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Endobiotic bacteria and their pathogenic potential in cnidarian tentacles

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Abstract Endobiotic bacteria colonize the tentacles of cnidaria. This paper provides first insight into the bacterial spectrum and its potential of pathogenic activities inside four cnidarian species. Sample material originating from Scottish waters comprises the jellyfish species *Cyanea capillata* and *C. lamarckii*, hydrozoa *Tubularia indivisa* and sea anemone *Sagartia elegans*. Mixed cultures of endobiotic bacteria, pure cultures selected on basis of haemolysis, but also lyophilized samples were prepared from tentacles and used for DGGE-profiling with subsequent phylogenetic analysis of 16S rDNA fragments. Bacteria were detected in each of the cnidarian species tested. Twenty-one bacterial species including four groups of closely related organisms were found in culture material. The species within these groups could not be differentiated from each other (one group of *Pseudoalteromonas* spp., two groups of *Shewanella* spp., one group of *Vibrio* spp.). Each of the hosts exhibits specific endobacterial spectra. Solely *Cyanea lamarckii* harboured *Moritella viscosa*. Only in *Cyanea capillata* members of the *Shewanella* group #2 and the species *Pseudoalteromonas arctica*, *Shewanella violacea*, *Sulfitobacter pontiacus* and *Arcobacter butzleri* were detected. Hydrozoa *Tubularia indivisa* provided an amazingly wide spectrum of nine bacterial species. Exclusively in the sea anemone *Sagartia elegans* the bacterial species *P. aliena* was found. Overall eleven bacterial species detected were described recently as novel species. Four 16S rDNA fragments generated from lyophilized material displayed extremely low relationship to their next neighbours. These organisms are regarded as members of the endobiotic “terra incognita”. Since the origin of cnidarian toxins is unclear, the possible pathogenic activity of endobiotic bacteria has to be taken into account. Literature data show that their next neighbours display an interesting diversity of haemolytic, septicaemic and necrotic actions including the production of cytotoxins, tetrodotoxin and R-toxin. Findings of haemolysis tests support the literature data. The potential producers are *Endozoicimonas elysicola*, *Moritella viscosa*, *Photobacterium profundum*, *P. aliena*, *P. tetraodonis*, *Shewanella waksmanii*, *Vibrio splendidus*, *V. aestuarius*, *Arcobacter butzleri*.

Keywords Cnidaria, Tentacles, Endobiotic bacteria, Pathogenic activity, PCR, DGGE

Introduction

Interactions between bacteria and invertebrates are common in marine environment (Deming and Colwell 1982; Paul et al. 1986; Cary et al. 1997; Burnett and McKenzie 1997; Althoff et al. 1998; Schuett et al. 2005; Wichels et al. 2006). Yet, the bacterial mode of association (symbiosis, commensalisms or parasitism) and particular physiological functions are often unidentified. This also applies to the scarcely investigated cnidaria from cold-moderate regions. Jellyfish represent a major zooplanktonic fraction in the North Sea and the Atlantic. Among them are dangerous stingers causing painful dermal injuries after contact with humans. Similarly, the sessile cnidarian sea anemones and hydrozoan species harbour complex toxin cocktails, mostly of unknown chemical structures and functions. Currently the structures and activities of jellyfish species *Cyanea capillata* and *C. lamarckii* collected from the North Sea and the Atlantic are under investigation (Helmholz et al. 2007). However, the origin of cnidarian toxins is still open. A hypothesis favors the involvement of endobiotic bacteria as producers of cnidarian pathogenic activities. Reports of Palincsar et al. (1989) and Schuett et al. (2007) allowed first insight into the occurrence and morphology of organ-like bacterial aggregates in the intra-tentacular epidermis region of the sea anemone species *Aiptasia pallida* and *Metridium senile*.

This report provides an overview of cnidarian endobiotic bacteria from cold-moderate regions by phylogenetic analysis and allows information on their pathogenic potential.

Material and Methods

Samples and preparation

Individuals of *Cyanea lamarckii*, *Cyanea capillata*, *Tubularia indivisa* and *Sagartia elegans* were collected by divers during research cruises (June/July 2005-2008) from waters around the Orkney Islands. Tips of fresh tentacles were clipped off and subjected to washing procedures and CTAB treatment (Schuett et al. 2007) in order to remove potentially contaminating epibiotic bacteria. Light microscopic inspection showed no bacteria attached to the surface of fresh tentacle material. After removing seawater, one set of tentacle material was placed in three liquid medium modifications of Medium #621 (DSMZ, German Type Culture Collection; Staley, 1968), shown in Tab. 1. Each test tube containing one tentacle tip was incubated under aerobic conditions for two months at 18 °C. A second set containing the same sample material and medium ingredients has been incubated at microaerophilic

conditions (90% N₂ and 10% CO₂). Additionally, cultures were tested for haemolytic activity according to Smibert and Krieg (1984) by using 5 % (v/v) sheep blood.

Positive haemolytic strains were tested for their cytolytic activity by agar diffusion test using cell culture L929 (protocol provided by Lindl, 2002).

Finally, washed tentacle material was lyophilized for further DNA extraction.

DNA extraction and PCR amplification of 16S rDNA fragments

One milliliter aliquots of aerobic and microaerophilic mixed cultures respectively picked colonies from blood agar plates were used for nucleic acid extraction according to Anderson & McKay (1983). Primers 341fc (5'-cgc ccg ccg cgc ccc gcg ccc ggc ccg ccg ccc ccg ccc ccc tac ggg agg cag cag-3', clamp region underlined) and 907rwob (5'-ccg tca att cct ttr agt tt-3'; Muyzer et al., 1995) were applied to PCR.

Reaction mixture (100 µl) contained 2 U of Taq DNA polymerase, 1x polymerase buffer and 1x Master Enhancer (Eppendorf), 100 µM each dNTP, 0.4 µM each Primer and 1 µl DNA. After an initial denaturation step at 94°C for three minutes 20 cycles (one minute each step) followed: denaturation 94°C, annealing temperature starting at 65°C and decreasing by 0.5°C per cycle, elongation 68°C. Another 12 cycles were added this time using 55°C as annealing temperature. A final incubation at 68°C for 6 minutes was followed by 18 cycles lowering the temperature for 1°C per minute in order to avoid the formation of heteroduplexes. Negative controls were carried out omitting template DNA; Escherichia coli J53 served as positive control. DNA quantity was checked on agarose gels.

FastDNA Kit (Bio101) and DNeasy Blood & Tissue Kit (Qiagen) were combined for DNA extraction of lyophilized tentacles. Lysing Matrix tubes including a ¼" Ceramic Sphere and Garnet Matrix (Bio101) were supplemented by 1 ml CLS-TC solution and 100 mg lyophilized material. The tube was placed in a Fast Prep Instrument (FP120, Bio101), processed for 30 sec at speed 4 and incubated according to the manufacturer's manual. After the first centrifugation step the supernatant was transferred to a new tube and complemented by 20 µl proteinase K of the DNeasy Blood & Tissue Kit. The extraction was continued following Qiagen instructions. PCR was performed using Hot Master Taq polymerase (Eppendorf) and adapting the protocol (initial denaturation 1 min., annealing 30 sec., elongation 65°C).

DGGE was performed using the DCode electrophoresis system (Biorad). Preparation of polyacrylamide gels (15-70 % resp. 25-55 % denaturant gradient) and electrophoresis

parameters (100 V, 15 h) were performed according to Muyzer et al. (1995). Bands were stained with SYBR-Gold (Molecular Probes) and excised. DNA was extracted from gel material and dissolved in 10 µl distilled water (Sambrook et al. 1989).

Re-amplification of DNA fragments from DGGE bands

In difficult cases a further purification of DNA fragments could be obtained by re-amplifying the extracted DNA using again the forward primer with clamp (341fc), checking the accurate position of the band and excising it after an additional DGGE run. The extracted DNA was re-amplified for sequencing by using the Taq DNA polymerase but this time applying the forward primer without clamp (341f) and omitting the enhancer. PCR products were purified by using the Qiaquick PCR Purification Kit (Qiagen) following the instructions of the manufacturer's protocol and eluted with 60 µl distilled water.

DNA sequencing of PCR products and comparative sequence analysis

Sequencing was carried out by Qiagen Sequencing Services / Hilden, Germany. Sequences were aligned by using the Align IR program and the advanced BLAST search program of the National Center of Biotechnology Information (NCBI) website <http://www.ncbi.nlm.nih.gov/Blast>) to find closely related sequences.

Results

Phylogenetic analysis of mixed cultures

Cultures were prepared at low concentrations of organic matter, applying aerobic and micro-aerophilic conditions. Different media were selected in order to widen the spectrum of culturable bacterial endobionts (Table 1). Bacterial cultures could be obtained from all four cnidarian species. Regarding DGGE patterns the influence of different media compositions on the bacterial spectrum was negligible.

Seventy-nine fragments of the 16S rDNA deriving from cultures of four cnidarian species were successfully sequenced, aligned and phylogenetically analyzed (consult Table 2). Most of the fragments displayed similarities between 98 and 100% to their next neighbours; exceptions were *Endozoicimonas elysicola* (97%), *Arcobacter butzleri* (97%) and *Mesorhizobium tianshanense* (89%). Seventeen bacterial species and four groups were found,

the latter containing different closely related bacterial species which could not be differentiated from each other. The four groups were: (#1) *Pseudoalteromonas tetraodonis* / *P. elyakovii* / *P. haloplantis*, (#2) *Shewanella sairae* / *S. marinintestina*, (#3) *S. waksmanii* / *S. surugaensis* / *S. kaireiae* and (#4) *Vibrio splendidus* / *V. lentus* / *V. tasmaniensis* / *V. kanaloae*. In these cases the used fragment size was inappropriate to discriminate between the species forming these groups. Except for the *Bacillus subtilis* (Fermicutes), *Ilyobacter psychrophilus* (Fusobacterium) and *Arcobacter butzleri* (ϵ -proteobacterium) all other of the endobacteria detected were members of γ -proteobacteria. The most abundant endobionts comprised the groups #4 and #1 as well as *Photobacterium profundum*.

Remarkably, eleven bacterial species were described recently as novel organisms, some of them also experienced several taxonomic rearrangements. There were *Cobetia marina* (Arahal et al. 2002), *Colwellia aestuarii* (Jung et al. 2006), *Endozoicimonas elysicola* (Kurahashi and Yokoto 2006), *Pseudoalteromonas aliena* (Ivanova et al. 2004), *Pseudoalteromonas marina* (Nam et al. 2007), *P. tetraodonis* (Ivanova et al. 2001), *Psychrobacter nivimaris* (Heuchert et al. 2004), *Shewanella livingstonensis* (Bozal et al. 2002), *S. sairae* (Satomi et al. 2003), *S. surugaensis* (Miyazaki et al. 2006), *S. violacea* (Nogi et al. 1998). Their physiological traits are only partially known, yet.

Concluded, *Tubularia indivisa* provides the habitat for a wide spectrum of twelve bacterial species / groups, *Cyanea capillata* nine, *Sagartia elegans* eight and *Cyanea lamarckii* three.

Host specific bacteria

All four of the cnidarian species tested harbored host specific bacteria. Thirteen endobiotic species and groups occurred as single bacterial tenants, which are regarded as “specialists” for their individual hosts. *Shewanella violacea*, *Sulfitobacter pontiacus*, *Arcobacter butzleri* and members of group #2 were detected exclusively in *C. capillata* and *Moritella viscosa* only in *C. lamarckii*. Remarkable similarities between the jellyfish hosts could not be detected. The hydrozoa *Tubularia indivisa* sheltered the endobacteria *Cobetia marina*, *Colwellia aestuarii*, *Endozoicimonas elysicola*, *Psychrobacter nivimaris*, *Shewanella livingstonensis*, *Vibrio aestuarianus*, *Bacillus subtilis* and *Ilyobacter psychrophilus*; the sea anemone *Sagartia elegans* had *Pseudoalteromonas aliena* as host specific tenant.

Phylogenetic analysis of lyophilized material

The phylogenetic analysis of six lyophilized samples comprising *Cyanea capillata*, *C. capillata* and *Tubularia indivisa* generated 10 bands. The band patterns displayed fewer bands than those of the cultures (Fig.1). *C. capillata* fragments displaying *Vibrio* species group #4 were found in both samples (similarity 99%). *Endozoicimonas elysicola* (similarity 97%) was spotted in lyophilized but also in mixed culture material of *Tubularia indivisa*. This novel organism has also been detected previously in the sea anemone *Metridium senile* (Schuett et al 2007). An exciting result was the discovery of endobacteria showing an extremely low relationship to their next neighbours. Because of high sequence quality strange microorganisms detected in both of the *Cyanea* species and *Tubularia indivisa* should be interpreted as unknown new species. Among them were bacteria distant to *Aquaspirillum itersonii* (similarity 90%, found in *Cyanea lamarckii*), *Francisella philomiragia*, (similarity 93%, both *Cyanea* species), and *Spiroplasma syrphidicola*, (89%, *Tubularia indivisa*). A definite phylogenetic allocation of these unambiguous sequence data to known bacteria is currently impossible.

Aggregates

Tubularia indivisa and *Sagartia elegans* contained organ-like bacterial aggregates in the tentacle's epidermis. The aggregates are smaller than those of *Aiptasia pallida* (Palincsar et al. 1989) and *Metridium senile* (Schuett et al. 2007). Nevertheless, each of these aggregate-bearing cnidaria harbor a different bacterial spectrum. *Cyanea* species showed only free floating endobiotic bacteria in their tentacles. A paper on aggregate visual nature and distribution by REM inspection is in preparation.

Pathogenic activities of bacterial endobionts

Nine bacterial species (out of 21) may possess pathogen activities and/or produce toxins (Table 3). This information has been taken from physiological literature data. It should be noted that the *Pseudoalteromonas* group #1 and the *Vibrio* group #4 contain members of pathogenic and non-pathogenic bacteria. All four cnidarian host organisms harbor potentially pathogen bacteria: *C. lamarckii* two species and *C. capillata* five, *Tubularia indivisa* and *Sagartia elegans* each five. *Vibrio splendidus* (the only pathogen species of group #4) has

been detected in all of the cnidaria tested. *Photobacterium profundum* and *Pseudoalteromonas tetraodonis* (group #1) have been found in *C. capillata*, *Tubularia indivisa* and *Sagartia elegans*.

Some remarkable examples in detail: Hydrozoa *Tubularia indivisa* harbors the potentially pathogen *Endozoicimonas elysicola* which is suspected to play a role in amoebic fish disease (Bowman and Nowak 2004). *Photobacterium profundum* produces ToxR genes that mediate virulence expression (Bidle and Bartlett 2001, Ivanova et al 2004). *Pseudoalteromonas tetraodonis* (group #1) is known to produce tetrodotoxin (Do et al. 1990; Ivanova et al. 2001). Metabolites of *Vibrio aestuarianus* may kill corals, oysters and turbot larvae. Extracellular toxic products of *Vibrio aestuarianus* (Labreuche et al. 2006, Garnier et al. 2007) may impair juvenile oysters. Members of *Vibrio splendidus* (group #4) can express septicaemic and necrotic activities (Sugumar et al. 1998, Thomson et al 2003, Koren and Rosenberg 2006). Yet, it is uncertain whether anaerobic *Ilyobacter psychrophilus*, member of the genus *Fusobacterium*, generates severe clinical infection.

Anthozoa *Sagartia elegans* harbors *Photobacterium profundum* and *Pseudoalteromonas aliena*, which produces cytotoxins and causes haemolytic activities (Ivanova et al. 2004). Additionally *Sagartia elegans* contains members of the groups #1 and #4 (activities see above).

Jellyfish *Cyanea lamarckii* provides the natural habitat for *Moritella viscosa*, which causes necrotic “winter ulcer” in salmon (Lunder et al. 2000, Heidarsdottir et al. 2008), additionally *Arcobacter butzleri*, a human pathogen species (Carbone et al. 2003, Gugliandolo et al. 2007), and finally members of group #4 (see above). The Lions mane jellyfish *C. capillata* carries the haemolytic species *Shewanella waksmanii*, a member of group #3 (Ivanova et al. 2003), and pathogenic endobionts of the already described *Photobacterium profundum* and the putatively pathogenic species of groups #1 and #4 (see above).

Haemolysis

Selection of mixed culture material based on haemolysis tests and subsequent phylogenetic analysis were applied to verify the pathogenic activities derived from literature data. The findings are in good correspondence with literature reports: Haemolytic positives were detected in *Cyanea capillata* (*Pseudoalteromonas* group1, 3 isolates; *Vibrio* group 4, 5 isolates), *Tubularia indivisa* (*Vibrio* group 4, 1 isolate), and in *Sagartia elegans* (*Pseudoalteromonas aliena*, 1 isolate; *Pseudoalteromonas* group 1, 2 isolates; *Vibrio* group 4,

4 isolates). In order to determine cytolytic activity, the agar diffusion test was applied to haemolysis positive pure cultures. In case of *Vibrio* group #4 strain, derived from tentacle material of *Cyanea capillata*, the cells were completely disintegrated and decolorized within a 1 cm zone.

Discussion

The spectrum of culturable bacterial species is remarkable: Some of them can be regarded as “all rounders”; others were host specific. An unambiguous identification on species level was excellent for seventeen different fragments (i.e. 80%). Unclear allocation of sequence data at species level led to the formation of groups # 1 to # 4. Some authors reported similar findings of different bacterial species (Ivanova et al., 2003 for *Shewanella* species, Marques et al., 2005, Venkateswaran et al., 1998 for *Vibrio* species, Mc Aucliffe et al., 2003 for *Mycoplasma* species). The application of larger 16S rDNA or 23S rDNA fragments might be more appropriate in future experiments. Physiological information concerning pathogenic activities was exclusively taken from literature data and therefore insufficient as proof for pathogenic traits but also unsuitable in cases of unknown new species.

Eighty-one percent of the species or groups found were host specific. This is a clear sign that bacterial contamination of the tentacle surface was avoided during experiments, which was confirmed by microscopic inspection subsequently to CTAB treatment.

Which approach reflects the actual presence of endobionts - phylogenetic data derived from cultures or lyophilized material? The strange, unidentified endobacteria may be regarded as the authentic, dominant tenants inhabiting the “exotic” tentacles whose physico-chemical conditions are still not discovered. Yet, PCR-processing of lyophilized material cannot detect low bacterial numbers. The major disadvantage of cultures is their medium dependence; the bacteria that grow may be of less endobiotic importance. In our experiments the only bacterial species detected in both of the approaches was *Endozoicimonas elysicola*. To meet the actual growth requirements, refined culture techniques are needed.

The organ-like bacterial aggregates detected in *Sagartia elgans* and *Tubularia indivisa* contain hundreds of tightly packed, differently shaped endobacteria. Their different shapes suggest that these organisms may be host-specific. The development and function of the organ-like aggregates are unclear. Do we presently observe here an evolutionary step during the formation of organ-like aggregate structures? Is that comparable to the developmental steps of prokaryotic mitochondria or chloroplasts in eukaryotes?

Data show that a considerable number of bacterial species living as tenants in cnidarian hosts may have pathogenic activity. The applied haemolysis and cell culture tests support the indirect approach using trait data from literature based on phylogenetic classification. Further testing of acute cell toxicity should be performed after calibrating bacterial protein concentration.

There is substantial knowledge of tetrodotoxin TTX, which is an extremely potent neurotoxin. Its chemical structure and activities at cellular level are well documented (Thuesen and Kogure 1989; Do et al. 1990; Ritchie et al. 2000). TTX-carrying animals are known from different marine, limnic and terrestrial habitats of warmer regions. Among them were the well known puffer fish species (*Tetraodontidae*) but also starfish, toads, flatworms and even chaetognathes. Interestingly the toxin is produced by endobiotic bacteria (Simidu et al. 1990, Yu et al. 2004). One of those endobionts is *Pseudoalteromonas tetraodonis* (Ivanova et al. 2001). Supposedly these bacteria are also common partners of cnidarian species. In order to generate answers to the bacterial role in the production of cnidarian toxins the investigation of their physiological traits is indispensable.

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Bacterial Species	Origin (literature data)	Toxic & Pathogenic Activities	Literature Data
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Medium DSMZ	Quantity	Notes	#621+M44	#621	#621- Glu
Peptone (Bacto)	0.25 g	pH 7,5 autoclaved	x	x	x
Yeast extract (Bacto)	0.25 g		x	x	x
Aged seawater	724.00 ml		x	725.00 ml	x
Distilled water	241.00 ml		x	x	251.00 ml
Mineral salt solution	19.00 ml	sterile filtered	x	x	x
Vitamin sol.(double conc.)	5.00 ml	sterile filtered	x	x	x
Metals 44 (DSMZ #590)	1.00 ml	sterile filtered	x	—	x
Glucose sol. (2,5 %)	10.00 ml	sterile filtered	x	x	—

Tab. 1 Medium DSMZ #621 and derivatives; metal composition M44 originates from Medium #590. Both media are provided by the German Collection of Microorganisms and Cell Cultures (www.dsmz.de). Negative symbol indicates substitution of distilled water

<i>Endozoicimonas elysicola</i>	nudibranch <i>Elysia ornata</i>	amoebic fish disease	Bowmann and Nowak 2004
<i>Moritella viscosa</i>	Atlantic salmon	necrotic, “winter ulcer”	Lunder et al. 2000
<i>Photobacterium profundum</i> SS9	deep sea crustaceans	ToxR-gene (mediating virulence expression)	Bidle and Bartlett 2001; Ivanova et al. 2004
<i>Pseudoalteromonas aliena</i>	seawater	cytotoxic, haemolytic	Ivanova et al. 2004
<i>Pseudoalteromonas tetraodonis</i> (group #1)	buffer-fish	produces tetrodotoxin	Simidu et al. 1990; Ivanova et al. 2001
<i>Shewanella wachsmannii</i>	marine worm <i>Phascolosoma sipuncula</i>	haemolytic	Ivanova et al. 2003
<i>Vibrio aestuarianus</i>	oyster juveniles, seawater	extracellular toxic products	Labreuche et al. 2006; Garnier et al. 2007
<i>Vibrio splendidus</i> (group #4)	mostly abundant in corals, oysters, turbot larval gut,	haemolytic, septicaemic, necrotic	Sugumar et al. 1998; Thomson et al. 2003; Koren and Rosenberg 2006
<i>Arcobacter butzleri</i>	brackish marine environment	human and animal pathogen, secretion of toxic factors	Carbone et al. 2003; Gugliandolo et al. 2007

Tab. 3 Pathogen potential of endobiotic bacteria detected in cnidarian tentacles. *Pseudoalteromonas* group #1 and *Vibrio* group #4 contain closely related pathogenic and non-pathogenic species