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Phylogenetic diversity and antimicrobial activities of bryozoan-associated bacteria isolated from Mediterranean and Baltic Sea habitats

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ABSTRACT

To date, only a small number of investigations covering microbe–bryozoa associations have been carried out. Most of them have focused on a few bryozoan species and none have covered the antibacterial activities of associated bacteria. In the current study, the proportion and phylogenetic classification of Bryozoan-associated bacteria with antimicrobial properties were investigated. Twenty-one specimens of 14 different bryozoan species were collected from several sites in the Baltic and the Mediterranean Sea. A total of 340 associated bacteria were isolated, and 101 displayed antibiotic activities. While antibiosis was predominantly directed against Gram-positive test strains, 16S rRNA gene sequencing revealed affiliation of the isolates to Gram-negative classes (*Flavobacteria*, *Alpha-* and *Gammaproteobacteria*). One isolate was related to the Gram-positive *Actinobacteria*. The sequences were grouped into 27 phylotypes on the basis of similarity values $\geq$ 99.5%. A host-specific affiliation was not revealed as members of the same phylotype were derived from different bryozoan species. Site-specific patterns, however, were demonstrated. Strains of the genera *Sphingomonas* and *Alteromonas* were exclusively isolated from Mediterranean sites, whereas *Shewanella*, *Marinomonas* and *Vibrio*-related isolates were only from Baltic sites. Although *Pseudoalteromonas* affiliated strains were found in both habitats, they were separated into respective phylotypes. Isolates with 16S rDNA similarity values $<$ 98%, which could possibly represent new species, belonged to the genera *Shewanella*, *Pseudoalteromonas* and *Tenacibaculum*.

Introduction

Bryozoans mainly populate marine habitats worldwide and they settle on surfaces of different macroorganisms, such as algae and mussels, as well as on non-living hard substrates. Although the bryozoans described so far mostly appear as important index fossils, present-day species still contribute to the discovery of new natural products. However, from more than 5000 estimated recent species [33,42] only 32 have been studied from the point of view as producers of natural substances, but they have yielded approximately 200 compounds [45]. As sessile organisms, which lack an immune system, bryozoans are exposed to grazing and overgrowing pressure and therefore rely on mechanical and chemical defence strategies [13]. With regard to the supposed ecological function of secondary metabolites as antagonistic compounds, many studies have been carried out to assess their possible impact on human health. Thus, screenings have focused on cytotoxic [53] and antibiotic [7] extracts obtained from bryozoans, with the bryostatins being shown as the most prominent elucidated compounds [29,38]. Bryostatin 1 is a potent protein kinase C modulator and is still undergoing clinical trials to consider its effectiveness in cancer and Alzheimer's disease treatments (see http://www.clinicaltrials.gov/ct2/results?term=Bryostatin for details).

Metabolites of bryozoans are able to influence their microbial population as well. In the case of *Flustra foliacea*, eleven compounds (ten brominated alkaloids and one diterpene) were isolated and tested for antibacterial activities and AHL-dependent quorum sensing (QS) antagonism [37]. Five compounds displayed significant antibiotic properties which, in addition, targeted marine-derived strains isolated from *F. foliacea* (*Roseobacter* sp., *Psychroserpens* sp., *Sulfitobacter* sp., and *Paenibacillus pabuli*) more favourably than terrestrial strains (*E. coli* and *Bacillus megaterium*). Two compounds exhibited QS inhibitory effects without inhibiting growth.

Microbial associations with bryozoans on their surfaces or in inner tissues were examined by microscopic methods [43,54]. Nevertheless, studies on the bacterial diversity found on bryozoans are scarce. Gerdes et al. [12] investigated microbial fouling on 92 bryozoan colonies (e.g. *Disporella* sp., *Callopora* sp., *Celleporella hyalina*, *Watersipora subtorquata*, *Bugula neritina*) sampled from Japan and New Zealand, at four out of six sites in both spring and

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autumn. Some samples were selected for further microbial and molecular analysis apart from SEM and thin section studies. The biofilms were quite heterogeneous and thus any specificity to a bryozoan species or sampling site could not be determined. Another cultivation-independent approach was pursued by Kittelmann et al. [21]. Four bryozoan species (*Aspidelectra melolontha*, *Conopeum reticulum*, *Electra monostachys*, and *Electra pilosa*) were collected at three sites in the Jade area (Southern North Sea), together with empty mussel shells as references. In a comparative DGGE profile analysis, three species (*A. melolontha*, *E. monostachys*, and *E. pilosa*) demonstrated host-specific microbial communities, whereas the fourth (*C. reticulum*) revealed site-specific influences on its epibionts. By cultivation experiments, Pukall et al. [39] classified bacterial isolates associated with *Flustra foliacea* at two sampling sites in the North Sea. Taxonomic affiliation of these 220 bacterial strains to commonly isolated bacteria from marine environments led to the conclusion that *F. foliacea* may accept colonization of the surface.

The attempt to cultivate bacterial strains isolated from bryozoans seems to be more and more crucial, since it is argued that these microorganisms are the real producers of compounds formerly attributed to their hosts [3]. In the case of the amathamide alkaloids from the bryozoan *Amathia wilsoni*, strong indications for a bacterial producer were found [52]. Regarding bryostatins from *Bugula neritina*, the microbial symbiont "*Candidatus* Endobugula sertula" was determined as the true producer [9], although it could not be cultured.

In this study, twenty-one specimens of 14 different bryozoan species were collected from the Baltic Sea and the Mediterranean Sea. More than 300 associated bacteria were cultivated and their antibiotic activity was assessed. Isolates showing antimicrobial traits were phylogenetically classified by 16S rRNA gene sequencing.

Materials and methods

Sampling of bryozoans

Three sites each in the Baltic Sea and the Adriatic Sea were chosen for sampling (Table 1). Samples collected by scuba diving were put in sterile plastic containers with screw caps and all samples were stored in local seawater at 4 °C in a cool box until further treatment. Encrusting specimens were cut from their substrata with sterile razorblades and, if necessary, dissected with sterile tweezers. Then, all samples were washed thoroughly with sterile seawater. All bryozoans from the Baltic Sea sites were encrusting species, while some of the Mediterranean species were also arborescent colonies (sample IDs 11–16). Each sample was photographed for later taxonomic classification. Ten samples were collected from the Baltic Sea and they comprised five different bryozoan species. Eleven samples from the Mediterranean Sea covered nine different species.

Scanning electron microscopy (SEM) of bryozoan surfaces

A piece of each Mediterranean bryozoan was fixed in sterile 2% glutaraldehyde in 25‰ saline. After dehydration in a gradient ethanol series (30%, 50%, 70%, 90%, and 100%; v/v) the samples were critical point dried with carbon dioxide (Balzers CPD030) and sputter coated with gold–palladium (Balzers Union SCD004). The specimens were examined with a scanning electron microscope (Zeiss DSM960). Pictures were taken with a Contax SLR camera.

Isolation and cultivation of bacteria

Each sample was crushed with a sterile micropestle, and a dilution series with sterile seawater was made (10^{-1}–10^{-5}). The serial dilutions (100 μl each), as well as pieces of the bryozoan samples, were spread on agar plates containing different media.

TSB3S25 medium (containing 0.3% (w/v) tryptic soy broth (Difco) and 2.5% (w/v) NaCl) and natural seawater media using Baltic or Mediterranean seawater depending on the sample source were used, and 1.5% (w/v) agar was added to the media for solidification. The plates were incubated at 25 °C for at least one week. Colonies were picked and sub-cultured on TSB3S25 agar plates until pure cultures could be obtained.

16S rRNA gene sequence-based identification of active bacteria

Isolates exhibiting antimicrobial activities were chosen for 16S rRNA gene sequence analysis provided they were both from different bryozoans and of diverse colonial morphology (the latter was considered within all isolates from one sample). Crude DNA of pure cultures suspended in DNA-free water was extracted either by freezing and boiling (−20 °C overnight, 90 °C for 3 min) or by using a Precellys 24 lysis and homogenization device (Bertin Technologies) with a 0961VK05 Precellys grinding kit (Bertin Technologies). These DNA extracts were used directly for PCR. The primer pairs 27f (5′-GAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) or 27f and 1387r (5′-CGGGCGG TGTGTACAAGG-3′) were used for amplification of the 16S rDNA sequences [24]. PCR reactions were performed as follows: initial denaturation at 93 °C for 2 min, 30 cycles of amplification (each cycle consisting of annealing at 55 °C for 30 s, elongation at 72 °C for 30 s, and denaturation at 93 °C for 30 s), followed by final annealing (42 °C for 1 min) and an elongation step (72 °C for 5 min). The amplified PCR products were verified by agarose gel electrophoresis. PCR product purification, as well as sequencing, was undertaken at the Institute for Clinical Molecular Biology, University Hospital Schleswig-Holstein, Kiel. For each purification reaction, a mix of 1.5 units Exonuclease I (GE Healthcare) and 0.3 units Shrimp Alkaline Phosphatase (SAP, Roche) was added to the PCR product and incubated for 15 min at 37 °C. The enzymes were inactivated at 72 °C for 15 min. A sequencing reaction was carried out with the BigDye® Terminator v1.1 Sequencing Kit (Applied Biosystems) and it was analyzed in a 3730*xl* DNA-Analyzer (Applied Biosystems), as specified by the manufacturer. Primers used for sequencing were 27f, 534r (5′-ATTAC CGCGGCTGCTGG-3′), and 342f (5′-TACGGGAGGCAGCAG-3′), as well as 790f (5′-GATACCCTGGTAGTCC-3′), 1387r, and 1492r. Results were compared with other sequences in the EMBL prokaryotes database using BLAST [1], which is available online at the European Bioinformatics Institute homepage, and they were classified with the tools on the RDP-II Project homepage [6].

The 16S rRNA gene sequences were submitted to the EMBL database with the accession numbers FN295743 to FN295831 and AM412314.

Phylogenetic analysis

Type strain relatives of all isolates were determined by comparison of 16S rRNA genes in the EMBL prokaryotes database using BLAST and the online database LPSN (http://www.bacterio.net) [11]. Isolates were grouped into phylotypes by sequence similarities ≥ 99.5%. One representative isolate of each phylotype was selected for phylogenetic calculation. All subsequent relative type strains, as well as selected non-type strains, were included in