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Short Communication

Characterization of the bacterial community associated with body wall lesions of *Tripneustes gratilla* (Echinoidea) using culture-independent methods

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ABSTRACT

The bacterial community associated with skin lesions of the sea urchin *Tripneustes gratilla* was investigated using 16S ribosomal RNA gene cloning and fluorescent *in situ* hybridization (FISH). All clones were classified in the *Alphaproteobacteria*, *Gammaproteobacteria* and *Cytophaga–Flexibacter–Bacteroides* (CFB) bacteria. Most of the *Alphaproteobacteria* were related to the *Roseobacter* lineage and to bacteria implicated in marine diseases. The majority of the *Gammaproteobacteria* were identified as *Vibrio* while CFB represented only 9% of the total clones. FISH analyses showed that *Alphaproteobacteria*, CFB bacteria and *Gammaproteobacteria* accounted respectively for 43%, 38% and 19% of the DAPI counts. The importance of the methods used is emphasized.

1. Introduction

Body wall lesions consisting of infected areas of the test with loss of epidermis and appendages are recurrently observed in reared echinoids (Tajima et al., 1997a; Takeuchi et al., 1999; Bauer and Young, 2000) and in wild populations (Jangoux, 1990; Nagelkerken et al., 1999; Becker et al., 2008). Bacteria involved in such lesions were usually identified using culture-dependent techniques and were assigned, among other, to *Vibrio alginolyticus* (Bauer and Young, 2000), *Flexibacter* sp. (Tajima et al., 1997b), *Vibrio anguillarum* and *Aeromonas salmonicida* (Gilles and Pearse, 1986).

Although bacteria are responsible for these infections, a preliminary abrasion of the integument is required (Jangoux and Maes, 1987; Becker et al., 2007). In the field, abrasions are induced by various factors including parasites. Recently, a skin disease initiated by a parasitic gastropod, *Vexilla vexillum,* to the sea urchin *Tripneustes gratilla* has been described in Madagascar (Vaïtilingon et al., 2004; Becker et al., 2007). The gastropod grazes the body surface that progressively turns black as the infection by microorganisms occurs while non-affected areas remain healthy and free of bacteria (Vaïtilingon et al., 2004; Becker et al., 2007). Four strains identified as *Vibrio* spp. and *Exiguobacterium* sp. were isolated from infected lesions and all induced symptoms when applied on healthy echinoids (Becker et al., 2007). In the present study, a culture-independent method (16S rRNA gene cloning) is used in order to obtain a thorough identification of the bacterial community associated with *T. gratilla* lesions. It is indeed known that less than 1% of the bacteria are cultivable. Moreover, preliminary culture-independent results on *T. gratilla* lesions using Denaturing Gradient Gel Electrophoresis (DGGE) detected the presence of *Alphaproteobacteria* and CFB (Becker et al., 2007). FISH experiments are then performed to estimate the proportion of the bacterial groups identified.

2. Materials and methods

2.1. Sampling

Tripneustes gratilla individuals showing infected lesions were collected by hand at low tide on the reef off Toliara (23°25'00" S, 43°39'23" E), Madagascar, in January 2006. Eight lesions from eight different individuals were sampled: four were fixed in absolute ethanol for cloning and four in 4% paraformaldehyde for 3 h, rinsed in phosphate buffer saline (PBS) and stored in a 1:1 mixture of PBS and absolute ethanol at −20 °C for FISH.

2.2. Cloning and 16S rRNA gene sequencing

Clones were obtained from samples according to Becker et al. (2008). Clones sequences were compared with those in the GenBank database with BLAST (Basic Local Alignment Search Tool) in

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order to find related species (Altschul et al., 1990). Each sequence was also checked for chimera formation using Chimera Check v2.7 (Cole et al., 2003). Coverage value of the clone library was calculated according to Good (1953) with 99% of sequence similarity used as the criterion for sequence uniqueness. The sequences obtained in this study have been deposited in the EMBL database under Accession Nos. AM930414–AM930504.

2.3. Fluorescence in situ hybridization (FISH)

The tested oligonucleotide probes were EUB338 for *Eubacteria* (Amann et al., 1990), ALF968 for *Alphaproteobacteria* (Neef, 1997), GAM42a for *Gammaproteobacteria* (Manz et al., 1992) and CF319a for the CFB bacteria (Manz et al., 1996). NON338 was used as a negative control (Wallner et al., 1993). The probes were labelled with Cy3 at the 5′ end. Samples were treated according to Gillan et al. (2005). The signal obtained with probe NON338 was 0% in all counts.

3. Results

3.1. 16S rRNA gene cloning

Cloning results are summarized in Table 1. A library of 91 clones (71% of coverage value) was obtained from four different lesions. Sequences with at least 99% similarity were gathered, giving 26 operational taxonomic units (OTU) sharing less than 99% similarity. A BLAST search indicated that sequences were *Alphaproteobacteria* (39 sequences), *Gammaproteobacteria* (43 sequences) or CFB bacteria (9 sequences). Among *Alphaproteobacteria*, several clones were closely related (96–99%) either to bacteria infecting black-band diseased corals or to a bacterium responsible for a sponge disease. Most of the other *Alphaproteobacteria* were related to the *Roseobacter* lineage. Nearly two third (65%) of the *Gammaproteobacteria* clones belonged to the *Vibrio* genus. The majority of these *Vibrio* were highly similar (99%) to *Vibrio harveyi* and were present

Table 1
16S rRNA gene sequence identities of clones associated with skin lesions of *Tripneustes gratilla*[a].

Clones (Nos.)	Nos. bases	Best-matched organism (GenBank Accession Nos.)	Source	ID%	Division	Sampling[b]				References
						A	B	C	D	
OTU-1 (13)	1385–1389	*Roseobacter* sp. isolate 27-4 (AJ536669)	Turbot larvae rearing unit	96	α		x	x	x	Hjelm et al. (2004)
OTU-2 (10)	1340–1389	Alpha proteobacterium NW4327 (AF384141)	Diseased sponge	97–98	α	x				Webster et al. (2002)
OTU-3 (6)	1389	Uncultured alpha proteobacterium clone WA_06f (EF123405)	Black band diseased coral	98–99	α	x		x	x	Unpublished
OTU-4 (2)	1389	Alpha proteobacterium MBIC1876 (AB026194)	Sponge tissue	99	α			x		Unpublished
OTU-5 (1)	1389	*Roseobacter* sp. isolate 8-1 (AJ536670)	Turbot larvae rearing unit	96	α	x				Hjelm et al. (2004)
OTU-6 (1)	1378	Uncultured alpha proteobacterium clone BBD_216_40 (DQ446109)	Black band diseased coral	96	α	x				Sekar et al. (2006)
OTU-7 (1)	1387	Alpha proteobacterium NW4327 (AF384141)	Diseased sponge	97	α	x				Webster et al. (2002)
OTU-8 (1)	1386	*Nautella italica* strain R-28753 (AM944522)	Marine biofilm	97	α	x				Unpublished
OTU-9 (1)	1391	Uncultured alpha proteobacterium clone BBD216b_11 (EF123360)	Black band diseased coral	96	α		x			Unpublished
OTU-10 (1)	1389	Alpha proteobacterium NW4327 (AF384141)	Diseased sponge	98	α			x		Webster et al. (2002)
OTU-11 (1)	1413	Uncultured alpha proteobacterium clone BBD_216_19 (DQ446093)	Black band diseased coral	98	α				x	Sekar et al. (2006)
OTU-12 (1)	1423	Rhizobiales bacterium CL-DNM10 (DQ401094)	Tidal flat sediments	92	α				x	Unpublished
OTU-13 (5)	1450–1451	Uncultured bacterium clone S26-53 (EU287353)	Arctic surface sediments	95	CFB		x		x	Unpublished
OTU-14 (3)	1344–1451	Cryomorphaceae bacterium CML50 (AB176674)	Marine	92–93	CFB	x				Lau et al. (2006)
OTU-15 (1)	1449	*Kordia algicida* (AY195836)	Marine	95	CFB		x			Sohn et al. (2004)
OTU-16 (22)	1436–1487	*Vibrio harveyi* strain S35 (AY750578)	Marine	99	γ		x	x	x	Fukui and Sawabe (2007)
OTU-17 (7)	1466	*Thalassolituus oleivorans* isolate SLHC162b (AM279755)	Seawater	95	γ	x	x	x	x	Unpublished
OTU-18 (4)	1457–1467	*Amphritea balenae* strain JAMM 1525 (AB330883)	Marine sediments	97	γ		x	x		Unpublished
OTU-19 (3)	1425–1468	Uncultured bacterium clone Osedax_sym1 (AY549004)	Marine	95	γ			x	x	Goffredi et al. (2005)
OTU-20 (1)	1479	Uncultured gamma proteobacterium clone DPC166 (DQ269084)	Surface of marine macro-alga	93	γ		x			Unpublished
OTU-21 (1)	1480	*Vibrio harveyi* strain SW-3 (AY911396)	Marine	99	γ			x		Zhang et al. (2006)
OTU-22 (1)	1479	*Vibrio* sp. FLTOD1 (DQ317678)	Fish gut	96	γ			x		Unpublished
OTU-23 (1)	1479	*Vibrio* sp. BWDY-57 (DQ328956)	Marine	99	γ				x	Unpublished
OTU-24 (1)	1479	*Vibrio* sp. LMG 20546 (AJ316172)	Unknown	98	γ				x	Thompson et al. (2001)
OTU-25 (1)	1480	*Vibrio* sp. BWDY-57 (DQ328956)	Marine	99	γ				x	Unpublished
OTU-26 (1)	1480	*Vibrio* sp. YASM14 (DQ314529)	Turbot *S. maximus*	98	γ				x	Unpublished

[a] Listed for each OTU are the numbers of corresponding clones (in brackets), the numbers of bases sequenced, the best-matched organisms in GenBank followed by their accession numbers, sources, percent identities, divisions and references.
[b] X signs indicate if the clone is present in sampled lesions A, B, C and/or D.

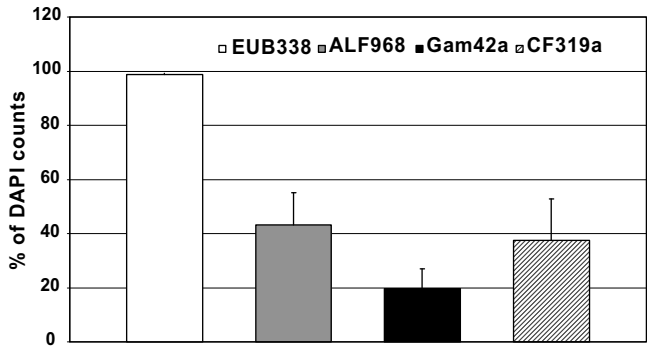

Fig. 1. Ratios between Cy3 and DAPI counts (expressed in percent) obtained in samples ($n = 4$) of infected lesion of *T. gratilla* for probes staining *Eubacteria* (EUB338), *Alphaproteobacteria* (ALF968), *Gammaproteobacteria* (GAM42a) and CFB bacteria (CF319a).

in three of the four lesions. Most of the other *Gammaproteobacteria* were related to *Thalassolituus oleivorans* or to *Amphritea balenae*. CFB bacteria were detected in three of the four lesions. One of the CFB clones was 95% similar to *Kordia algicida* while other CFB clones were related either to a *cryomorphaceae* or to an unidentified bacterium associated with marine sediments. Seven OTU were found in at least two different samples, showing some homogeneity in the bacterial composition of the microbiota infecting the lesions.

3.2. FISH

Fig. 1 details FISH results. In all samples, *Eubacteria* cells accounted for 98% of the DAPI counts. Percentages of *Alphaproteobacteria* varied between 26% and 53% of the DAPI counts (mean value 43 ± 12%). Proportions of CFB bacteria were between 23% and 57% (mean value 38 ± 15%) while *Gammaproteobacteria* varied between 9% and 25% (mean value 19 ± 7%). In three of the four samples, *Alphaproteobacteria* were the most abundant and reached about a half of the DAPI counts. In all samples, *Gammaproteobacteria* were the less numerous and never exceeded a quarter of the DAPI counts.

4. Discussion

The skin disease in *T. gratilla* has been analysed by bacterial cultures and DGGE in a previous study (Becker et al., 2007) and by cloning and FISH in the present work. The results obtained with these methods differ in some respect. The four strains isolated from bacterial cultures were three *Vibrio* sp. and *Exiguobacterium* sp. while DGGE identified CFB bacteria and *Alphaproteobacteria* (Becker et al., 2007). Cloning analysis suggests that the bacterial community associated with *T. gratilla* lesions consists of *Alphaproteobacteria*, *Gammaproteobacteria* and CFB bacteria, providing a more complete identification as it reveals bacterial species, such as non-*Vibrio Gammaproteobacteria*, that were not detected with previous methods. It is however noteworthy that culturing approaches suggest the presence of *Exiguobacterium* sp. which was not retrieved with cloning and DGGE, probably due to a limited presence of this bacterium in the lesions. Furthermore, the FISH results obtained in this study clearly illustrate the biases of the PCR-dependent methods that should not be considered for quantitative results. For example, the CFB bacteria represent less than 10% of the clones but more than a third of the DAPI counts. Inversely, *Gammaproteobacteria* account for nearly a half of the clones but for less than 25% of the DAPI counts.

FISH analyses show that *Alphaproteobacteria* are numerically the most abundant and some *Alphaproteobacteria* clones are related to bacteria implicated in the black-band disease of the coral *Siderastrea siderea* (Sekar et al., 2006) or to a pathogenic bacterium infecting the sponge *Rhopaloides odorabile* (Webster et al., 2002). Other *Alphaproteobacteria* clones are assigned to the *Roseobacter* lineage. The latter is an exclusively marine and physiologically heterogeneous group that is well represented in coastal waters (Wagner-Döbler and Biebl, 2006). Moreover, a *Roseobacter* species, *Roseovarius crassostreae*, is responsible for the juvenile oyster disease affecting juvenile *Crassostrea virginica* and causing losses exceeding 90% of the production in American hatcheries (Boettcher et al., 2005; Davis and Barber, 1994). Given the relative high abundance and affiliations of the *Alphaproteobacteria* from *T. gratilla* lesions and the fact that they are present in all samples, they could be considered as key members of the bacterial community infecting these lesions. Nearly two third of the *Gammaproteobacteria* clones identified in the present study are assigned to *Vibrio* species. The latter are the most commonly found bacteria in echinoids infections. Representatives of this genus were isolated from diseased *Strongylocentrotus purpuratus* in California (Gilles and Pearse, 1986), *Paleopneustes cristatus* and *Archaopneustes hystrix* in the Bahamas (Bauer and Young, 2000), *Strongylocentrotus intermedius* in Japan (Takeuchi et al., 1999) and *Paracentrotus lividus* in France (Becker et al. 2008). FISH experiments however show that *Gammaproteobacteria* were the less numerous in lesions of *T. gratilla*. Consequently, they may play a less significant role in the infection than suggested by bacterial cultures and infection assays. Few CFB clones were obtained and they were not related to bacteria implicated in marine diseases. However, FISH analyses show that these bacteria are relatively abundant with a mean of 38% of the DAPI counts, thus forming a important part of the microbiota associated with the lesions.

The present study thus put in evidence that skin lesions of *T. gratilla* are infected by complex microbial communities without dominant etiological agent, although *Alphaproteobacteria* seem to prevail. Infecting bacteria are probably opportunistic invaders that benefit from the weak immune response of the echinoid to develop within the affected integument. This would explain the differences that occur between bacterial communities from a lesion to another. Compared to bacteria that were found in a single sample, the few common pathogens would be more abundant in the environment and/or more able to grow on the lesions. Consequently, they could statistically be the first invaders although our results do not permit to establish a chronology of the infection.

In addition to analyse the structure of the bacterial community associated with *Tripneustes gratilla* lesions, this work also emphasizes the importance of choosing cloning coupled with FISH to investigate sea urchin (and probably other marine animals) skin lesions, rather than bacterial cultures as in previous works.

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