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TROPHOLOGY

Bacterial Phylotypes Associated with the Digestive Tract of the Sea Urchin Paracentrotus lividus and the Ascidian *Microcosmus* sp.[1]

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Abstracts—We used sequencing and phylogenetic analysis of PCR-amplified 16S rRNA genes from bacteria that are associated with the esophagus/pharynx, stomach and intestine of two marine sympatric invertebrates but with different feeding mechanisms, namely the sea urchin Paracentrotus lividus (grazer) and the ascidian *Microcosmus* sp. (suspension feeder). Amplifiable DNA was retrieved from all sections except the pharynx of the ascidian. Based on the inferred phylogeny of the retrieved sequences, the sea urchin's esophagus is mainly characterized mostly by bacteria belonging to α-, γ-Proteobacteria and Bacteriodetes, most probably originating from the surrounding environment. The stomach revealed phylotypes that belonged to γ- and δ-Proteobacteria, Verrucomicrobia and Fusobacteria. Since the majority of their closest relatives are anaerobic species and they could be putative symbionts of the *P. lividus* stomach, in which anaerobic conditions also prevail. Seven out of eight phylotypes found in the sea urchin's intestine belonged to sulfate reducing δ-Proteobacteria, and one to γ-Proteobacteria, with possible nutritional activities, i.e. degradation of complex organic compounds which is beneficial for the animal. The bacterial phylotypes of the ascidian digestive tract belonged only to the phyla of Actinobacteria and Proteobacteria. The stomach phylotypes of the ascidian were related to pathogenic bacteria possibly originating from the water column, while the intestine seemed to harbour putative symbiotic bacteria that are involved in the degradation of nitrogenous and other organic compounds, thus assisting ascidian nutrition.

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INTRODUCTION

The presence of prokaryotes in the digestive tract of marine invertebrates has advantages for both sides. The microorganisms may provide alternative sources of carbon and energy to the host cells and the hydrolysis of complex organic compounds and production of microbial biomass could provide the host cells with protein (Mayer et al. 2001). In return, the host supplies the microorganisms with a steady environment free of predators.

Fairly recently, molecular methods were developed to overcome problems related with the non-culturability of microorganisms from environmental samples (Amann et al. 1995). Although problems with the polymerase chain reaction (PCR), such as the formation of chimeric sequences and PCR selectivity, have already been recognized (Ravenschlag et al. 1999), analysis of the 16S rDNA phylotype libraries still remains the most widely used tool for the general description of microbial diversity.

The microorganisms occurring in the hindgut of sea urchins have been studied so far only in *Echinocardium cordatum*, focusing on sulfur oxidizers and sulfate reducers (Thorsen et al. 2003). The study of microorganisms associated with ascidians has targeted the subcuticular area. The most striking results thus far were the discovery of microorganisms involved in the detoxification of vanadium (Lyalikova and Yurkova 1992) and of those producing compounds with antibiotic and antitumor activity (Aassila et al. 2003).

The habitat of the sea urchin *Paracentrotus lividus* is primarily hard substrata (e.g. pebbles, boulders, rocks) covered with encrusting algae as well as seagrass beds, mostly of *Cymodocea* and *Posidonia*. *Paracentrotus lividus* is a scraper that feeds on attached material on hard substrata and on seagrass. *Microcosmus* sp. is a solitary filter-feeding ascidian attached to a hard substrata (Ruppert and Barnes 1994). By using molecular

[1] The text was submitted by the authors in English.

fingerprinting methods, our study focused on the characterization of the bacterial phylotypes occurring in the digestive tract of these two sympatric marine invertebrates taken from a coastal site.

MATERIAL AND METHODS

Three specimens of each species were collected from the rocky (boulders) substrate of a small Aegean Sea cove (Agios Nikolaos, Anavissos, Greece) at a depth of 2 m in June 2003. The water temperature was 20°C and salinity was 30 psu. The specimens were transferred immediately at the laboratory to a cool box, where, after dissection, their digestive tract with its content was split in three sections: upper (esophagus in *Paracentrotus lividus* and pharynx in *Microcosmus* sp.), middle (stomach) and lower (intestine). The corresponding sections of the three specimens for each species were pooled and DNA from approximately 1.5 g of the material was extracted using a RNA/DNA isolation kit (Qiagen Inc., USA) according to the manufacturer's protocol.

Either ~900 or ~1400 bp of the 16S rDNA were amplified by nested PCR. For the first amplification we used the bacterial primers BAC8f (5'-AGAGTTTGATCCTGGCTCAG-3') and BAC1492r (5'-CGGCTACCTTGTTACGACTT-3') in a MyCyler (Bio-Rad Inc., USA) thermal cycler. For the second amplification we used either the BAC8f – BAC1390r (5'-TGTACACACCGCCCGTC-3') or the BAC8f – BAC907r (5'-CCCGTCAATTCCTTTGAGTTT-3') primer pairs. Each run consisted of a 1-min pre-PCR hold at 94°C, followed by 30 cycles consisting of a 45-sec denaturation step at 94°C, a 45-sec annealing step at 52.5°C, a 2-min elongation step at 72°C, and at the end of the 30 cycles, and a final 7-min finishing step at 72°C. All PCR ingredients were prepared with twice-autoclaved ultra-pure water, and stringent anti-contamination controls were used during PCR preparation. PCR products were visualized on a 1.2% agarose gel under UV light, bands were excised, and PCR products were extracted with the Wizard SV Gel and PCR Clean-up kit (Promega Inc., USA) following the manufacturer's protocol. The purified products were A-tailed to improve cloning efficiency by mixing 2.5 µl 10X PCR buffer (200 mM Tris, pH 8.55), 2.5 µl deoxynucleoside triphosphate 2mM, 1.5 µl $MgCl_2$ 25mM, 5.9 µl PCR water, 0.1 µl Taq polymerase and 12.5 µl of the purified sample. The mixture was incubated at 72°C for 10 minutes in a water-bath or thermal cycler. The PCR products were cloned using the TOPO XL PCR cloning kit (Invitrogen Co., USA) using chemically competent cells according to the manufacturer's specifications. For each sample, 20–40 clones were analyzed. These clones were screened for unique restriction fragment length polymorphism (RFLP) patterns after digestion with the RsaI and AluI (Fermentas UAB, Lithuania) enzymes. Clones with unique RFLP patterns were grown in liquid cultures and their plasmids were purified using the Nucleospin Plasmid QuickPure kit (Macherey-Nagel GmbH & Co. KG, Germany) for DNA sequencing. Sequence data were obtained by Macrogen Inc. (Korea) using capillary electrophoresis by the BigDye Terminator kit (Applied Biosystems Inc., USA) with the primers M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3'). Each time ~900 bp were sequenced. For each individual clone, forward and reverse sequences were assembled. The assembled sequences were checked for chimeras using the CHIMERA_CHECK function of the Ribosomal Database Project II (Maidak et al. 2001).

For identification of closest relatives, all sequences were compared with the BLAST function (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned using the ARB FastAligner utility (www.arb-home.de) and by manual aligning according to secondary structure in ARB as well. Analyses were performed using minimum evolution and parsimony methods implemented in PAUP* version 4.08b (Swofford 2000). Heuristic searches under minimum evolution criteria used 1000 random-addition replicates per data set, each followed by tree bisection-reconnection topological rearrangements. The topology of the tree was based on neighbor–joining according to Jukes-Cantor. Bootstrapping under minimum evolution and parsimony criteria was performed with 1000 replicates for both the sea urchin and ascidian data sets. Sequences of unique phylotypes found in this study have GenBank numbers AY770692-AY770729.

RESULTS AND DISCUSSION

Methodological considerations. The frequency of the retrieved clones indicates that the bacterial communities of both animals studied did not completely describe the bacterial diversity of their digestive tracts, and that these communities were not sampled to exhaustion. Redundancy analysis showed that, for all samples, the number of clones analyzed were at the beginning of the plateau of the cumulative frequency of the observed phylotypes (data not shown). Nevertheless, to avoid any misleading conclusion about the presence of a 100% complete bacterial community survey, only the unique phylotypes which were included in the phylogenetic analysis are discussed in this study.

Paracentrotus lividus. The phylotypes from the esophagus, stomach and intestine of the sea urchin *P. lividus* belonged to the bacterial phyla of Proteobacteria, Fusobacteria, Bacteriodetes, Actinobacteria and Verrucomicrobia (Table 1, Fig. 1).

The occurrence of symbionts or bacterial cells in high abundances in the esophagus is very unlikely as the ingested food does not remain there long enough and it quickly passes through (Ruppert and Barnes 1994, Hickman et al. 2000). Indeed, most of the phylotypes found in this section were related to the wide-

Bacterial 16S rDNA phylotypes of the sea urchin *Paracentrotus lividus* (Pl) and the ascidian *Microcosmus* sp. (Msp) digestive tracts

Phylotype	Closest relative (GenBank access No.)	Similarity (%)	Reference
Esophagus			
Pl139	Uncultured clone, Suruga Bay, Japan, sediments (AB015520)	88	Li et al. (1999)
Pl142	Uncultured clone, Suruga Bay, Japan, sediments (AB015520)	88	Li et al. (1999)
Pl137	*Defluvibacter lusatiae* (AJ132378)	88	Fritsche et al (1999)
Pl138	Uncultured Bacteroidetes clone, sulfidogenic enrichments (AF121885)	89	Knight et al. (1999)
Pl144	*Propionibacterium acnes* (AB042289)	99	Skerman et al. (1980)
Pl140	Uncultured clone, oligotrophic marine γ-group (OMG) (AY216447)	93	Cho and Giovannoni (2004)
Pl143	Uncultured *Acinetobacter*, deep western Pacific Ocean sediments (AJ551115)	99	Wang et al. (2004)
Stomach			
Pl51	*Vibrio orientalis* (X74719)	95	Yang et al. (1983)
Pl52	*Vibrio splendidus* (AB038030)	96	Urakawa et al. (1999)
Pl53	*Colwellia* sp., marine invertebrate symbiont (AY198115)	96	Unpublished, in GenBank
Pl59	*Photobacterium lipolyticum* (AY554009)	97	Yoon et al. (2005)
Pl48	*Verrucomicrobium* sp., hydrothermal vent polychaete (AJ441222)	93	Alain et al (2002)
Pl50	*Desulfotalea arctica* (AF099061)	96	Knoblauch et al. (1999)
Pl57	*Desulfotalea arctica* (AF099061)	95	Knoblauch et al. (1999)
Pl49	*Propionigenium maris* (X84049)	97	Watson et al. (2000)
Intestine			
Pl93	*Desulfotalea arctica* (AF099061)	96	Knoblauch et al. (1999)
Pl94	Uncultured *Desulforhopalus* clone, Antarctic sediment (AY177796)	93	Purdy et al. (2003)
Pl95	*Desulfotalea psychrofila* (AF099062)	96	Knoblauch et al. (1999)
Pl96	*Desulfovibrio gracilis* (U53464)	89	Magot et al. (2004)
Pl99	*Desulfotalea psychrofila* (AF099062)	95	Knoblauch et al. (1999)
Pl100	*Desulfotalea arctica* (AF099061)	94	Knoblauch et al. (1999)
Pl102	*Desulfotalea psychrofila* (AF099062)	94	Knoblauch et al. (1999)
Pl103	*Photobacterium lipolyticum* (AY554009)	97	Yoon et al. (2005)
Stomach			
Msp111	*Propionibacterium acnes* (AB042289)	99	Skerman et al. (1980)
Msp113	Uncultured Actinobacterium clone, aphid symbiont (AB075644)	99	Nakabachi et al. (2003)
Msp126	*Tsukamurella paurometabola* (AF283280)	99	Katar et al. (2001)
Msp112	*Achromobacter xylosoxidans* (AF411021)	99	Busse and Stolz (2001)
Msp123	*Acinetobacter* sp. (AY043369)	99	Coenye et al. (2001)
Intestine			
Msp75	Uncultured γ-Proteobacteria, swine manure (AY167969)	99	Whitehead & Cotta (2004)
Msp83	*Stenotrophomonas maltophilia* (AJ293468)	97	Minkwitz & Berg (2001)
Msp76	*Agrobacterium* sp. (AY174112)	99	Trott et al. (2003)
Msp87	*Phyllobacterium myrsinacearum* (D12789)	99	Knösel (1984)
Msp26	*Rothia* sp. (AJ131121)	96	Collins et al (2000)
Msp77	*Rothia* sp. (AJ131121)	98	Collins et al (2000)
Msp79	*Rothia* sp. (AJ131121)	95	Collins et al (2000)
Msp84	*Rothia* sp. (AJ131121)	98	Collins et al (2000)
Msp90	*Rothia* sp. (AJ131121)	98	Collins et al (2000)

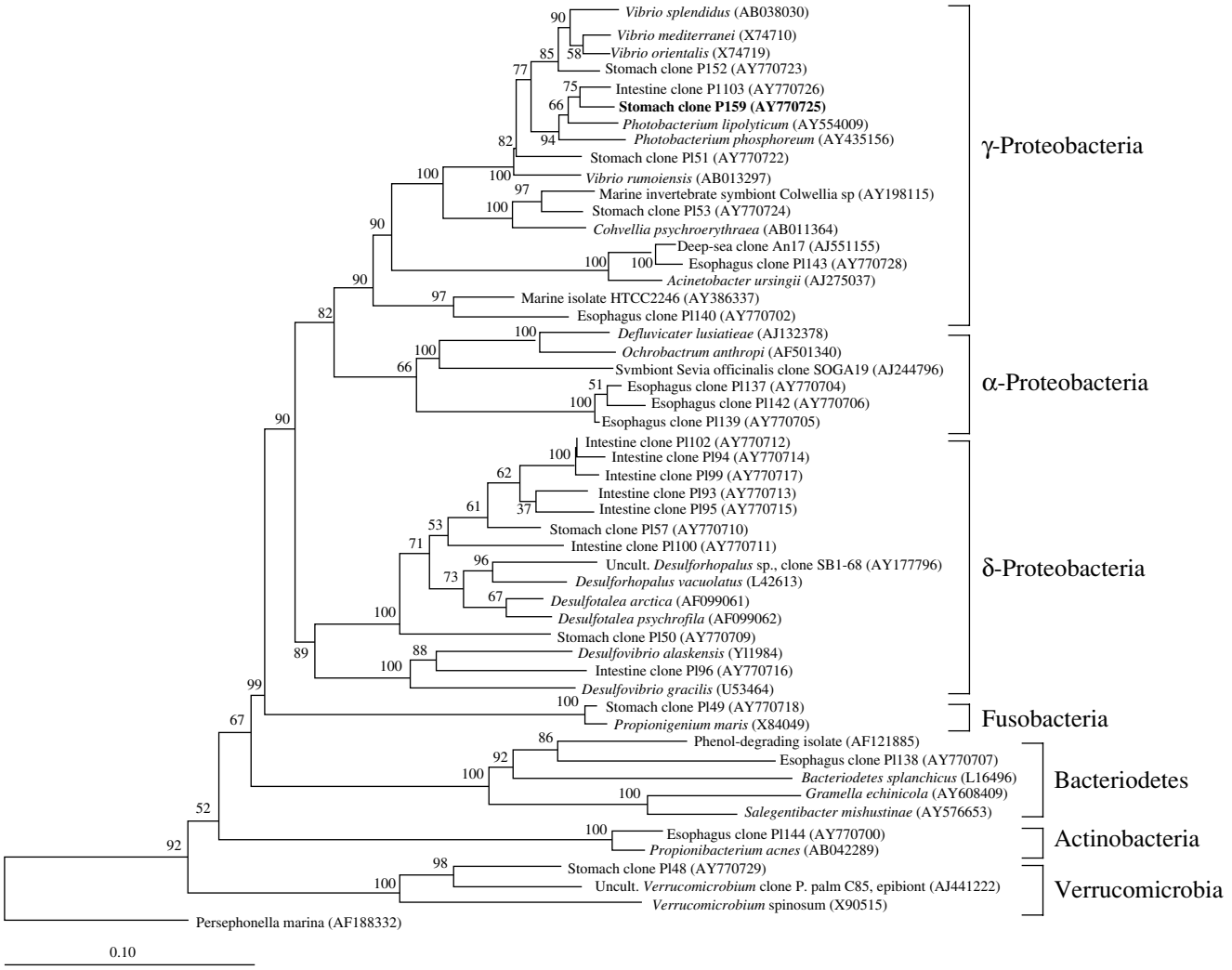

Fig. 1. 16S rRNA-based minimum evolution distance tree showing the phylogenetic relationships of bacterial clones from the digestive tract of *Paracentrotus lividus* (in bold) to cultured bacterial species and related environmental clones. The tree is based on *Escherichia coli* positions 28–1390 of the 16S rRNA gene. Bootstrap (1000 replicates) support values (%) are given at nodes for minimum evolution distance. Bar indicates the number of substitutions per site.

spread marine α- and γ-Proteobacteria (Kirchman 2000). Phylotypes Pl139 and Pl142 were related to unknown bacteria from marine sediments and Pl137 and Pl138 to microorganisms degrading phenolic compounds (Table 1). The occurrence of high concentrations of haloaromatic compounds could be derived from the scrapped algal material (Paul and Puglisi 2004). Phylotype Pl144 is almost identical to an anaerobic human pathogen, possibly originating from a pollution source in the surrounding environment. Phylotypes Pl140 and Pl143 are closely related to anaerobic microorganisms, suggesting their origin from sediment particles or processed food pellets.

Eight bacterial phylotypes were found in the stomach, where most of the food digestion takes place (Ruppert and Barnes 1994, Hickman et al. 2000). Moreover, the occurrence of planktic microorganisms is not expected in this section because water bypasses the stomach and diverts directly from the esophagus to the intestine (Hickman et al. 2000). Phylotype Pl48 is related to a sequence retrieved from the hydrothermal vent polychaete *Paralvinella palmiformis*. This sequence was marginally related to *Verrucomicrobium spinosum* and is believed to be involved in the decomposition of mucous secretions and other organic debris (Alain et al. 2002). In addition, *Verrucomicrobium* includes heterotrophic bacteria fermenting polysaccharides and occurs primarily in aquatic ecosystems, although there are some representatives from soil (Schlesner 1999). The ability to decompose organic compounds, as well as the fact that *Verrucomicrobium spinosum*, the only known species of the genus, is facultatively anaerobic (Schlesner 1999), assigns this phylotype as a possible symbiont of the digestive tract that could grow in the anaerobic microniches of the food pellets and contribute to the decomposition of complex

sugars. Phylotype Pl49 is related to the anaerobic *Propionigenium maris*, which debrominates the burrows of bromophenol-producing marine infauna (Watson et al. 2000); its detoxification ability for high bromophenol sediment concentrations, in addition to the presence of phylotypes Pl137 and Pl138, shows that there may be high concentrations of aromatic compounds in the stomach of *P. lividus* originating from algal material (Paul and Puglisi 2004). Phylotypes Pl51 and Pl52 are closely related to *Vibrio* spp., a genus with many parasitic or symbiotic representatives in marine organisms (Farmer 2004). The halophilic, facultative anaerobic *V. splendidus* is a symbiont of the marine bivalve *Crassostrea* gigas and is capable of fermentative as well as oxidative metabolism in anaerobic conditions using NO^{3-} as an alternative electron acceptor. As such, it could prevail in the anaerobic microniches in the stomach (Pujalte et al. 1999) along with phylotypes Pl50 and Pl57, which are related to anaerobic sulfate reducing bacteria. As well, it has been suggested that symbiotic strains produce a great number of proteolytic and lipolytic extracellular enzymes that allow the host to use a wide array of complex compounds (Harris 1993). Consequently, the lysates of these enzymes would nutritionally benefit the sea urchin as a host. A similar lipolytic role beneficial for the sea urchin is also inferred from phylotype Pl59, closely related to the anaerobic, lipolytic *Photobacterium lipolyticum* (Yoon et al. 2005). The symbiotic way of life of *Photobacterium* sp. is supported by the fact that it is a symbiont or parasite for some marine organisms (Euzeby 2004). Phylotype Pl53 is related to the genus *Colwellia*, known mostly for its planktic psychrophilic representatives, and its closest relative is a sequence retrieved from a marine invertebrate.

The majority of the intestine phylotypes belong to the genera *Desulfotalea*, *Desulforhopalus* and *Desulfovibrio* of the δ-Proteobacteria. All these genera are anaerobic sulfate-reducing bacteria (SRB). Sulfate reduction (SR) is responsible for a large portion of the organic decomposition in sediments (Ravenschlag et al. 1999). The possible role of SRB in the gut of *P. lividus* is of particular interest. It has been shown that microbial function in the gut, and especially the products of fermentation, are very important for the nutrition of the sea urchin *Echinocardium cordatum* (Thorsen 1998). The products of fermentation provide the sea urchin with 10% of the energy required for its metabolism. The occurrence of SRB has been demonstrated in *E. cordatum*, suggesting that decomposition of food aggregates takes place in the animal's hindgut (Thorsen et al. 2003). Accumulated fermentation products during anaerobic metabolism would provide the SRB with an ideal energy source, assist the host in the removal of these undesirable end-products and recycle metabolites that would otherwise be lost to symbiosis, as has been suggested for the syntrophic sulfur cycle in the oligochaete worm *Olarius algarvensis* (Dubilier et al. 2001). The build-up of H_2S as a result of SR can be toxic to the urchin, but there are means of overcoming this problem. The produced sulfide can react with reduced iron Fe(II) and the resulting FeS can act as a buffer limiting the toxic impact of H_2S. Moreover, the presence of sulfur oxidizers (SO) could be implied that would detoxify H_2S thus making possible a full S cycle in the gut of *P. lividus*. The presence of SO has been investigated previously in *E. cordatum* (Brigmon and Ridder 1998, Thorsen et al. 2003), but SO were not found in our study, possibly due to their low presence and/or by PCR biases. Also, this absence could be attributed to the different food sources of *E. cordatum* and *P. lividus* and to the fact that the latter lacks a cecum or a similar structure where, as in *E. cordatum*, SO bacteria develop on the nodules of the cecum and SRB within the nodules (Thorsen et al. 2003). Further investigation, mainly with FISH using probes specific for SO, is required.

Microcosmus sp. No phylotypes were found in the pharynx, since ascidians are suspension feeders and this section acts solely as an effective water filter with very low residence time for filtered particles and has no digestive function (Ruppert and Barnes 1994, Hickman et al 2000). The sequences found in the stomach and intestine belonged to the Actinobacteria and Proteobacteria phyla (Table 1, Fig. 2).

The stomach phylotypes may reflect some of the microorganisms occurring in the surrounding water. The location and depth of specimen collection may play an important role, as the ascidians were close to a shore influenced by humans both directly and indirectly. Phylotypes Msp113 and Msp126 are almost identical to the actinobacterial representatives of an aphid symbiont and a human pathogen. However, as Actinobacteria are common in the marine environment (Bull et al. 2005), it is possible that they were ingested through feeding. The human impact on ascidian feeding is also revealed by phylotypes Msp123, Msp111 and Msp112, which were identical to pathogenic species (*Propionibacterium acnes*, *Acinetobacter* sp. and *Achromobacter xylosoxidans*, respectively).

In the intestine, the presence of *Phyllobacterium myrsinacearum* could be attributed to sample contamination as its 16s rDNA sequence is strongly amplified by PCR (Elmer and Cranton 2004), or to transportation from the land. Phylotype Msp83 is closely related to *Stenotrophomonas maltophilia*, a ubiquitously occurring species which produces antibiotics, siderophores, protease and chitinases (Minkwitz and Berg 2001) and could contribute to the animal's feeding and defense mechanisms. Phylotype Msp75, is related to a swine manure bacterium (AY167969) which has been suggested to play an important role in the digestion and fermentation of proteinaceous material in manure (Whitehead and Cotta 2004), and a possible similar role in the gut of *Microcosmus* sp. Another relative was an uncultured γ-Proteobacterium found in soil samples from uranium waste piles (Selenska-Pobell et al. 2001). This

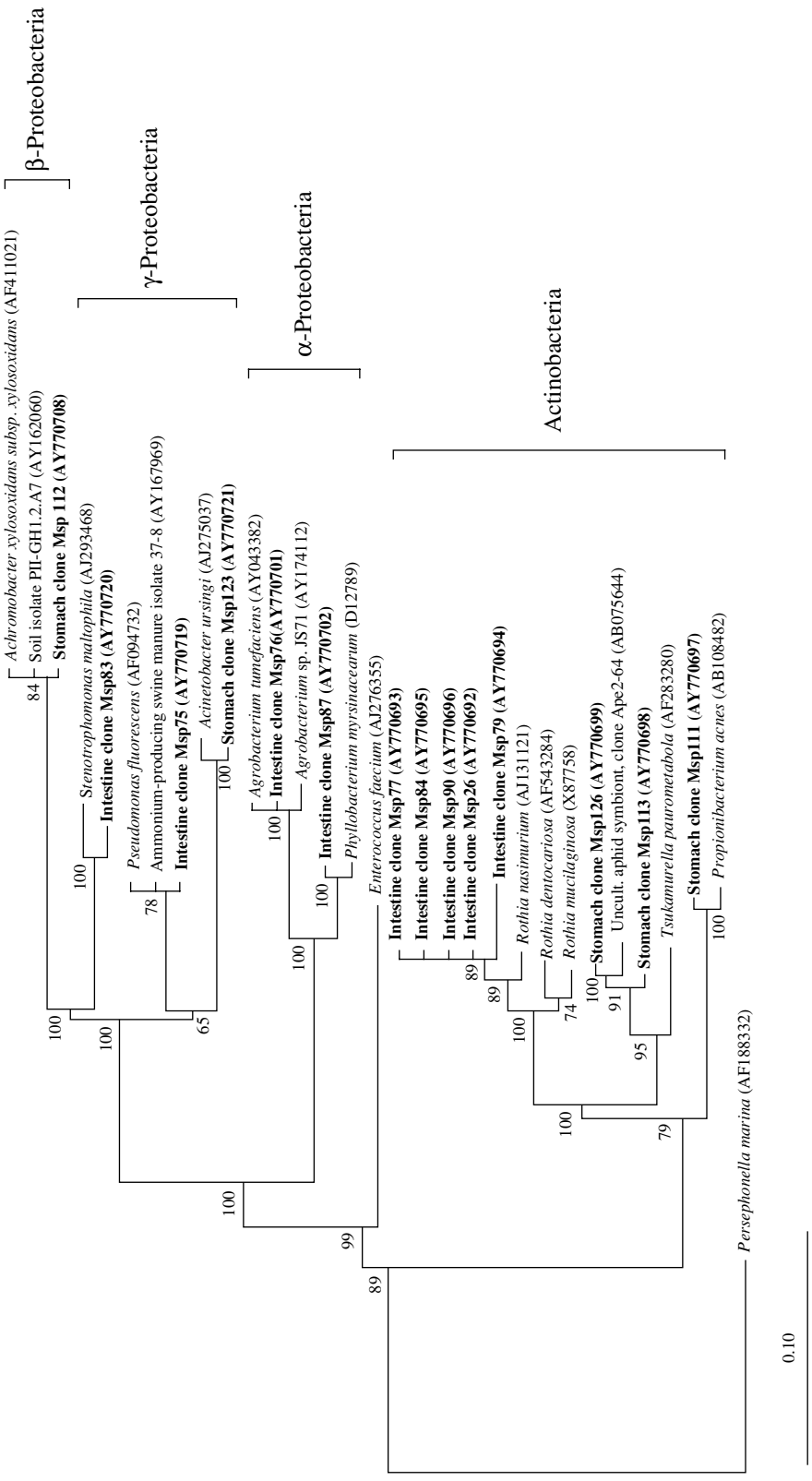

Fig. 2. 16S rRNA-based minimum evolution distance tree showing the phylogenetic relationships of bacterial clones from the digestive tract of *Microcosmus sp*. (in bold) to cultured bacterial species and related environmental clones. The tree is based on *Escherichia coli* positions 2–907 of the 16S rRNA gene. Bootstrap (1000 replicates) support values (%) are given at nodes for minimum evolution distance. Bar indicates the number of substitutions per site.

strain is adapted to various uranium concentrations and it could assist the ascidian in the detoxification of heavy metals, since it is known that marine ascidians accumulate high concentrations of heavy metals in their blood (Hickman et al. 2000). Phylotype Msp76 was related to an *Agrobacterium* sp., a nitramine explosive degrader (Trott et al. 2003) that could serve the animal in the biodegradation of complex nitrogenous compounds.

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