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ORIGINAL ARTICLE

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Diversity of intratunical bacteria in the tunic matrix of the colonial ascidian *Diplosoma migrans*

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Abstract This paper provides the first information on diversity based on sequence data of the 16S rDNA of intratunical bacteria in the colonial ascidian *Diplosoma migrans* and its embryonic offspring. Ascidians were collected from waters near Helgoland (German Bight, North Sea). Sample material comprised tunic tissue, bacteria collected from tunic tissue, eggs with single embryos at different developmental stages, and free-swimming larvae. Bacterial 16S rDNA from *D. migrans* was directly amplified using PCR. DNA species were separated using denaturing gradient gel electrophoresis (DGGE). DGGE profiles generated ca. ten different distinguishable operational taxonomic units. Eleven bands from different sample materials were successfully re-amplified and sequenced. Sequence data generated five different subgroups of intratunical proteobacteria. The dominant band, detected in all of the samples tested, showed a low degree of relationship (84–86%) to *Ruminococcus flavefaciens* (δ-subgroup). A weaker band, located above, which was not detected in all of the samples, was also similarly related to *R. flavefaciens*. Other bands derived from tunic material and embryonic stages showed closer relationship (ca. 97–99%) to *Pseudomonas saccherophilia*, a knallgas bacterium, and *Ralstonia pickettii*, a pathogen bacterium (both members of the β-subgroup). A solitary band generated from tunic material was assigned to a typical marine *Flavobacterium* symbiont (95%). Finally, a band from isolated bacteria was related (96%) to pathogen *Arcobacter butzleri* (ε-subgroup). At this state of the investigation, a reliable interpretation of the ecological functions of intratunical bacteria cannot yet be given. This is due to the low degree of relationship of some of the bacteria and the fact that not all of the characteristic bands were successfully sequenced. However, the intratunical bacteria represent a unique bacterial community. Their DGGE profiles do not correspond to the profiles of the planktonic bacteria generated from surface seawater close to the ascidian habitat. The allocation of DNA sequences to the different morphotypes, their isolation and culturing, and the elucidation of the physiological functions of intratunical bacteria are in progress.

Keywords Ascidians · Didemnidae · *Diplosoma migrans* · Tunic · Intratunical bacteria · Diversity · PCR · DGGE · Sequencing

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Introduction

Molecular approaches allow close insight into the fascinating and widely unexplored marine world of interactions between bacteria and invertebrates in their habitats. Current literature describes "endobiotic" bacteria in various eukaryotic host organisms, such as protozoans, sponges, cnidaria, annelids, echinoderms, and ascidians (Deming and Colwell 1982; Paul et al. 1986; Cary et al. 1997; Burnett and McKenzie 1997; Althoff et al. 1998). However, key questions concerning phylogenetic relationships of bacterial symbionts (Kirchner et al. 1999; Seibold et al. 2001; Moss et al. 2003; Wichels et al. 2004), their ecological and physiological functions, their secondary metabolites, their chemical structures and the effects of these compounds on host tissue remain mostly unanswered. This is particularly true for the intratunical bacteria associated with ascidians (Mackie and Singla 1987; Hirose and Saito 1992; Hirose et al. 1996, 1998). Groepler and Kümmel (1988)

observed bacteria in the tunic of *Diplosoma migrans* for the first time. Later investigations (Groepler 1994; Groepler and Schuett 2003) showed that the tunic harbours high numbers of conspicuous, long, needle-like rods and some other bacterial morphotypes in its interstitial space (Fig. 1). Microscopic observations indicate that the intratunical bacterial community is stable and seasonally independent. Bacteria were found also in the different embryonic stages. Presumably, these bacteria are transferred during sexual propagation from the parental colony to its offspring. The present report provides initial information about the diversity of the intratunical bacterial community in the colonial ascidian *D. migrans* and its embryonic offspring using 16S rDNA sequencing.

Materials and methods

Samples and preparation

Divers from Biologische Anstalt Helgoland provided fresh *Laminaria* sp. from the shallow waters around Helgoland. The claw-like holdfasts are often settled by *D. migrans* colonies. After collecting ascidians from holdfasts, colonies were subjected to washing procedures in order to remove contaminating epibiotic bacteria. Samples were washed in seawater for 5 min in cetyltrimethyl-ammonium-bromide (CTAB; final concentration 10 μg/ml). Light microscopic controls showed no bacteria attached to the cell surface of *D. migrans*. In a subsequent step, CTAB was washed away three times with sterile seawater (5 min each). After removing the seawater from *D. migrans* colonies, samples were directly used for further procedures. For molecular detection and identification of intratunical bacteria, samples of different tissue material were aseptically taken from the washed material during microscopic inspection: (1) tunic matrix tissue which contained exclusively intratunical bacteria; (2) bacterial cells drawn from tunic tissue; and (3) developmental stages, i.e. eggs with embryos of different age collected from the common test and free-swimming larvae. For a comparison of intratunical bacteria with the planktonic bacteria, surface water samples from Helgoland Roads were collected. Two filter fractions (3–60 μm and 0.2–3 μm) from the surface water samples were prepared.

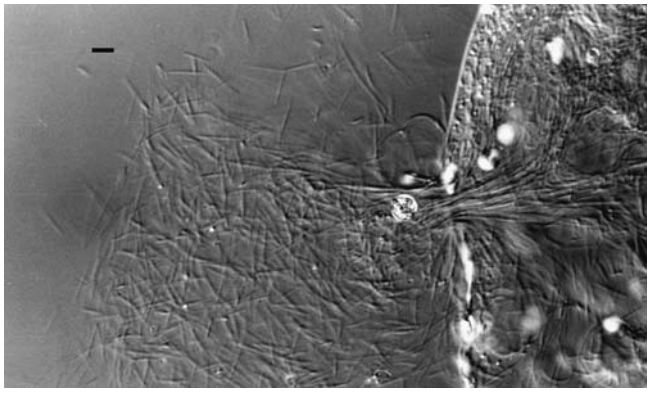

Fig. 1 Intratunical needle-like bacteria in the colonial ascidian *Diplosoma migrans* released through a lesion in the cuticle. *Bar* 5 μm

Direct PCR amplification of 16S rDNA fragments

As the bacterial DNA concentration in the tunic matrix is too low for DNA extraction, prokaryotic DNA was directly amplified without prior extraction. Sample material of ca.10 mg in 100 μl distilled water (d.w.) was centrifuged for 5 min at 10,000 *g*. Pellets were resuspended in 100 μl $CaCl_2$ solution (1 mM), and incubated with 1 μl proteinase K (10 mg/ml) for 2 h at 55°C. After a freeze-thaw lysing treatment, samples were centrifuged for 5 min at 10,000 *g*. In order to improve direct PCR conditions within the complex tissue material of the ascidians, 10 μl Lyse-N-Go solution (Pierce) was added to the pellets (incubation according to manufacturer's instructions). PCR amplifications were performed in 100 μl volumes containing ca.10 μl tissue with prokaryotic DNA in Lyse-N-Go solution; 10 μl buffer (Eppendorf, 10× concentrated); 15 μl Master Enhancer (Eppendorf, 5× concentrated); 75 μM final concentration each of the dNTP species (Promega); 0.4 μM final concentration of primer 341f-cl (5′-cgc ccg ccg cgc ccc gcg ccc ggc ccg ccg ccc ccg ccc ccc tac ggg agg cag cag-3′) primer 341f-cl has been suggested as p3 by Muyzer et al. (1995), clamp region underlined; 0.4 μM final concentration of primer 907rwob (5′-ccg tca att cct ttr agt tt-3′). The primers correspond to positions 341 and 907 in *Escherichia coli*. Finally, 2.5 units polymerase (Eppendorf) were added. Amplification was performed in a Mastercycler (Eppendorf): one initial pre-incubation step at 94°C for 3 min followed by 30 cycles (denaturing at 94°C for 3 min, annealing at 55°C for 1 min, extension at 72°C for 3 min). A final extension step at 72°C for 6 min completed the amplification. Negative controls were carried out without template DNA; *E. coli* J53 served as positive control.

DGGE analysis of PCR products

Prior to DGGE the quantity of amplified PCR products was determined by analyzing 5 μl PCR product on 1.2% agarose gels. Bands stained with ethidium bromide were visualized by using a transilluminator (Pharmacia) and documented with a MP-4 camera (Polaroid Corp. Espanol, Spain).

Denaturing gradient gel electrophoresis (DGGE) with prokaryotic PCR samples (30–60 μl) was performed by using the Dcode electrophoresis system (Biorad). Preparation of polyacrylamide gels and electrophoresis parameters were performed according to Muyzer et al. (1995). In our experiments, denaturant

gradients from 15% to 70% were chosen (100% denaturant corresponds to 7 M urea and 40% (v/v) formamide). Electrophoresis was performed at 60°C in 0.5×TAE buffer, and 100 V was applied to the gels for 15 h. Gels were stained with SYBR Gold (Molecular Probes) and documented as described above. Bands were excised from polyacrylamide gels and DNA was extracted from gel material and dissolved in 10 µl d.w. (Sambrook et al. 1989).

Re-amplification of DNA fragments from DGGE bands

Sequencing of DNA from DGGE bands required its reamplification. PCR cocktails of 100 µl contained following components: 1 µl DNA; 10 µl buffer (Perkin Elmer, 10× concentrated); 75 µM final concentration each of the dNTP species (Promega); 0.3 µM final concentration of primer 341f (5′-cct acg gga ggc agc ag-3′, Muyzer et al. 1993); 0.3 µM final concentration of primer 907rwob (5′-ccg tca att cct ttr agt tt-3′), 2 units polymerase (Perkin Elmer); amplification was generated by using a touchdown program (Don et al. 1991).

Quantities of amplified DNA samples were tested by electrophoresis on 1.2% agarose gels. DNA samples (ca. 95 µl each) were purified using the Qiaquick PCR Purification Kit (Qiagen) and eluted with 30 µl d.w. For sequencing, samples of 3 µl were used.

DNA sequencing of PCR products and comparative sequence analysis

Purified DNA samples were sequenced according to the manufacturer's instruction on a Liqor DNA 4200 sequencer using the SequiTherm EXEL II long read sequencing Kit-LC (Biozym) as described by Wichels et al. (2004).

Comparative sequence analysis

Sequences were aligned using the advanced BLAST search program from the National Center of Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST) to find closely related sequences. Data were screened for existing chimera by applying the "Ribosomal Database Project" (http://rdp.cme.msu.edu/index.jsp).

Results

DGGE profiles of intratunical bacteria and their sequencing data

The diversity of intratunical bacteria was investigated in 41 samples from *D. migrans*. Sample material comprised bacteria collected from tunic tissue (16 samples), tunic tissue material (10), and embryos as well as larvae of different developmental stages (15). Bacterial 16S rDNA from sample material was directly amplified using PCR. DGGE profiles generated ca. ten different distinguishable OTUs. Figure 2 shows the DGGE profiles from eleven samples: (1) tunic matrix, lanes 1–3; (2) bacteria drawn from tunic, lanes 4–7; and (3) different developmental stages of *D. migrans*, lanes 8–11. The eleven bands (here artificially marked as bold bands) were excised, re-amplified and successfully sequenced. These sequence data suggest the presence of four different subgroups of intratunical proteobacteria and one of the *Cytophaga/Flavobacterium* group (Table 1) displaying a wide spectrum of relationship from 84% to 99% to the type strains reported in the literature. Table 2 shows the distribution of the intratunical bacterial species identified in the different sample materials. The dominant bands (Rfa-22, Rfa-39) were detected in all of the 41 samples tested. They displayed a low degree of relationship (ca. 84%) with *Ruminococcus flavefaciens* (δ-subgroup of proteobacteria). Surprisingly, the weaker bands Rfb-72 and Rfb-74, located well above the main bands, showed the same similarity to *R. flavefaciens* type strain ATCC 19208 (accession no. AF030450). These DGGE fragments were not detected in every single sample. The bands Ps-71, Ps-9, and Ps-42, derived from tunic material and larval stages, showed a close relationship (92–97%) with *P. saccherophilia* (β-subgroup). Another DGGE fragment (Rp-70, Rp-79), found in the tunic matrix and embryonic stages, displayed a close relationship (98–99%) with *R. pickettii* (β-subgroup). A solitary band (Fb-69) generated from tunic material was assigned to the typical marine *Flavobacterium* symbiont (95%). Finally, a band (Ab-37) from bacteria drawn from the tunic matrix was related (96%) to the pathogen *A. butzleri* (ε-subgroup). A comparison between the

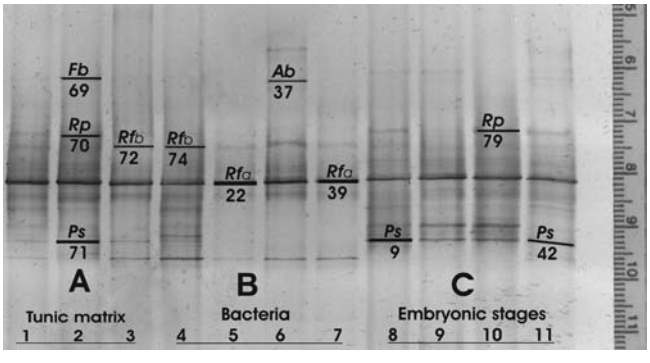

Fig. 2 DGGE profiles of intratunical bacteria (assigned to literature type strains) from different sample materials from *D. migrans*. **A** Tunic matrix material, lanes 1–3; **B** bacteria drawn from tunic matrix, lanes 4–7; **C** developmental stages, lanes 8–11 (lane 8 embryo of late tail-bud stage, in egg envelope; lanes 9 and 10 larvae before hatching, in egg envelope; lane 11 hatched larva). *Marked bands* (selected eleven DNA-fragments were re-amplified and sequenced) represent next neighbors to *Ab Arcobacter* sp.; *Fb Flavobacterium* sp.; *Ps Pseudomonas saccherophilia*; *Rf(a,b) Ruminococcus flavefaciens* (a, b); *Rp Ralstonia pickettii*

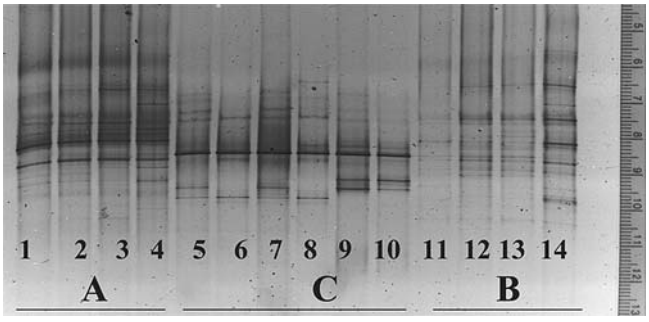

Fig. 3 Comparison between DGGE profiles of planktonic bacteria from Helgoland Roads (1 m depth) and intratunical bacteria from *D. migrans*. **A** Planktonic bacteria (filter fraction 0.2–3.0 μm) lanes 1–4; **B** planktonic bacteria (filter fraction 3.0–60 μm) lanes 11–14; **C** intratunical bacteria from different sources of *D. migrans* (lanes 5–6 tunic matrix; lanes 7–8 bacteria collected from tunic; lanes 9–10 larvae shortly before hatching)

DGGE profiles of intratunical bacteria and the bacterioplankton of the surrounding surface water from the Helgoland Roads (Fig. 3) exhibited no similarity to the DGGE profiles of intratunical bacteria from *D. migrans*.

Discussion

It is noticeable that no members of the γ-subgroup of proteobacteria were detected among the intratunical bacteria. *R. flavefaciens* (represented by the dominant *Rfa* bands and the weaker *Rfb* bands located above them) has the ability to degrade recalcitrant polysaccharides (Ding et al. 2001). *P. saccherophilia* is known as "knallgas bacterium" and has the specific ability to hydrolyse starch and gelatin (Aragno and Schlegel 1992). *R. pickettii* (Coenye et al. 2003) and *A. butzleri* are known as pathogen organisms of clinical sources (On et al. 2003). Unfortunately, these data do not allow a reliable interpretation of the ecological functions of intratunical bacteria. This is due to the low degree of relationship of some of the intratunical bacteria, and to the fact that some of the characteristic bands could not be successfully sequenced so far. Nevertheless, the data strongly suggest that intratunical bacteria represent a unique bacterial community, which is underlined by the fact that the DGGE profiles of intratunical bacteria do not correspond to the profiles of the planktonic bacteria generated from the seawater close to the ascidian habitat.

The survey of the distribution of intratunical bacteria in different sample material shows that the major bacterial species in adult *D. migrans* comprise all bacterial groups except for *A. butzleri*. The bacterial spectrum in the developmental stages investigated is similar, except for *A. butzleri* and *Flavobacterium* sp. This suggests that intratunical bacteria are transferred from the tunic matrix to the offspring, which corresponds to microscopic observations (Groepler and Schuett 2003). The occurrence of vertical transfer of bacteria from the adult ascidian colony to its offspring is demonstrated by the DGGE profile (data not shown) of a fully developed larva, freed from the egg envelope and all bacteria of the perivitelline space, which shows a distinct main band.

The unambiguousness of nearest-neighbour data analysis of prokaryotic 16S rDNA fragments from intratunical bacteria generated by direct PCR and separated by DGGE from eukaryotic tissue material is sometimes problematic. Direct PCR of prokaryotic fragments embedded in eukaryotic tissue material is not always successful, bands of the same sequences may occur at different positions like in the case of *Rfa* and *Rfb*, weak bands may occur, or bands are too closely located for further processing. The latter may be true for the main *Rfa* band position; a second band is located close to the main band (conspicuously in lane 9, Fig. 2). Additionally, different gel positions of DGGE fragments of the same species may occur occasionally. This may be due to the heterogeneity of the 16S rDNA operons (Nübel et al. 1996). The assignment of the five different groups of bands (representing the different intratunical bacterial groups) to the microscopically observed morphotypes is currently not possible. However, it is most likely that the main band (*Rfa*) (Fig. 2 shows 11 samples) corresponds to the extremely high numbers of the bipolarly monotrichously flagellated needle-like rods (Groepler and Schuett 2003). These rods were found exclusively inside the tunic. Hence, the *Rfa* band was

Table 1 Diversity of eleven bacterial PCR fragments, separated by DGGE and sequenced

DGGE fragment numbers & (sample material)	Best match [Accession No.] for 16S rDNA;	Similarity (%)	Numbers of nucleotides compared	Division
Rfb72 (A); *Rfb74* (B)	*Ruminococcus flavefaciens (b)* [AF030450]	84, 86	509, 495	δ-proteobacteria
Rfa22 (B); *Rfa39* (B)	*Ruminococcus flavefaciens (a)* [AF030450]	85, 85	515, 537	δ-proteobacteria
Fb69 (A)	*Flavobacterium*, symbiont [AF459795]	95	435	*Cytophaga*/*Flavobacterium* group
Ps71 (A); *Ps9* (C); *Ps42* (C)	*Pseudomonas saccherophilia* [AF368755]	92, 97, 97	528, 484, 507	β-proteobacteria
Rp70 (A); *Rp79* (C)	*Ralstonia pickettii* [AY268180]	99, 98	532, 432	β-proteobacteria
Ab37 (B)	*Arcobacter butzleri* [AF314538]	96	511	ε-proteobacteria

DGGE fragment numbers refer to excised and re-amplified PCR products. Accession numbers refer to 16S rDNA sequences from fragments analyzed in this study. Sample material comprises bacteria from the following sources: *A* tunic material; *B* bacteria drawn from tunic material; *C* different developemental stages of *D. migrans*

Table 2 Distribution of intratunical bacteria (assigned to type strains) detected in different sample sources

	Rf(a)	Rf(b)	Ps	Rp	Fb	Ab
A	+	+	+	+	+	–
B	+	+	–	–	–	+
C	+	+	+	+	–	–

Sample material comprises bacteria from *A* tunic material, *B* bacteria drawn from tunic material; *C* different developemental stages of *D. migrans*. *Rf(a) Ruminococcus flavefaciens*; *Rf(b) Ruminococcus flavefaciens*; *Ps Pseudomonas saccherophilia*; *Rp Ralstonia pickettii*; *Fb Flavobacterium* sp.; *Ab Arcobacter butzleri*

detected in all of the 41 *D. migrans* samples tested. The other bands were not detected in every single sample. In comparison to the other morpho-types, the microscopic observation showed an explicit predominance of the needle-like rods, which is emphasized by the thickness of band *Rfa*. The next major objective is the elucidation of the ecological functions of intratunical bacteria, as well as the unambiguous assignment of sequenced bands to the microscopically observed morphotypes.

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