal section and size class, and (b) mean OTU number (±SE) per individual for each intestinal section and size class. Legend indicates size class of fish (mm SL); *ns*: no sample obtained; refer to text for sample sizes.

from low G+C gram-positive bacteria that clustered within the clostridia (Fig. 3). The largest of these clades contained six clones and was the sister group to a clade containing *Clostridium lituseburense*; three clones formed a clade with *C. orbiscindens/*; and two clones formed a clade with *C. leptum*. The remaining clone formed a clade within the low G+C cell wall-less mycoplasmas and was most closely associated with *Acholeplasma vituli* (Fig. 4). There was no pattern between phylogenetic relationship of clones and host size class or intestinal section.

A *Hae*III *in silico* digest of clones forming a clade with *C. lituseburense* resulted in a T-RF of 289 bp from the 5' (PB 36) end of the partial sequence. *In silico* digests using the TAP application indicated that *C. lituseburense* and *C. difficile* both had T-RFs of 290 bp. None of the sequenced clones had a T-RF corresponding to the other dominant OTU comprising fragments of 220–225 bp.

Short-Chain Fatty Acid Analysis.   The <100 mm SL and 100–199 mm SL size classes had similar con-

centrations of SCFAs in intestinal section V (~15–20 mM). These concentrations were much lower than those found by Mountfort et al. [22] for specimens >300 mm SL (~52 mM, Table 1). The proportions of acetate:propionate:butyrate were broadly similar for all size classes (Table 1).

Discussion

The trend of increasing bacterial diversity along the intestine found in this study is consistent with the microscopic observations of Rimmer and Wiebe [26]. This trend was evident in all but the smallest size class, where microbial diversity was low in both of the intestinal sections surveyed. This is consistent with the hindgut being the predominant site of microbial fermentation [22]. The T-RFLP results indicate that there is an increase in bacterial diversity with fish size class in all intestinal sections, though a few samples were atypically diverse or depauperate, skewing the data. The atypical depauperate samples were most probably due to inhibition of PCR by algal metabolites, as microscopic observations revealed dense microbial communities (data not shown). The difficulty in deriving T-RFLP profiles for the <100 mm SL size class was likely due to a combination of factors, including low diversity, low genomic DNA yield, and PCR inhibition.

Along with an increase in diversity, the gastrointestinal microbiota of *K. sydneyanus* may also increase in abundance with fish size. Although no quantitative measures were made, the DNA extraction yields and SCFA concentrations indicate that bacterial abundance was positively correlated with fish size class. Peak heights and areas of GeneScan profiles used in T-RFLP analysis can be used as proxies of *in situ* abundance, but this was not employed because of the uncertainties associated with the quantitative nature of PCR-based assays [13, 19, 29, 34].

No OTUs or clones appeared to be specific to a particular fish size or intestinal section. The fact that the microbial communities did not differ between gut sections may reflect incomplete partitioning between the proximal intestine and the hindgut chamber, although a sphincter separates the hindgut chamber from the rest of the gut in fish as small as 20 mm SL (pers. obs.). The presence of the 220–225 and 290–295 bp OTU in most samples is interesting, especially as these OTUs solely composed the microbiota of intestinal section III and V, respectively, of the <100 mm SL specimens. The bacteria represented by these OTUs were found in most intestinal samples and appear to be the initial colonizers of the intestine in juvenile fish, supporting the notion that they may be functionally important for digestion.

The TAP analysis indicated that >50 bacteria gave a T-RF within the bin size 290–295 bp, including several species of the genus *Clostridium*. *Clostridium difficile* and
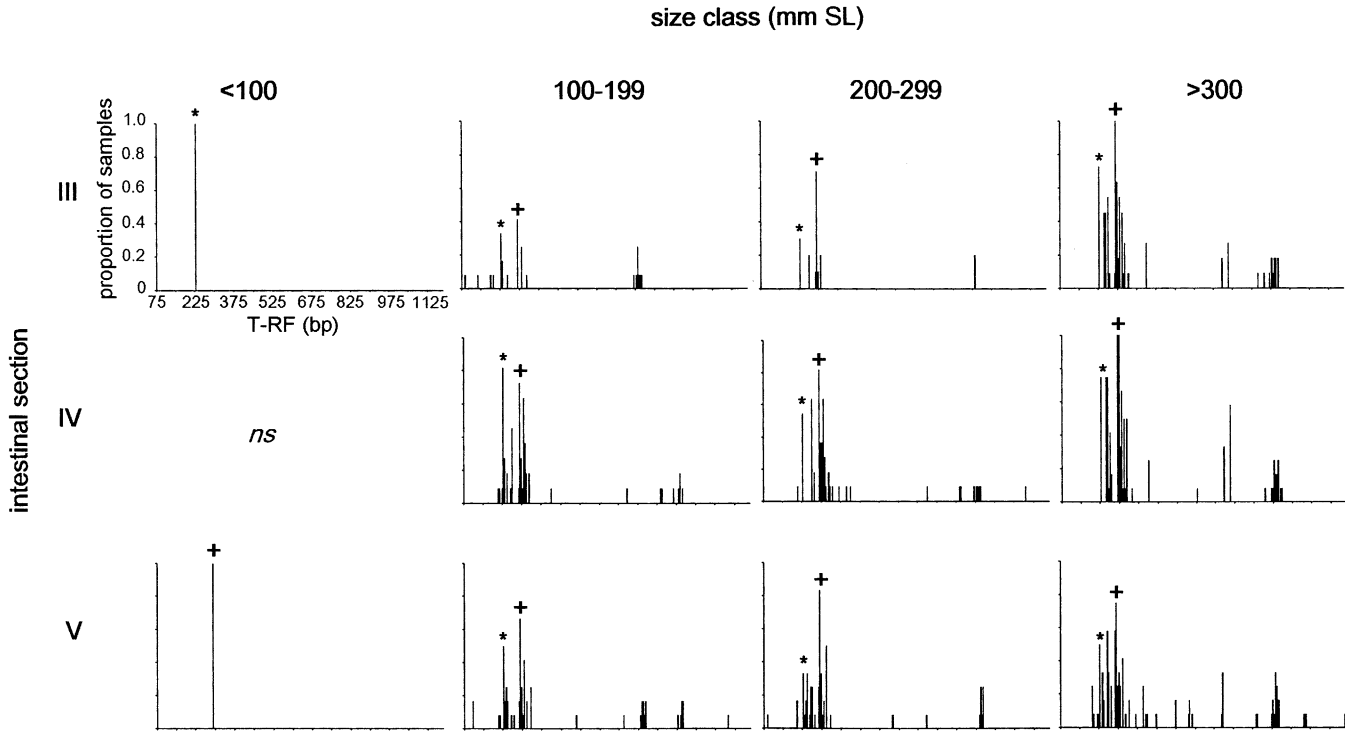
Figure 2. T-RFLP distribution across intestinal section and size class (mm SL) of *K. sydneyanus*. *x*-Axis represents terminal restriction fragment length (bp); *y*-axis represents proportion of samples found to possess OTU; *: 220–225 bp OTU; +: 290–295 bp OTU; *ns*: no sample obtained; refer to text for sample sizes.

C. lituseburense both yielded *in silico* T-RFs of 290 bp and formed a clade with several clones from the 16S clone library. Based on these observations, it is reasonable to assume that the 290–295 bp OTU included the clones from this clade. The 1 bp discrepancy between the *in silico* T-RF of these clones (289 bp) and the 290–295 bp OTU can be explained by the observation that both the molecular size and nucleotide composition influence the migration of T-RFs along a denaturing gel [13]. Evidence for this comes from the results from the T-RFLP analysis and clones derived from samples taken from intestinal section V of fish <100 mm SL, where the only OTU derived in the T-RFLP analysis was 290–295 bp, and the only clones derived were those which formed a clade with *C. lituseburense* and gave an *in silico* T-RF of 289 bp. Additionally, the dominance of this particular OTU and clone is corroborated by the dominance of the *C. lituseburense* clone group in the gut of adult *K. sydneyanus* reported by IBY Pasch (2002, unpublished MSc thesis, The University of Auckland, New Zealand).

Unfortunately, none of the sequenced clones gave a terminal restriction fragment that matched the 220–225 bp OTU, so presumptive identification of this bacterium or group of bacteria was not possible. The finding of two clones affiliated with *C. leptum* supports the finding by IBY Pasch (2002, unpublished MSc thesis, The University of Auckland, New Zealand) that *K. sydneyanus*

and two other species of New Zealand herbivorous fishes (*Aplodactylus arctidens* and *Odax pullus*) harbored clones closely related to *C. leptum*. In contrast, the three clones forming a clade with *C. orbiscindens* found in the present study were not found in the aforementioned study.

It is highly significant that the majority of the clones found formed a clade within the clostridia, as members of this genus are characteristic gastrointestinal symbionts in a diverse range of vertebrate herbivores (Fig. 3, [17, 18, 23]). Interestingly, the well-studied intestinal bacterium *Epulospiscium fishelsoni* from the gut of the Red Sea surgeonfish *Acanthurus nigrofuscus* [2, 14] is not closely related to any of the clones in the present study on the basis of our sequence data. Our phylogenetic results support the results of functional studies in suggesting that the primary role of most of the bacteria in the intestine of *K. sydneyanus* is the fermentation of algal metabolites into nutritionally useful end products for the host (i.e., SCFAs).

One clone in our study fell out with the cell wall-less mycoplasma group, which are typically pathogenic or parasitic [17]. The closest known species to this clone was a member of the genus *Acholeplasma*, which are facultative anaerobes with a fermentative type metabolism and are known to be parasites of vertebrate hosts [17]. A recent survey of the gastrointestinal microbial community of a marine salmonid by Holben et al. [16] showed that
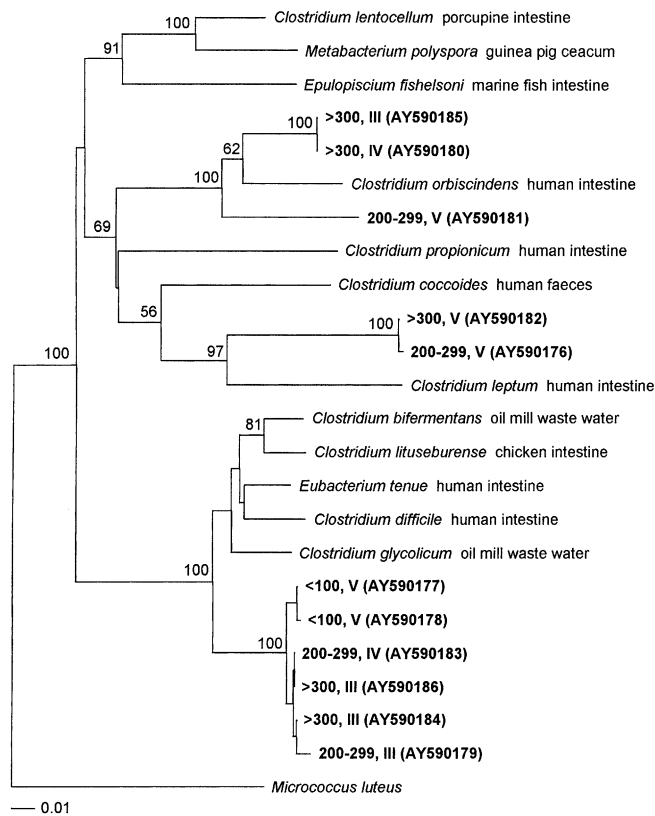
Figure 3. Phylogenetic tree derived by neighbor joining of clostridial clones from intestinal content samples of *K. sydneyanus*. Bootstrap values >50% for 1000 replicates are indicated at each node. Clones are denoted in bold text by sample size class (mm SL) and intestinal section (III–V) of origin, and also by GenBank accession number in parentheses. The scale bar equals 0.01 changes per nucleotide position. Comparative sequences are shown with typical habitat, and the GenBank accession numbers from top to bottom are as follows: X71851, U22331, AF067413, Y18187, X77841, MS9090, M59095, AF320283, M59107, M59118, AF072474, AY007244, AJ536198.

Figure 4. Phylogenetic tree derived by neighbor joining of a mycoplasma clone derived from an intestinal content sample of *K. sydneyanus*. Bootstrap values >50% for 1000 replicates are indicated at each node. Clones are denoted in bold text by sample size class (mm SL) and intestinal section (III–V) of origin, and also by GenBank accession number in parentheses. The scale bar equals 0.05 changes per nucleotide position. Comparative sequences are shown with an example of the habitat derived from, and the GenBank accession numbers from top to bottom are as follows: AF031479, AF085350, X63781, D78650, M59090.

~96% of the microbes were a mycoplasma phylotype, leading the authors to speculate that mycoplasmas may actually have an unknown commensal role in the host gut.

In summary, the intestinal microbiota of *K. sydneyanus* appears to develop via an increase in diversity. Levels of SCFA increase between juvenile and adult fish, although data on rates of fermentation are only available for adult fish [22]. The majority of clones isolated from
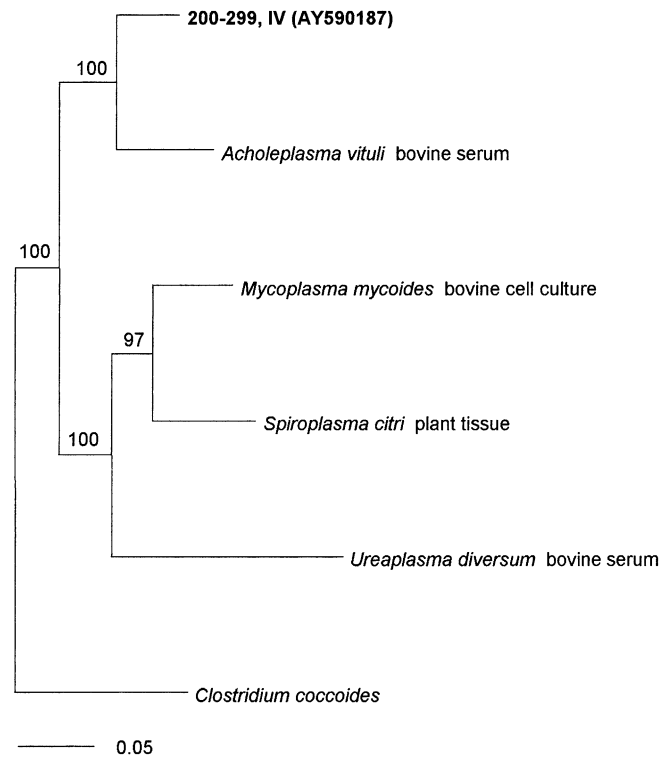
intestinal samples showed that the bacteria were most closely related to members of the genus *Clostridium*, indicative of a role that involves fermentation of refractory parts of the host diet into more readily assimilable nutrients for the host. These results support the suggestion that there is an ontogenetic diet shift by *K. sydneyanus* from a diet of chlorophyte and rhodophtye algae that is digested endogenously, to a diet of phaeophyte algae that is high in refractory algal metabolites and is partially digested exogenously by microbial symbionts in the hindgut. Further work is needed to characterize more completely the gastrointestinal symbiotic community of

Table 1. Ontogenetic variation in the concentration (mM) and proportions (in parentheses) of SCFA in intestinal section V of *K. sydneyanus* (mean ± SE)

	Acetate	Propionate	Butyrate	Total
<100 mm SL[a] ($n = 3$)	11.4 ± 2.4 (73.3 ± 15.2)	3.9 ± 0.2 (25.1 ± 5.6)	0.2 ± 0.1 (1.6 ± 1.1)	15.5 ± 2.70
100–199 mm SL[a] ($n = 6$)	11.4 ± 1.2 (59.5 ± 6.0)	6.9 ± 0.9 (35.9 ± 4.9)	0.9 ± 0.1 (4.6 ± 0.5)	19.2 ± 2.2
>300 mm SL[b] ($n = 3$)	37.5 ± 4.9 (72.2 ± 2.1)	12.8 ± 0.5 (25.3 ± 2.4)	1.3 ± 0.3 (2.5 ± 0.3)	51.6 ± 5.5

[a]Taken from present study.
[b]Taken from Mountfort et al. [22].

this fish species, and to determine the metabolic pathways used by the dominant bacteria. Such studies will provide a better understanding of the dynamics of the host/symbiont relationship, and the role that microbial processes play in the nutrition of marine herbivorous fishes.

Acknowledgments

References

1. Angert, ER, Brooks, AE, Pace, NR (1996) Phylogenetic analysis of *Metabacterium polyspora*: clues to the evolutionary origin of daughter cell production in *Epulopiscium* species, the largest bacteria. J Bacteriol 178: 1451–1456

2. Angert, ER, Clements, KD, Pace, NR (1993) The largest bacterium. Nature 362: 239–241

3. Bjorndal, KA (1997) Fermentation in reptile and amphibians. In: Mackie, RI, White, BI (Eds.) Gastrointestinal Microbiology, Chapman and Hall, New York, pp 199–230

4. Brosius, J, Dull, TL, Sleeter, DD, Noller, HF (1981) Gene organisation and primary structure of a ribosomal RNA operon from *Escherichia coli*. J Mol Biol 148: 107–127

5. Clements, KD (1991) Endosymbiotic communities of two herbivorous labroid fishes, *Odax cyanomelas* and *Odax pullus*. Mar Biol 109: 223–230

6. Clements, KD (1997) Fermentation and gastrointestinal microorganisms in fishes. In: Mackie, RI, White, BI (Eds.) Gastrointestinal Microbiology, Chapman and Hall, New York, pp 156–198

7. Clements, KD, Choat, JH (1993) The influence of season, ontogeny and tide on the diet of the temperate marine herbivorous fish *Odax pullus* (Odacidae). Mar Biol 117: 213–220

8. Clements, KD, Choat, JH (1995) Fermentation in tropical marine herbivorous fishes. Physiol Zool 68: 355–378

9. Clements, KD, Choat, JH (1997) Comparison of herbivory in the closely-related marine fish genera *Girella* and *Kyphosus*. Mar Biol 127: 579–586

10. Clements, KD, Glesson, VP, Slaytor, M (1994) Short-chain fatty acid metabolism in temperate marine herbivorous fish. J Comp Physiol B 164: 372–377

11. Clench, MH, Mathias, JR (1995) The avian cecum: a review. Wilson Bull 107: 93–121

12. Cole, JR, Chai, B, Marsh, TL, Farris, RJ, Wang, Q, Kulam, SA, Chandra, S, McGarrell, DM, Schmidt, TM, Garrity, GM, Tiedje, JM (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31: 442–443

13. Dunbar, J, Ticknor, LO, Kruske, CR (2001) Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. Appl Environ Microbiol 67: 190–197

14. Fishelson, L, Montgomery, WL, Myrberg, AAJ (1985) A unique symbiosis in the gut of tropical herbivorous surgeonfish *Acanthuridae teleostei* from the Red Sea. Science 229: 49–51

15. Hall, TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95–98

16. Holben, WE, Williams, P, Saarinen, M, Särkilahti, LK, Apajalahti, JHA (2002) Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma* phylotype in farmed and wild salmon. Microb Ecol 44: 175–185

17. Holt, JG, Krieg, NR, Sneath, PHA, Staley, JT, Williams, ST (Eds.) (1994) Bergey's Manual of Determinative Bacteriology. Williams and Wilkins, Baltimore

18. Krause, DO, Dalrymple, BP, Smith, WJ, Mackie, RI, McSweeney, CS (1999) 16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: design of a signature probe and its application in adult sheep. Microbiology 145: 1797–1807

19. Marsh, TL, Saxman, P, Cole, J, Tiedje, J (2000) Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. Appl Environ Microbiol 66: 3616–3620

20. Moran, D, Clements, KD (2002) Diet and endogenous carbohydrases in the temperate marine herbivorous fish *Kyphosus sydneyanus*. J Fish Biol 60: 1190–1203

21. Moré, MI, Herrick, JB, Silva, MC, Ghiorse, WC, Madsen, EL (1994) Quantitative cell lysis of indigenous mircoorganisms and rapid extraction of microbial DNA from sediment. Appl Environ Microbiol 60: 1572–1580

22. Mountfort, DO, Campbell, J, Clements, KD (2002) Hindgut fermentation in three species of marine herbivorous fish. Appl Environ Microbiol 68: 1374–1380

23. Nelson, KE, Thonney, ML, Woolston, TK, Zinder, SH, Pell, AN (1998) Phenotypic and phylogenetic characterization of ruminal tannin-tolerant bacteria. Appl Environ Microbiol 64: 3824–3830

24. Rake, AV (1972) Isopropanol preservation of biological samples for subsequent DNA extraction and reassociation studies. Anal Biochem 48: 365–368

25. Rimmer, DW (1986) Changes in diet and the development of microbial digestion in juvenile buffalo bream *Kyphosus cornelii*. Mar Biol 92: 443–448

26. Rimmer, DW, Wiebe, WJ (1987) Fermentative microbial digestion in herbivorous fishes. J Fish Biol 31: 229–236

27. Russell, JB, Rychlik, JL (2001) Factors that alter rumen microbial ecology. Science 292: 1119–1122

28. Stevens, CE, Hume, ID (1995) Comparative Physiology of the Vertebrate Digestive System, 2nd ed, Cambridge University Press, New York

29. Suzuki, MT, Giovannoni, SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 62: 625–630

30. Thompson, JD, Gibson, TJ, Plewniak, F, Jeanmougin, F, Higgins, DG (1997) The ClustalX-Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876–4882

31. Titus, E, Ahearn, GA (1988) Short-chain fatty acid transport in the intestine of a herbivorous teleost. J Exp Biol 135: 77–94

32. Titus, E, Ahearn, GA (1991) Transintestinal acetate transport in a herbivorous teleost anion exchange at the basolateral membrane. J Exp Biol 156: 41–62

33. Titus, E, Ahearn, GA (1992) Vertebrate gastrointestinal fermentation: transport mechanisms for volatile fatty acids. Am J Physiol 262: R547–R553

34. Wintzingerode, FV, Gobel, UB, Stackebrandt, E (1997) Determination of microbial diversity on environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21: 213–229

35. Zar, JH (1999) Biostatistical Analysis, 4th ed, Prentice Hall, Upper Saddle River, NJ