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Genetic diversity of bacteria associated with the hindgut of the terrestrial crustacean *Porcellio scaber* (Crustacea: Isopoda)

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Abstract

Molecular approaches were used to examine the genetic diversity of bacteria associated with the gut wall of the terrestrial isopod *Porcellio scaber* and to determine whether an autochthonous microflora exists in the *P. scaber* hindgut. 16S ribosomal genes were amplified from the total DNA isolated from thoroughly washed papillate regions of the hindgut, where the highest concentrations of bacteria are commonly found. The amplified genes were cloned, sequenced and phylogenetically analysed. The results implied an unexpectedly large diversity of microflora associated with the cuticle of the hindgut. Almost half of the retrieved sequences were found to be less than 80% homologous with any of the known sequences available at DNA data banks. Most of these sequences were clustered in one of three groups, and were clearly distant from the sequences of other bacterial taxa, indicating that they could represent novel bacterial species or even genera. More than two thirds of the sequences were found to be phylogenetically related to sequences from bacteria typically isolated from human and animal intestines, e.g. streptococci, enterococci, and members of the genus *Bacteroides*. The majority of the remaining sequences were most closely related to typical soil bacteria, e.g. bacilli and pseudomonads. The facts that a large proportion of the retrieved sequences was related to the sequences of bacteria, which are autochthonous to intestinal ecosystems, and that bacteria, specifically attached to the cuticular spines, were observed, indicate that truly autochthonous bacteria may well be present in the hindgut of *P. scaber*.

Keywords: Terrestrial isopod; Hindgut; Intestinal bacteria; Indigenous microbial flora; Genetic diversity; 16S rDNA

1. Introduction

The common woodlouse *Porcellio scaber*, Latreille 1804 (Crustacea: Isopoda) is a widely distributed terrestrial isopod. Terrestrial isopods are believed to play an important role in decomposition processes and global carbon cycling. In spite of its omnivorous nutritional habits, the diet of *P. scaber*, as that of most terrestrial isopods, consists mainly of decayed plant material composed of cellulose and other polysaccharides [1]. Although the role of microorganisms is clearly crucial in the process of degradation of complex polysaccharides such as cellulose, it has not been as thoroughly investigated in the intestinal tract of *P. scaber* as in some other arthropods which feed on cellulose-rich diets [2,3].

The digestive system of *P. scaber* consists of a foregut (stomodeum) and a hindgut (proctodeum), both lined with a cuticle. The midgut (mesenteron) is reduced to two pairs of tubular midgut glandular caeca, also known as hepatopancreas, which is the key site for the secretion of digestive enzymes and for the absorption of soluble nutrients [4] (Fig. 1). Fragments of food pass through the foregut where they are briefly masticated, compressed and filtered into the hindgut. During this process, the food is mixed with secretions derived from the hepatopancreas, before passing into the long hindgut. In contrast to other arthropods, the hindgut of isopods is a simple straight tube subdivided along its length into an anterior region, with a pair of dorsal channels (a typhlosole), a median papillate region, which ends with a muscular sphincter, and a short posterior rectum. Absorptive properties have been attributed to both the anterior hindgut and the hepatopancreas [5–7] whereas in the papillate region water and ions are reabsorbed [8–10].

Microbes in the gut of terrestrial isopods have been

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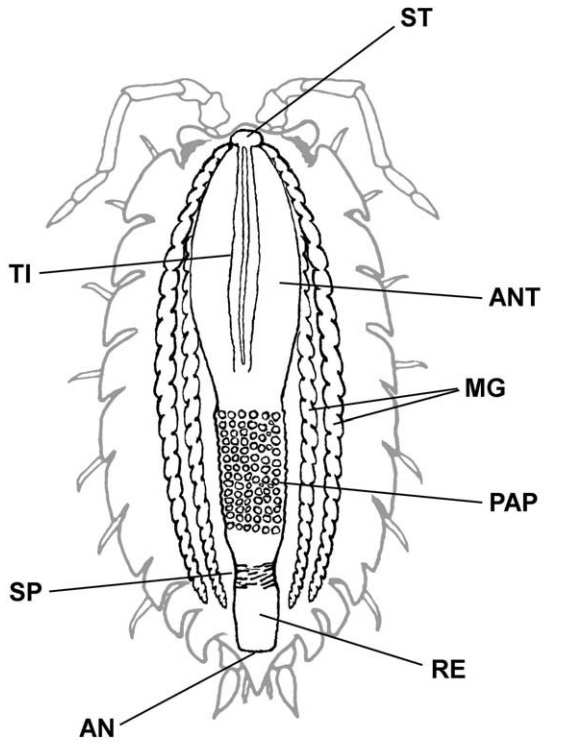


Fig. 1. Anatomy of the digestive system of an adult *P. scaber*. ST – stomach, MG – midgut glands (hepatopancreas), ANT – anterior region of the hindgut, TI – typhlosole, PAP – papillate region of the hindgut, SP – sphincter, RE – rectum, AN–anus. Scale bar: 5 mm.

observed and studied by several authors [11–20]. Their presence indicates that the isopod gut represents a suitable environment for microbial colonisation, although it lacks specialised structures or modifications of the gut, which would facilitate colonisation [11,12,14]. The majority of the microbial flora is found in the hindgut, whereas the foreguts of terrestrial isopods are generally poorly colonised [15]. Data on the microflora inhabiting the digestive system of terrestrial isopods were acquired mainly through observations by means of light and electron microscopy, and by isolation and further chemotaxonomic studies of microorganisms from food, intestines and faeces of the animal [11–14,16–20]. Culture-dependent methods are biased, however, since the proportion of cultivable microorganisms in natural environments is often very low [21]. Such approaches are also not suitable if one wants to determine wether a part of the microbial community is truly autochthonous to the given environment.

Data concerning the presence of autochthonous bacterial flora in the isopod gut are contradictory. According to Dubos et al. [22] the term autochthonous flora is appropriate for describing the microorganisms resident in the gut and possessing mechanisms which enable them to adapt to the gut environment. These mechanisms were developed during co-evolution with the host, and do not occur in other environments. Autochthonous microorganisms can live either in a mutualistic or commensalistic relationship with the host [23]. Different authors have used various terms to describe microorganisms discovered in the isopod gut. Whereas some authors support the idea of the presence of autochthonous microbial flora in the isopod gut [12–14], others suggest the absence of such flora [6,15] as a consequence of the simple anatomy of the gut, the short retention time of the food and the moulting of the hindgut cuticle, which occurs approximately once a month.

In the work reported here, cloning and sequencing of PCR-amplified 16S rRNA genes was applied in order to screen the genetic diversity of bacteria resident in the *P. scaber* hindgut and to resolve their taxonomic status. It was anticipated that some of the bacteria associated with the cuticular lining of the papillate region, where the highest density of microorganisms occurs, could represent the autochthonous bacterial flora, which has not yet been proven.

2. Materials and methods

2.1. Animals

The isopods examined in this study were collected in the spring of 1999 in woods near Cerknica in Slovenia. The animals were kept at 20°C in glass tanks filled with soil, under conditions of high humidity and 12–12 h day/night illumination. The animals were fed on leaf litter from the collection site for at least 1 month prior to the experiment. Adult animals of both sexes were used in the experiment.

2.2. Gut preparation and DNA extraction

The guts of five healthy adult isopods were extracted with fine-tipped sterile forceps and the papillate regions of the hindguts were removed. The papillate regions were opened with a sterile needle, transferred to a 0.5-ml sterile phosphate-buffered saline (PBS buffer; 130 mM NaCl, 3 mM NaH_2PO_4, 7 mM Na_2HPO_4; [pH 7.4]), and washed vigorously for 10 s. Washing was repeated three times.

Total DNA was extracted from the isolated papillate regions by the modified method proposed by Mäntynen and Lindstrom [24]. The papillate regions and the attached microorganisms were crushed using a teflon homogeniser in 0.5 ml of PBS buffer. Tissue particles and bacterial cells were harvested by centrifugation and than resuspended in 100 μl of TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA) in the presence of 1 μl of proteinase K (20 mg ml^{-1}) and 8 μl of lysozyme (50 mg ml^{-1}). Following incubation at 37°C for 1 h, 60 μl of 10% sodium dodecyl sulfate and 20 μl of 10% *N*-lauroylsarcosine were added, and the mixture was incubated for further 30 min at 37°C. A 60 μl aliquot of preheated (65°C) 10% CTAB in 0.7%

NaCl was then added, and the mixture was incubated for 10 min at 65°C. The sample was further treated by adding an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1), and then centrifuged at 10 000 × *g* for 20 min. The DNA was precipitated with an equal volume of isopropyl alcohol, centrifuged at 20 000 × *g* for 15 min, and rinsed with 70% ethanol. After centrifuging at 13 000 × *g* for 10 min, the DNA was air dried and resuspended in 20 μl of the TE buffer, pH 8.0.

2.3. PCR amplification and cloning of 16S rDNA

16S rRNA genes from total DNA were amplified using the modified evolutionary conserved eubacterial primers fD1 [25] and 1392r [26] as described elsewhere [27] with minor modifications. The sample DNA (1 μl) was amplified in a reaction mixture that consisted of 1× reaction buffer (Gibco BRL, Carlsbad, CA, USA), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 6 pmol of each primer and 1 U of *Taq* polymerase (Gibco BRL) in a final volume of 20 μl. The reaction mixture was first denatured at 94°C for 5 min, and then subjected to 30 PCR cycles (40 s at 94°C, 30 s at 60°C, and 80 s at 72°C) followed by 10 min at 72°C.

The amplified 16S rRNA genes were separated by agarose gel electrophoresis, cut from the gel and purified using a QIAquick Gel Extraction Kit (Qiagen, Germantown, MD, USA), according to the manufacturer's recommendations. The purified amplicons were digested with *Sal*I (Promega, Madison, WI, USA) and *Not*I (New England Biolabs, Beverly, MA, USA) restriction endonucleases, as recommended by the manufacturers, and subsequently cloned in the phagemid pBluescript KS II+(Stratagene, La Jolla, CA, USA). The ligated vectors carrying 16s rRNA amplicons were used for the transformation of *Escherichia coli* DH-5-α recipient cells (Gibco BRL) by the $CaCl_2$ method [28].

2.4. Restriction fragment length polymorphism (RFLP) analysis and sequencing

The white colonies grown on LB/xgal plates were further analysed by restriction analysis. The recombinant plasmids were purified by a 'mini-prep method' with 5% CTAB and ethanol precipitation [28], and digested with *Taq*I or *Dde*I (Boehringer Mannheim, Germany) restriction endonucleases according to the manufacturer's recommendations. Jaccard's coefficients were calculated on the basis of the observed RFLP profiles from 29 randomly selected recombinant plasmids, and a phenogram was constructed with the UPGMA method, using the NTSYS pc 1.80 package (Applied Biostatistics Inc.) [29]. Representative clones from established groups were chosen for further analysis, and the cloned inserts were sequenced with the fD1 sequencing primer [25] by Mycrosynth GmbH (http://www.microsynth.ch/) or Colorado State University, Department of Biochemistry (http://mmr.bmb.colostate.edu/dna/dnaseq.html) on our request. Some of the cloned inserts were also sequenced with the sequencing primers 338f [21] and 1100r [26], thus providing almost total sequence length information.

2.5. Phylogenetic analysis and nucleotide sequence accession numbers

The phylogenetic analysis was done essentially as described previously [27], using programmes available at the Ribosomal Database Project site (http://www.cme.msu.edu/RDP/html/analyses.html) [30], BLAST and FASTA homology search algorithms [31,32], and the CLUSTAL X [33] and PHYLIP (Phylogeny Inference Package version 3.57c) [34] packages. The nucleotide sequence data have been deposited in the GenBank nucleotide sequence database under accession numbers AF395310–AF395327.

3. Results

Following the amplification and cloning of the 16S rRNA genes, 29 randomly selected clones, carrying recombinant plasmids with inserts of the expected size, i.e. 1400 bp, were subjected to RFLP analysis. The RFLP analysis showed that most clones clustered in one of three larger groups (not shown). The first group (RFLP-1) consisted of 10 clones, whereas the second and the third group (RFLP-2 and RFLP-3) consisted of six clones each. The rest of the clones remained ungrouped, indicating the large diversity of the amplified and cloned 16S rRNA genes and thus of the bacteria associated with the gut wall of *P. scaber*.

A total of 18 clones representing different groups and lineages in the RFLP dendrogram were chosen for partial sequencing with the fD1 sequencing primer. The names of the obtained sequences were created in a way that various alphabetical letters were attached to the common prefix RKPs. Sequences with the highest homologies to cloned sequences were retrieved from DNA data bank using homology search algorithms. The sequences were than pairwise aligned, and similarity values were calculated (Table 1). Almost half of the analysed sequences (8 out of 19) were less than 80% similar to the most homologous sequence deposited in the data bank, whereas only three sequences were more than 95% similar to the best-matched sequence from the data bank.

The obtained sequences were then further compared with sequences of bacteria from known taxa, and a phylogenetic tree was constructed (Fig. 2). The phylogenetic analysis clustered most of the cloned 16S rRNA sequences in similar groups to those of the RFLP analysis. The sequences of eight clones from the group RFLP-1 (RKPsBD, RKPsAL, RKPsB, RKPsA, RKPsAV, RKPsG, RKPsAA and RKPsAN) formed a deeply branched cluster (Fig. 2)

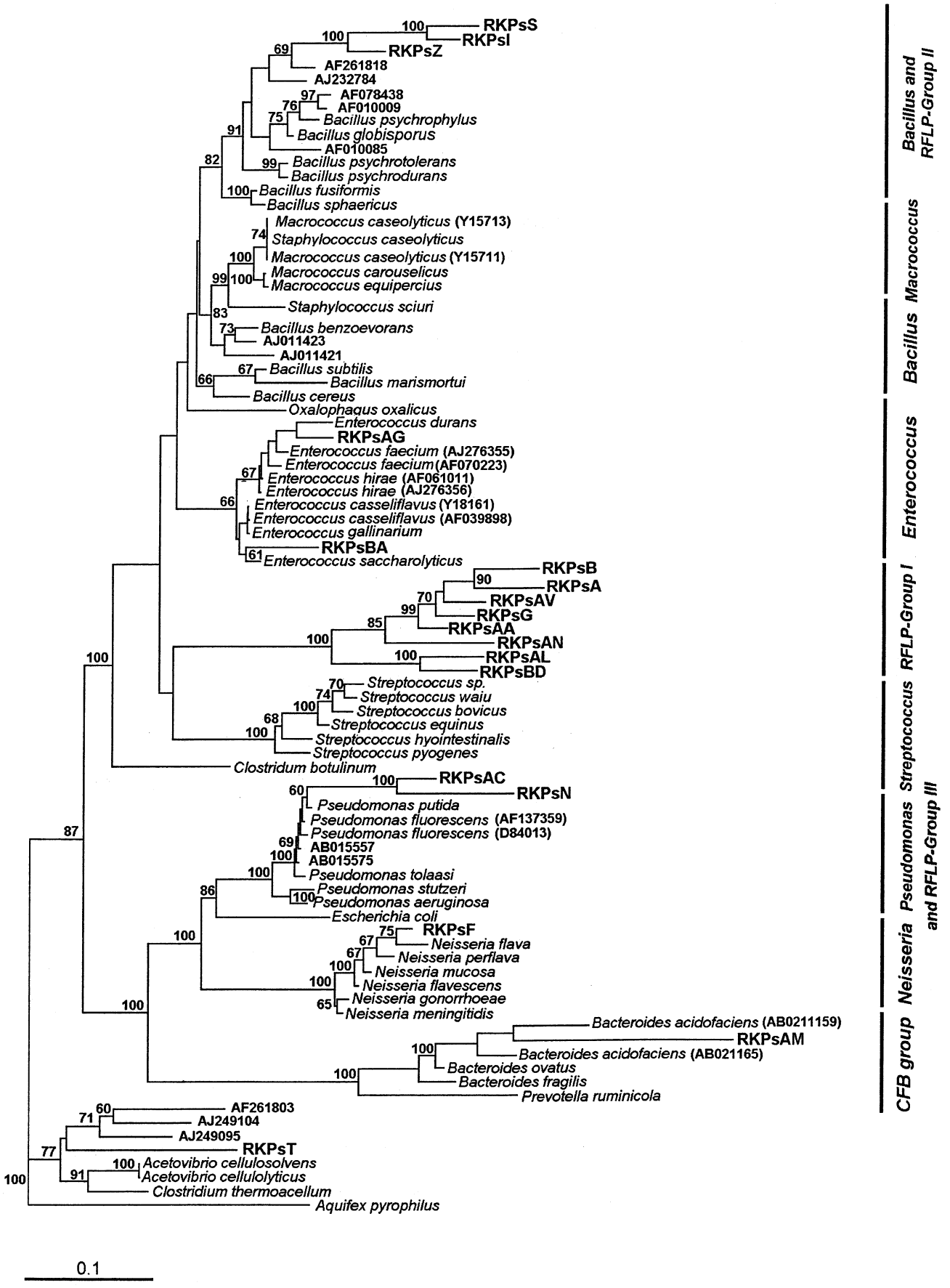
Table 1
List of the nearest known sequences to the hindgut clones (named RKPs plus additional letters) obtained from the data bases

Clone	Group	Closest known sequences	Percentage of similarity	Length of aligned sequences
RKPsA	RFLP-1	*Macrococcus caseolyticus* (Y15711)	74.9	928
		Staphylococcus caseolyticus (D83359)	74.9	925
		Streptococcus hyointestinalis (AF201898)	74.8	928
RKPsB	RFLP-1	U.C. bacterium (firmicute) (AF141540)	80.9	513
		Streptococcus waiu (AF088900)	77.1	513
		Streptococcus hyointestinalis (AF201898)	76.8	514
RKPsG	RFLP-1	*Macrococcus carouselicus* (Y15713)	72.7	821
		Macrococcus equipercius (Y15712)	72.6	821
		Staphylococcus caseolyticus (D83359)	72.6	821
RKPsAA	RFLP-1	*Staphylococcus sciuri* (S83569)	78.1	657
		Macrococcus bovicus (Y15714)	77.5	657
		Macrococcus carouselicus (Y15713)	77.4	657
RKPsAL	RFLP-1	U.I. bacterium (soil) (AJ011421)	77.9	938
		Enterococcus hirae (AF061011)	74.9	938
		E. faecium (AF070223)	74.8	938
RKPsAN	RFLP-1	*Bacillus benzovorans* (Y14693)	73.1	940
		Bacillus psychrodurans (AJ277984)	73.5	940
		U.I. bacterium (hizosphere) (AJ011421)	77.5	687
RKPsAV	RFLP-1	*S. equinus* (AB002514)	78.1	739
		Streptococcus hyointestinalis (AF201898)	77.6	739
		Streptococcus waiu (AF088900)	74.7	822
RKPsBD	RFLP-1	*Bacillus psychrodurans* (AJ277984)	79.8	584
		Bacillus psychrotolerans (AJ277983)	79.5	577
		Bacillus benzovorans (Y14693)	79.2	584
RKPsI	RFLP-2	*B. fusiformis* (M77486)	77.0	820
		U.C. bacterium (manure pit) (AF261818)	84.2	495
		U.I. bacterium (soil) (AF010085)	84.3	461
RKPsS	RFLP-2	*B. fusiformis* (M77486)	76.0	831
		U.C. bacterium (manure pit) (AF261818)	86.1	489
		U.I. bacterium (soil) (AF010085)	85.4	457
RKPsZ	RFLP-2	U.I. bacterium (manure pit) (AF261818)	92.3	505
		U.I. bacterium (soil) (AJ232784)	92.2	510
		B. psychrophilus (D16277)	92.0	501
RKPsN	RFLP-3	*P. fluorescens* (AF094725)	83.1	958
		U.I. bacterium (sea sediment) γ Proteobcteria (AB015575)	83.1	958
		Pseudomonas putida (D86001)	83.0	958
RKPsAC	RFLP-3	*P. fluorescens* (AF094725)	94.0	769
		U.I. bacterium (sea sediment) γ Proteobcteria (AB015575)	94.0	769
		Pseudomonas putida (D86001)	93.7	763
RKPsAG	*Enterococcus*	*E. faecium* (AJ276355)	99.1	1415
		Enterococcus durans (AJ276354)	98.9	1418
		Enterococcus hirae (AJ276356)	98.7	1417
RKPsBA	*Enterococcus*	*E. saccharolyticus* (Y18357)	96.8	1695
		Enterococcus gallinarum (AF039898)	96.3	1395
		Eubacterium sp. (AF135452)	96.2	1405
RKPsAM	Bacteroides	U.I. bacterium (river organic aggregate) (AF150715)	93.6	1033
		U.I. bacterium (AF227834)	88.4	1418
		Bacteroides acidofaciens (AB021159)	87.9	1419
RKPsF	*Neisseria*	*Neisseria perflava* (AJ239295)	97.7	478
		Neisseria mucosa (AJ239279)	97.7	476
		Neisseria flavescens (L06168)	96.1	512
RKPsT		U.I. bacterium (anaerobic consortia) (AJ249104)	81.9	482
		C. thermocellum (L09173)	80.1	482
		Acetovibrio cellulolyticus (L35516)	79.6	482

The percentage of similarity was calculated on the basis of pairwise alignment. U.C., uncultivated; U.I., unidentified.

→

Fig. 2. Phylogenetic tree including partial 16S rDNA sequences retrieved from hindgut clones (named RKPs plus additional letters) and the closest sequences to hindgut clones from the clone library. The 16S rDNA sequence of *Aquifex pyrophilus* was used as the outgroup. The scale bar represents 0.1 substitution per nucleotide position. The numbers at the nodes indicate bootstrap values based on 1000 resamplings (values below 60 are not shown).


RKPsS
RKPsI
RKPsZ
AF261818
AJ232784
AF078438
AF010009
Bacillus psychrophylus
Bacillus globisporus
AF010085
Bacillus psychrotolerans
Bacillus psychrodurans
Bacillus fusiformis
Bacillus sphaericus
Macrococcus caseolyticus (Y15713)
Staphylococcus caseolyticus
Macrococcus caseolyticus (Y15711)
Macrococcus carouselicus
Macrococcus equipercius
Staphylococcus sciuri
Bacillus benzoevorans
AJ011423
AJ011421
Bacillus subtilis
Bacillus marismortui
Bacillus cereus
Oxalophagus oxalicus
Enterococcus durans
RKPsAG
Enterococcus faecium (AJ276355)
Enterococcus faecium (AF070223)
Enterococcus hirae (AF061011)
Enterococcus hirae (AJ276356)
Enterococcus casseliflavus (Y18161)
Enterococcus casseliflavus (AF039898)
Enterococcus gallinarium
RKPsBA
Enterococcus saccharolyticus
RKPsB
RKPsA
RKPsAV
RKPsG
RKPsAA
RKPsAN
RKPsAL
RKPsBD
Streptococcus sp.
Streptococcus waiu
Streptococcus bovicus
Streptococcus equinus
Streptococcus hyointestinalis
Streptococcus pyogenes
Clostridum botulinum
RKPsAC
RKPsN
Pseudomonas putida
Pseudomonas fluorescens (AF137359)
Pseudomonas fluorescens (D84013)
AB015557
AB015575
Pseudomonas tolaasi
Pseudomonas stutzeri
Pseudomonas aeruginosa
Escherichia coli
RKPsF
Neisseria flava
Neisseria perflava
Neisseria mucosa
Neisseria flavescens
Neisseria gonorrhoeae
Neisseria meningitidis
Bacteroides acidofaciens (AB0211159)
RKPsAM
Bacteroides acidofaciens (AB021165)
Bacteroides ovatus
Bacteroides fragilis
Prevotella ruminicola
AF261803
AJ249104
AJ249095
RKPsT
Acetovibrio cellulosolvens
Acetovibrio cellulolyticus
Clostridium thermoacellum
Aquifex pyrophilus
0.1
Bacillus and RFLP-Group II
Bacillus Macrococcus
Enterococcus
Streptococcus RFLP-Group I
Pseudomonas and RFLP-Group III
Neisseria
CFB group

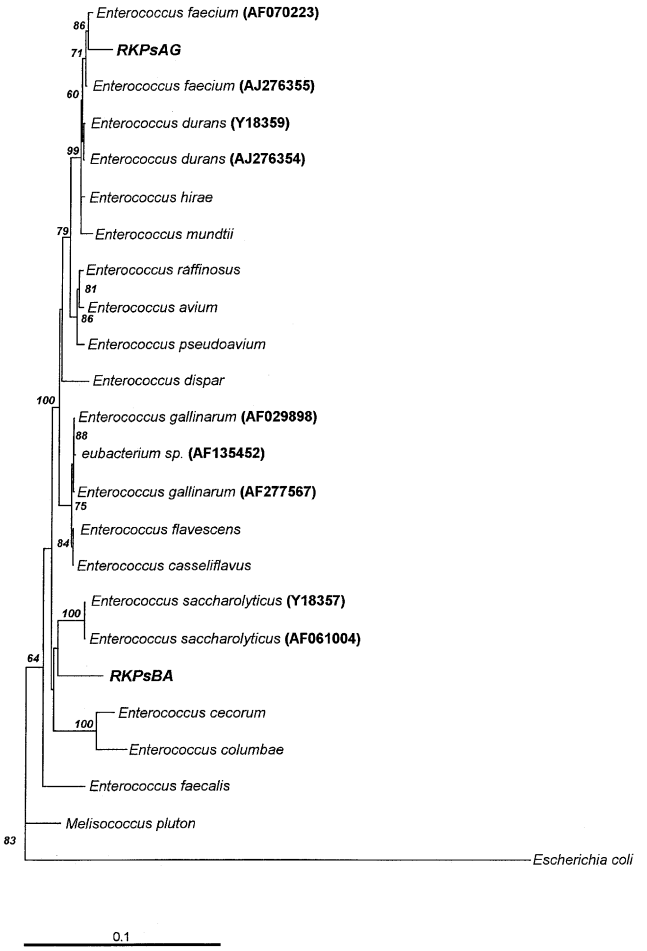


Fig. 3. Phylogenetic tree showing the relationships among 16S rDNA sequences from two hindgut clones, RKPsAG and RKPsBA, and the members of the genus *Enterococcus*. The 16S rDNA sequence of *E. coli* was used as the outgroup. The scale bar represents 0.1 substitution per nucleotide position. The numbers at the nodes indicate bootstrap values based on 1000 resamplings (values below 60 are not shown).

with the nearest known taxon being the genus *Streptococcus*. The similarity values to the best-matched sequences from the data bank, obtained by the pairwise sequence alignment analysis, were all below 80%. The unique line of descent was further strengthened by fairly high confidence values as revealed by the bootstrap analysis.

Three clones from the group RFLP-2 (RKPsS, RKPsI and RKPsZ) were grouped together again, as with the RFLP analysis, and the phylogenetically closest sequences were from unidentified bacteria isolated from the manure pit (AF261818) and soil (AJ232784) [35]. Sequences from this group were clustered together with sequences from members of the genus *Bacillus*, which was again supported by relatively high (91%) bootstrap values.

Two sequences from the RFLP-3 group (RKPsAC and RKPsN) clustered together with sequences from the bacteria of the genus *Pseudomonas*. The bootstrap value of 100% for the node clustering clones RKPsAC, RKPsN and members of the genus *Pseudomonas*, supports the assignment of these clones to the *Pseudomonas* group (Fig. 2).

The sequences from the clones RKPsAG, RKPsBA and RKPsAM were, as in the case of group RFLP-1, related to bacterial genera commonly inhabiting the gut of vertebrates and insects rather than to typical soil bacteria. Sequences from the clones RKPsAG and RKPsBA were placed within the cluster comprising sequences from the genus *Enterococcus*, whereas the sequence from the clone RKPsAM was placed within the cluster of sequences from the genus *Bacteroides* (Fig. 2). Since some members of the genus *Enterococcus* and most of the bacteria from the genus *Bacteroides* are commonly found in higher animals as part of the autochthonous intestinal flora, the cloned 16S rDNAs were further sequenced with additional sequencing primers. The more accurate phylogenetic positions of these three clones were therefore determined on the basis of almost complete sequences of their 16S RNA genes.

The sequence from the clone RKPsAG shared the highest homology with the sequence from *Enterococcus faecium* (99.1%), whereas the RKPsBA insert shared the highest homology with the sequence from *Enterococcus saccharolyticus* (96.8%) (Table 1). This was further confirmed by the phylogenetic analysis (Figs. 2 and 3), and supported by high confidence values (86 and 100% respectively).

The ribosomal gene sequence from the clone RKPsAM was most similar to the sequence from an unidentified bacterium from Elbe river organic aggregate (AF150715) [36]. These two sequences were placed within the *Cytophaga–Flexibacter–Bacteroides* phylogenetic group (Fig. 4), and they clearly belong to the *Bacteroides fragilis* subgroup as defined by the Ribosomal Database Project characterisation [30]. Within the *B. fragilis* subgroup, the RKPsAM and the Elbe river isolate formed a deep and distinct lineage supported by a high confidence level (85%).

Low sequence similarity (below 82%) between the sequence of the clone RKPsT and the best-matched sequences from the data bank (Table 1) suggests that this organism is very distant to major known bacterial taxa. Deep branching of the sequence (Fig. 2) indicates that RKPsT, same as members of the RFLP-1 group, could represent a distinct and evolutionary divergent taxonomic group within the domain Bacteria.

4. Discussion

The hindgut of various soil invertebrates is a favourable environment for bacterial attachment, activation and growth [37,38]. These animals are considered to form 'hot-spots' of enhanced bacterial growth, activity and species interactions in the soil habitat [37] and are therefore very important partners in the efficient decomposition of organic matter and in mineral cycling. One example, the common woodlouse *P. scaber*, has also often been as-

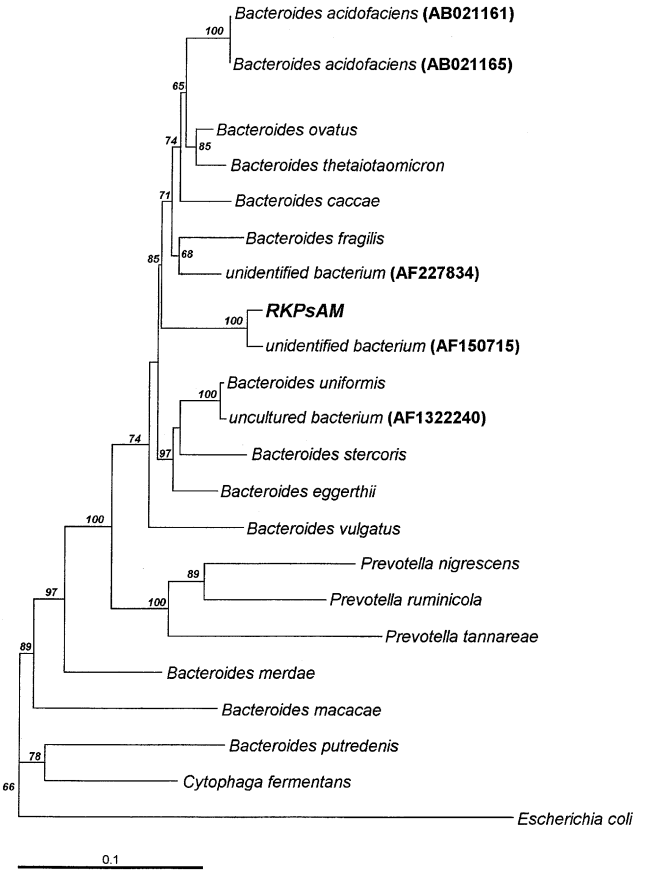


Fig. 4. Phylogenetic tree showing the relationships between 16S rDNA sequence from the hindgut clone RKPsAM and the members of the *Cytophaga–Flexibacter–Bacteroides* group. The 16S rDNA sequence of *E. coli* was used as the outgroup. The scale bar represents 0.1 substitution per nucleotide position. The numbers at the nodes indicate bootstrap values based on 1000 resamplings (values below 60 are not shown).

sumed to play an important role in the fragmentation of litter and in the incorporation of decomposition products in the soil profile.

Since it is assumed that terrestrial isopods do not possess endogenous cellulases, these crucial depolymerising enzymes must originate from microorganisms that are either indigenous to the ecosystem or ingested with food. The majority of authors support the latter [6,17] and some have even suggested that the gut of the woodlouse is actually not a favourable environment for microbial growth, as demonstrated by studies on the typical soil bacterium *Pseudomonas fluorescens* [19,20,39,40]. However, a process of intensified bacterial growth was observed in the faeces of *P. scaber*, and this is assumed to be the net effect of lysis during food transit through the gut and growth in the faeces after voiding. This process appears to be initiated within the hindgut [41]. Also, the retention of ingested food particles for prolonged periods of time in the hindgut of *P. scaber* is probable, as fluorescent microbeads were found in the faeces up to 16 days after feeding [20], indicating that there is enough time for microbial growth to occur. The beads were caught in the structure of the gut, between the folds of the exocuticle lining the hindgut region and trapped in mucopolysaccharides within the hindgut. It is therefore likely that some bacterial cells become trapped in the hindgut structures too, and develop into microcolonies in such niches. Other bacterial cells may have developed specific associations with the gut wall. Drobne [16] showed that the majority of bacteria associated with the gut wall of *P. scaber* are found in the papillate region of the hindgut. Bacterial cells remained associated with the gut wall even after several consecutive and vigorous washings and some of them were attached specifically to the cuticular spines protruding into the lumen of the gut. The existence of autochthonous microflora is, in the light of the observed attachment, therefore quite plausible.

The aim of the present study was to examine the taxonomic structure of the microbial community inhabiting the gut of *P. scaber* by molecular means. The investigation was focused on microorganisms that are potentially associated with the gut wall and are capable of remaining in the gut after the removal of the gut contents and thorough washing of the inner surface. To our knowledge, a molecular investigation of the structure of the microbial community inhabiting the *P. scaber* gut has not yet been employed. By such means autochthonous bacteria, or novel bacterial species or even genera, living in the soil and proliferating in the gut of the isopods could be discovered. The latter possibility actually seemed very probable due to the known low culturability of soil bacteria [42,21].

Phylogenetic analysis indicated the presence of a diverse bacterial flora in the *P. scaber* hindgut. The RFLP analysis was applied first, and it was found that most of the cloned sequences can be clustered in three groups, whereas approximately 25% remained ungrouped. This finding was strongly confirmed by sequence analysis of the representative clones, indicating the suitability of the RFLP approach in the initial steps of such studies. Almost half of the amplified rRNA genes were less than 80% homologous with the best-matched sequences deposited in the DNA data bank, indicating that they come from bacteria that have not yet been cultivated and very likely represent novel bacterial species or even genera. This is in agreement with observations of low percentage of cultivable bacteria from a variety of natural microbial ecosystems, such as soil [21,42,43].

More than 70% of the analysed rRNA genes were found to be homologous with the ribosomal genes from Gram-positive bacteria. Eight out of 18 rRNA genes positioned together and formed a deeply branched cluster, distantly related to the genus *Streptococcus*. Some of the most closely related streptococci, e.g. *Streptococcus bovis* and *Streptococcus equinus* are anaerobes that are typically isolated from the intestines of man and animals [44–46]. The same is true for the enterococci, that were most closely related to two of the analysed clones (RKPsAG and

RKPsBA), and for the members of the genus *Bacteroides*, that were most closely related to the rRNA gene sequence RKPsAM. Hence, 11 out of 18 retrieved rRNA gene sequences were related to bacteria that typically inhabit the animal gastrointestinal tract and are generally unable to survive outside their animal host. This could indicate that a specific, presumably anaerobic microflora exists in the hindgut of *P. scaber*, and that a constant, i.e. symbiotic, type of interaction between these microorganisms and the host has developed.

Most of the remaining recovered rDNA sequences were most closely related to typical soil bacteria. Three out of six ribotypes were sequenced from the RFLP-2 group, and their closest relatives were uncultivated bacteria from soil or the manure pit (Table 1). The cultivated bacteria with the most homologous 16S rDNA sequences to those of the RFLP-2 group were *Bacillus fusiformis* and *Bacillus psychrophilus*. However, the retrieved ribotypes from *P. scaber* formed a distinct group in the phylogenetic tree together with rDNA sequences from other uncultivable bacteria distantly related to cultivable bacilli. Two out of six ribotypes were sequenced from the RFLP-3 group and they were related to rDNA sequences from bacteria belonging to the genus *Pseudomonas*. The two remaining sequences, i.e. RKPsT and RKPsF, were most homologous with the rDNA sequences from uncultivated anaerobic bacteria and *Clostridium thermocellum*, and to species from the genus *Neisseria*, respectively. The former sequence, in particular, appears to be important due to the known cellulolytic potential of the anaerobic thermophilic clostridia.

Different bacteria resisted the applied washing procedure and we may speculate that they could also withstand the rapid flow of the food through the hindgut or the moulting of the hindgut cuticle. We can therefore assume that these bacteria may be capable of remaining in the gut of *P. scaber* for prolonged periods of time. Since a good proportion of the recovered ribotypes are related to bacteria that typically inhabit the human or animal gut and represent the true autochthonous microflora of these ecosystems, the existence of autochthonous bacteria within the gut of *P. scaber* is plausible. The contradictory evidence has been acquired with traditional microbiological approaches and is likely biased due to the dependence of these methods on in vitro cultivation. It appears possible that the hindgut of *P. scaber* is inhabited by truly autochthonous bacteria that are fastidious and do not grow under defined laboratory conditions.

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