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Aquaculture 242 (2004) 13–30

Aquaculture

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The skin ulceration disease in cultivated juveniles of *Holothuria scabra* (Holothuroidea, Echinodermata)

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Received 7 July 2003; received in revised form 10 November 2003; accepted 20 November 2003

Abstract

It is frequently reported that cultivated holothuroids can suffer from a disease affecting their integument. We report here on a disease of juvenile *Holothuria scabra*, the widely marketed edible sea cucumber, reared in the Aqua-Lab hatchery of Toliara, Madagascar. This disease, which has been called skin ulceration disease, is very contagious and results from a severe bacterial infection that causes death within 3 days. The first sign of the infection is a white spot that appears on the integument of individuals, close to the cloacal aperture. The spot extends quickly onto the whole integument leading to the death of individuals. Microscopic (histology, scanning and transmission electron microscopies) and biomolecular (denaturing gradient gel electrophoresis (DGGE) and sequencing) techniques have been used to describe the lesions and to investigate the infecting microbial communities. The lesions consist in a zone where the epidermis is totally destroyed and where collagen fibres and ossicles are exposed to the external medium. This zone is surrounded by a border line where degrading epidermis is mixed with the connective tissue. Lesions include three bacterial morphotypes: rod-shaped bacteria, rough ovoid bacteria, and smooth ovoid bacteria. The last morphotype is the only one found on the ossicles and is assumed to be responsible for their degradation. Three species of bacteria have been put

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doi:10.1016/j.aquaculture.2003.11.018

in evidence in the lesions thanks to biomolecular analyses: *Vibrio* sp., *Bacteroides* sp., and an α-Proteobacterium. Infection assays of healthy holothuroids have been performed from lesions and from bacterial cultures but the causative agent has not been identified. It is suggested that combined events or agents, including bacteria, are required to induce the disease.
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Keywords: Disease; Bacteria; Holothuroidea; DGGE

1. Introduction

Bacterial diseases in echinoderms are known in echinoids, holothuroids and asteroids (see Jangoux, 1990 for review). In the latter, only the species *Asterina gibbosa* has been reported to suffer from a bacterial infection (Delavault and Leclerc, 1969). The disease has only been observed in aquarium-reared asteroids and consisted in epidermal necroses that progressively extended and caused the death of individuals (Delavault and Leclerc, 1969). Much more documented are sea urchin bacterial diseases among which are the bald sea urchin disease and the black sea urchin plague, both occurring in the field and affecting regular echinoid populations where they produce mass mortality (Jangoux, 1990; Liddell and Ohlhorst, 1988). Infections cause the loss of spines and test lesions resulting in the death of individuals (Maes and Jangoux, 1984; Hughes et al., 1985). *Vibrio anguillarum* and *Aeromonas salmonicida*, two well known pathogenic marine bacteria, were able to initiate bald sea urchin lesions (Gilles and Pearse, 1986). The agents of the black sea urchin plague are supposed to be *Clostridium perfringens* and *Clostridium sordelli* (Bauer and Argerter, 1987). The plague disease has been especially intense in *Diadema antillarum* populations in the Caribbean during the years 1983 and 1984 when nearly 100% of individuals died in an epizootie that spread over three and a half million km^2 (Lessios et al., 1984). The *Diadema* die-off provoked the expansion of algae that started to colonise coral reefs (Liddell and Ohlhorst, 1988).

Almost nothing is known about bacterial diseases in holothuroids. There is a single note reporting that a broodstock of *Holothuria scabra* from Bribie Island (Australia) suffered from a bacterial disease (Morgan, 2000). The infection involved the loss of epidermal pigmentation associated with the presence of huge amount of viscous mucus. The lesions first arose around the mouth and/or cloacal openings of animals and then spread on both sides of the individuals before possibly encompassing the whole body. If infected animals were not eliminated from the broodstock, up to 95% of the reared individuals died (Morgan, 2000). According to this author, *Vibrio harveyi* was the predominant bacterium in the lesions of affected holothuroids. We report here on a disease affecting juvenile *H. scabra* reared in the hatchery of Toliara, Madagascar. Microscopic and biomolecular techniques (denaturing gradient gel electrophoresis and sequencing) together with bacterial cultures and infection assays were used to characterise the disease and to investigate the microbial communities of the lesions.

2. Materials and methods

Diseased and healthy juveniles of *H. scabra* Jaeger, 1833 (1 to 3 cm in length) were sampled from the aquaria of the Aqua-Lab hatchery of Toliara (Madagascar) in February 2001, May 2001 and February 2002.

2.1. Microscopic techniques

For light microscopy, diseased individuals were fixed for 8 h in Bouin's fluid (without acetic acid) and decalcified overnight in a 1:1 mixture of a 2% ascorbic acid solution and NaCl 0.3 M at 4 °C (Dietrich and Fontaine, 1975). Specimens were then dehydrated through a graded series of ethanol (50%, 70%, 90% and 100%), embedded in paraplast and cut into 7 μm thick sections that were stained with a Masson trichrome (Ganter and Jollès, 1969–1970).

For transmission electron microscopy (TEM), diseased individuals were fixed for 3 to 24 h in a 3% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.8) at 4 °C. Pieces of the body wall were washed in the buffer before post fixation in 1% osmium tetroxide solution in the same buffer at 4 °C for 1 h. Pieces were then decalcified for 1 week in a 1:1 mixture of a 2% ascorbic acid solution and NaCl 0.3 M at 4 °C (Dietrich and Fontaine, 1975). Decalcified samples were dehydrated through a graded series of ethanol (50%, 70%, 90% and 100%), transferred into Spuur resin at room temperature for 15 h follow by another 15 h at 60 °C and cut with a Leica ultracut UCT. Sections were stained with lead citrate and uranyl acetate and observed with a Zeiss LEO 906E electron microscope.

For scanning electron microscopy (SEM), diseased individuals were fixed for 8 h in Bouin's fluid (without acetic acid) or for 3 to 24 h in a 3% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.8) at 4 °C, dehydrated through a graded series of ethanol (50%, 70%, 90% and 100%), critical-point dried, mounted on stubs and coated with gold. Diseased and healthy parts of the body wall were also immersed overnight into a 2 mg ml^{-1} proteinase K solution in MilliQ water at 37 °C. Once all the tissues were digested, the remaining ossicles were placed on stubs and coated with gold. All samples were examined with a Jeol JSM-6100 electron microscope.

2.2. Biomolecular techniques

The number of phylotypes (i.e., nucleotide sequences obtained from an environmental sample and having phylogenetic affiliation with sequences from known species; Muyzer and de Waal, 1994) occurring in the lesions, was determined by denaturing gradient gel electrophoresis (DGGE hereafter; Muyzer et al., 1993; Muyzer and de Waal, 1994). Early lesions from a single 1-day-diseased individual, late lesions from a single 3-days-diseased individual, a mixture of early and late lesions from several individuals, and, as a control, a healthy part of the body wall of a single unaffected individual were taken as samples. Total DNA was extracted from the samples with a DNeasy (Qiagen) extraction kit. A 16S rDNA gene fragment was specifically amplified by PCR using the bacterial-specific primers GM5F-GC clamp and DS907R (Muyzer et al., 1993; Teske et al., 1996). These primers

amplify a 550 bp fragment of the 16S rDNA suitable for subsequent DGGE analysis and sequencing (Table 1).

Touchdown-PCR amplifications were performed with the kit Ready-To-Go PCR Beads (Amersham Pharmacia) in a Thermal iCycler (Bio-Rad). After a denaturation step of 4 min at 95 °C, the annealing temperature was decreased from 65 to 55 °C within 20 cycles. The cycles consisted of a 30 s denaturing step at 95 °C, a 30 s annealing step (from 65 to 55 °C) and a 30 s elongation step at 72 °C. After reaching the temperature of 55 °C, 10 additional cycles were performed under identical conditions (annealing temperature of 55 °C). The amplification results were checked on a 1% agarose gel stained with ethidium bromide (0.5 mg l^{-1}).

DGGE were performed with a Bio-Rad Protean II system and 8% (w/v) polyacrylamide gels in a 0.5 × TAE buffer (20 mM Tris–acetate [pH 7.4], 10 mM acetate, 0.5 mM disodium EDTA) with a denaturing gradient ranging from 25% to 75% of denaturant (100% corresponds to 7 M urea and 40% [v/v] formamide). The gradient was performed using a gradient-maker (Bio-Rad) and a Gilson Minipuls II pump. Electrophoresis was performed for 16h at a constant 75 V and a temperature of 60 °C (Muyzer et al., 1993). After electrophoresis, the gels were incubated for 30 min in MilliQ water containing ethidium bromide (0.5 mg l^{-1}), photographed and analysed with the Gel Doc System 1000/2000 of Bio Rad. The number of bands per lane was determined with the Quantity One 4.1 program. This program allows the user to select a level of sensitivity (based on the optical density) to determine the number of bands per lane and to compare the lanes with more objectivity. Bands taken into account were those for which the corresponding pike has an optical density of at least 10 units more than the average count of the whole lane.

DGGE bands were excised from the gels for sequencing and identification. The acrylamide with the DNA was crushed in Ependorff tubes containing 300 μl of Tris–EDTA. After a night at 4 °C, the tubes were centrifuged and the DNA, present in the supernatant, was precipitated with ethanol. DNA obtained after precipitation was used for a new touchdown-PCR amplification using the same primers as previously. After a denaturation step of 2 min at 94 °C, the annealing temperature was decreased from 70 to 60 °C within 10 cycles. The cycles consisted of a 30 s denaturation step at 94°C, a 40 s annealing step (from 70 to 60 °C) and a 30 s elongation step at 72 °C. After the annealing temperature reached 60 °C, an additional 15 cycles were performed under identical conditions (annealing temperature of 60 °C). The amplified products were then purified with a QIAQuick Purification kit (Qiagen) and ready to be sequenced. Sequences were obtained with the dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) in an automated sequencer 377 (ABI). The cycle

Table 1
Sequences of the primers used for PCR amplification of the 16S rDNA fragment

Primers	Positions	Sequences
DS907R	907–927	5′ -ccgtcaattccttragttt-3′
GM5F	341–357	5′ -cctacgggaggcagcag-3
GC-clamp		5′ -cgcccgccgcgcgcggcgggcggggcgggggcacggggggg-3′
GM5F-GC clamp		5′ -cctacgggaggcagcagcgcccgccgcgcgcggcgggcggggcgggggcacggggggg-3′

sequencing reaction consisted of 25 cycles with a 30 s denaturation step at 95 °C, a 15 s annealing step at 50 °C and a 4 min elongation step at 60 °C. The primers used were the same as previously. The sequences determined for this study have been deposited at the EMBL database under accession numbers AJ609636 (alpha-Proteobacterium), AJ609637 (Bacteroides sp.) and AJ609638 (Vibrio sp.).

The sequences obtained were checked against the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST) in order to find related species (Altschul et al., 1990). The 16S rDNA of 44 related species (Table 2) were taken from the GenBank database www.ncbi.nlm.nih.gov/GenBank) and aligned with the sequences obtained in this study using ClustalX (Thompson et al., 1994). Phylogenetic analyses were performed with Paup* (Swofford, 1998). Maximum parsimony analysis (MP hereafter) was used with the tree-bisection reconnection (TBR) heuristic algorithm and neighbour-joining analysis (NJ hereafter) with the Jukes and Cantor distance (Jukes and Cantor, 1969). Reliability of various inferred phylogenetic nodes were estimated by bootstrapping (1000 replicates) (Felsenstein, 1985).

2.3. Bacterial cultures and infection assays

Lesions of a few micrometers in diameter were isolated from diseased juveniles and cultivated on Plate Count Agar (PCA) media (DIFCO Laboratories) with 30‰ NaCl. Bacterial population were stained with a Gram coloration or fixed in non-acetic Bouin's fluid for SEM observations or in 100% ethanol for sequencing. Four experiments were performed in order to better understand the transmission of the

Table 2
List of the 44 related species (and their accession number), taken from the GenBank database, with 16S rDNA sequences close to the ones of the bacteria found in the lesions of *Holothuria scabra*

Names	Accession no	Names	Accession no	Names	Accession no
Bacteroides		*F. litoralis*	M58784	*R. euryhalium*	D16426
B. caccae	X83951	*F. polymorpus*	M58786	*R. strictum*	D16419
B. fragilis	X83943	*F. roseolus*	M58787	*R. sulfidophilum*	D16422
B. merdae	X83854	*F. ruber*	M58788	*Roseobacter*	
B. uniformis	L16486	*F. sanci*	M58795	*R. gallaeciensis*	Y13244
Chlorobium		*F. tractuosus*	M58789	*R. litoralis*	X78312
C. ferrooxidants	Y18253	*Jannaschia*		*Spirochaeta*	
C. limicola	Y10640	*J. helgolandensis*	AJ534224	*S. smaragdinae*	U80597
C. vibrioforme	M62791	*Marinilabilia*		*Tenacibaculum*	
Cytophaga		*M. salmonicolor*	D12672	*T. maritimum*	D14023
C. fermentants	M58766	*Planctomyces*		*Vibrio*	
Flavobacterium		*P. brasiliensis*	X85247	*V. alginolyticus*	X56576
F. aquatile	M62797	*P. limnophilus*	X62911	*V. campbellii*	X74692
F. ferrugineum	M62798	*P. maris*	X62910	*V. harveyi*	X74693
F. johnsoniae	M33886	*Rhodobacter*		*V. natriegens*	X74714
Flexibacter		*R. blasticus*	D16429	*V. parahaemolyticus*	X74721
F. canadensis	M62793	*R. capsulatus*	D16428	*V. proteolyticus*	X74723
F. echinicida	AY006470	*R. sphaeroides*	D16424	*Zobellia*	
F. elegans	M58782	*R. veldkampii*	D16421	*Z. uliginosa*	M62799
F. flexilis	AB078050	*Rhodovulum*			

disease. In each experiment, 5 to 10 healthy juveniles of *H. scabra* were used as treated groups and placed in 2 l aerated aquaria filled with 0.2 μm filtered sea water. The treatments consisted in placing the holothuroids of the groups (1) with five sick juveniles or (2) in a suspension of bacteria diluted in filtered sea water (concentration of bacteria: 90×10^6 ml^{-1}); in painting the integument of juveniles (3) with lesions freshly extracted from diseased individuals or (4) with a fragment of a bacterial culture. Treatments 2, 3 and 4 were performed with holothuroids slightly wounded with a razor blade. Juveniles of the treated groups were compared during 12 days to controls that consisted in healthy holothuroids placed in aquaria filled with 0.2 μm filtered sea water.

3. Results

3.1. Morphological observations

The skin ulceration disease, that affects the body wall of juvenile *H. scabra*, appeared for the first time in September 2000 and then in February 2001, May 2001, February 2002 and July 2002. The infection extended very quickly in the aquaria, being highly contagious: in February 2001, the skin ulceration disease reached a maximum infestation frequency by affecting two thirds of the juveniles 2 days after its appearance. The disease is also highly virulent causing death 3 days after detection of the first symptoms. Most of the time, diseased holothuroids were found lying on the sand while those that were completely buried were not infected.

The body wall of healthy juvenile holothuroids is ca. 800 μm thick and consists in an epidermis with cuticle, a sub-epidermal connective tissue layer, a layer of circular musculature and a mesothelium (Fig. 1E). Five pairs of longitudinal muscles are also observed on a transverse section of holothuroids (Fig. 1E,F). The first obvious signs of the disease was the appearance of little, white, rounded spots of ca. 1 mm in diameter corresponding to areas where the epidermis was destroyed (Fig. 1A). The white colour of the lesions is due to the exposure of the connective tissue that follows the destruction of the cuticle, the epidermis and the upper part of the connective tissue. Yet, the mesothelium, the muscles and the internal organs remained unaffected (Fig. 1F). First lesions always appeared close to the cloacal opening and this was quickly followed by the outbreak of other lesions similar in size, shape and colour in the posterior half of individuals. The rest of the body remained healthy and the behaviour of diseased and healthy holothuroids was unchanged except that the diseased holothuroids do not seemed to burrow anymore. Twenty-four hours later, lesions spread and joined together to form a large, posterior lesion which covered about a fifth of the body surface (Fig. 1B). Podia inside this lesion were totally destroyed (Fig. 1B). Other spots then appeared on the anterior part of the body and ossicles became visible externally, some of them separating from the integument. Forty-eight hours later, more than a half of the body surface was affected (Fig. 1C). Juveniles were much less active and became almost translucent, their internal organs being seen through the body wall. The latter was reduced to a thin layer of no more than 200 μm thick (Fig. 1F) and could be easily disrupted with forceps. Three days after the beginning of the disease, the whole body surface was

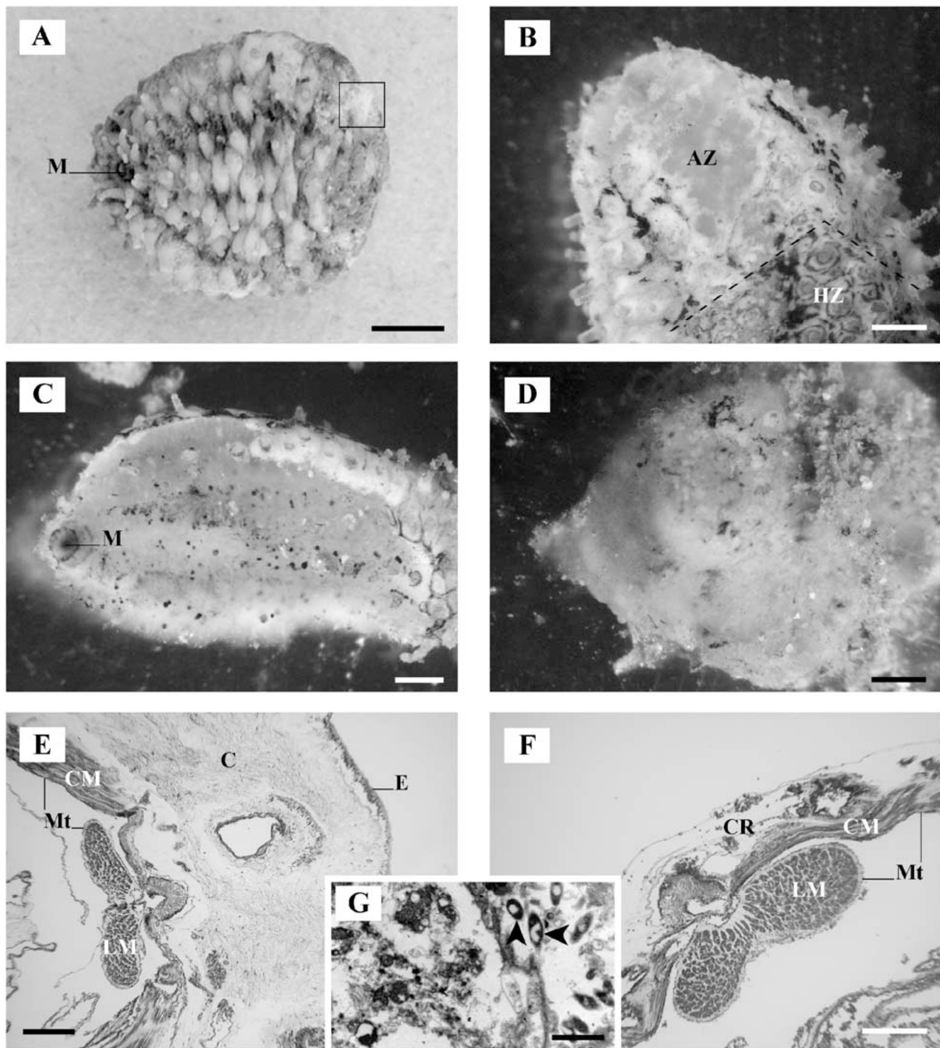

Fig. 1. Juveniles of *Holothuria scabra* affected by the skin ulceration disease. (A) Juvenile at the beginning of the disease when a white spot (surrounded) appear near the cloacal opening. (B) A large white lesion covers the posterior half of the dorsal part of the juvenile. (C) More than a half of the integument is affected. (D) The whole body surface is destroyed. (E) and (F) Cross sections of a 2 days-diseased juvenile showing an unaffected part (E) and an affected part (F) of the body wall. (G) TEM section of a lesion showing vacuoled cytoplasmic remnants and bacteria (arrowheads). Scale bars = 3.5 mm for A-D, 150 μm for E-F and 3 μm for G. AZ: affected zone, C: connective tissue, CM: circular muscles, CR: connective remnants, E: epidermis, HZ: healthy zone, LM: longitudinal muscles, M: mouth, Mt: mesothelium.

affected (Fig. 1D). Dead individuals were reduced to pellets of tissues heavily infested by microorganisms (Fig. 1D).

As the epidermis is rapidly destroyed during the infection, only the connective tissue is visible on TEM sections of the lesions. No intact connective cells have been observed but

vacuoled cytoplasmic remnants and disorganised collagen fibres. Between them, lie bacteria of 2 μm long that show an electron clear central part surrounded by an electron dense periphery (Fig. 1G). No signs of a viral infection have been detected within the affected tissues.

With the SEM, two different zones of the body surface may be observed in the lesions of diseased holothuroids: (1) the affected zone, colonised by microorganisms, where the epidermis and cuticle was totally destroyed and where a disorganised connective tissue was exposed to the medium (Fig. 2C,D) and (2) a border line of a

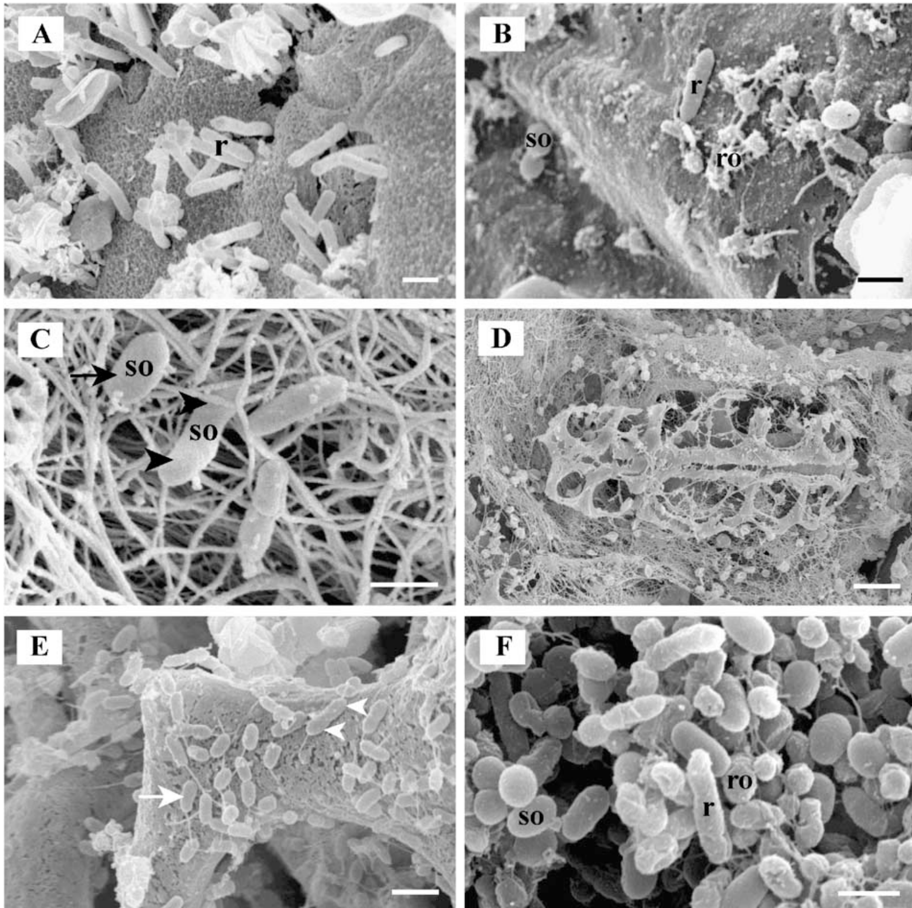

Fig. 2. Bacteria from the lesions of juveniles of *Holothuria scabra* (SEM observations). (A) Rod-shaped bacteria (r) in the border line. (B) The three bacterial morphotypes, observed in the border line: smooth ovoid bacteria (so), rod-shaped bacteria (r) and rough ovoid bacteria (ro). (C) Smooth ovoid bacteria (so), single (arrow) or in division (arrowhead), lying on collagen fibres in the affected zone. (D) Degraded ossicle in the affected zone. (E) Smooth ovoid bacteria, single (arrow) or in division (arrowhead), on an ossicle. (F) Bacterial culture showing smooth ovoid (so), rod-shaped (r) and rough ovoid (ro) bacteria. Scale bars = 10 μm for (D), 2 μm for (E) and 1 μm for other pictures.

few tens of micrometers wide where the surface was colonised by microorganisms and where patches of degrading epidermis were mixed with degrading, exposed connective tissue (Figs 2A,B). In the affected zone, collagen fibres ran in all directions, breaking off from each other (Fig. 2C) and ossicles, some of them being highly degraded, were exposed to the external medium (Fig. 2D). Three bacterial morphotypes were observed in these two zones: rod-shaped bacteria, rough ovoid bacteria and smooth ovoid bacteria. Rod-shaped bacteria (Fig. 2A,B), of 2 to 3 μm long, were abundant in the border line where they usually lay in groups of more than five individuals. They were never observed at the surface of the healthy integument nor on ossicles or on collagen fibres. Rough ovoid bacteria (Fig. 2B), of 300 to 800 nm in diameter, were occasionally found on the healthy part of the integument. They were, however, much more numerous in the border line and in the affected zone. Beside their small size, they were also characterised by their rough surface and by the fact that they lay within bacterial filaments (Fig. 2B). Smooth ovoid bacteria (Fig. 2B,C,E), of 1 to 2 μm long, were occasional on the healthy integument but were frequently observed in the border line and in the affected zone. The surface of these bacteria was smooth. Some of them divided in two, giving them a twisted rod shaped aspect (Fig. 2C). In the affected zone, they lay on collagen fibres (Fig. 2C) but also on ossicles where they were the only bacteria that have been observed (Fig. 2E). Degradation of the ossicles inside the lesions was very dramatic (Fig 2D). It led us to isolate them from affected tissues in order to compare them to intact ossicles and to follow the course of

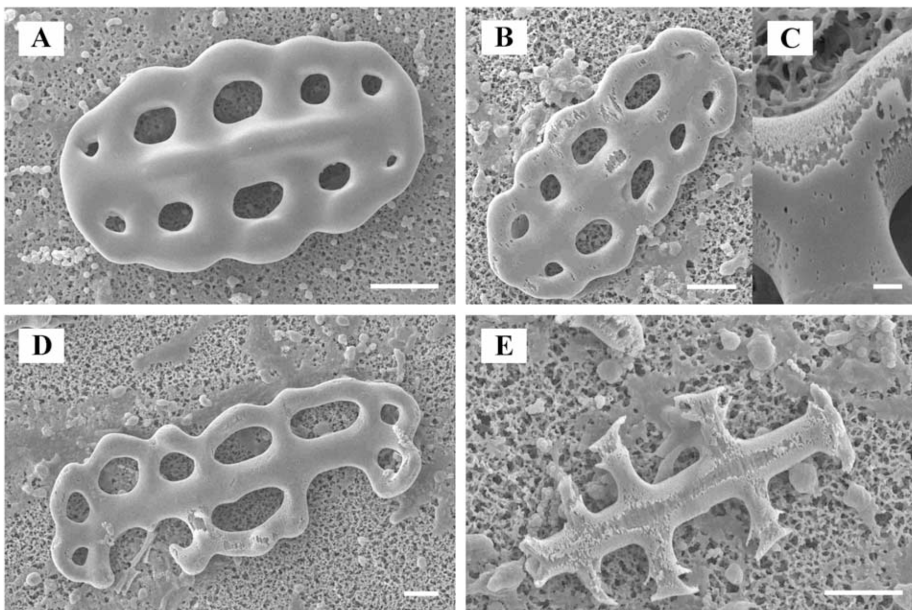

Fig. 3. Button ossicles isolated from the integument of healthy juveniles of *Holothuria scabra* (A) and from lesions of diseased juveniles (B to E). Figs. B, D and E show a succession of ossicles more and more degraded. Fig. C is a high magnification of a zone of degradation (SEM observations). Scale bars = 10 μm except for (C) = 1 μm.

their destruction. The surface of healthy ossicles was sleek (Fig. 3A) while the one of recently attacked ossicles contained holes of a few tens of micrometers long (Fig. 3B,C). The first sign of the degradation was the appearance of these holes mainly at the periphery of the ossicles (Fig. 3B,C). The peripheral trabeculae were often initially eroded (Fig. 3D) giving rise to ossicles with a central trabeculae and small lateral projections (Fig. 3E) before disappearing totally.

3.2. Observations of denaturing gradient gels and sequencing

Fig. 4 illustrates a gel obtained after electrophoresis of the four samples studied (A: mixture of early and late lesions, B: early lesion, C: late lesion, D: part of the integument of a healthy juvenile). Two other replicate gels were made and their band pattern was similar except for small intensity differences. At the level of detection chosen (see Materials and methods), three bands (bands 1, 2 and 3) were revealed in lane A. These three bands were also present in lane C which showed an additional band below band 3 (named band 5). No band was detected in lane B; however a few areas darker than the background (including areas at the level of bands 1 and 2) were very faint. Two bands

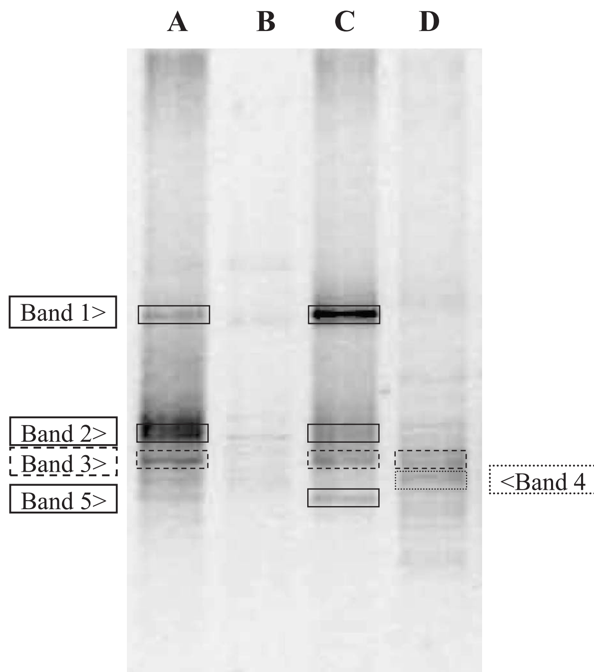

Fig. 4. Gel profile obtained after DGGE of the 16S rDNA fragments of bacteria observed in diseased and healthy juveniles of *Holothuria scabra*. Lane A: mixture of early and late lesions; lane B: early lesion; lane C: late lesion; lane D: healthy part of the body wall of the juveniles. Continuous line: bands only observed in lesions samples; dashed line: bands observed in lesions and in healthy integument samples; dotted line: bands only observed in healthy integument samples.

(bands 3 and 4) occurred in lane D. A total of five bands were thus detected on the gels: three of them were specific to the lesions (bands 1, 2 and 5), one (band 3) was present in the lesions and in or on the healthy integument, and one (band 4) was specific to the healthy integument. From the sequencing, only bands 1 and 5 have been identified: a fragment of 523 and 239 bases was obtained for band 1 and band 5, respectively. The sequences from the other bands were not reliable because the fragments obtained were too

Table 3
Results of a BLAST analysis on the three bacterial sequences obtained after sequencing their 16S rDNA gene

Bacterium	Nearest phylogenetic relative, description and accession no.	% Similarity
Phylotype 1	(1) Unidentified bacterium from marine sediment [AJ011657]	91
	(2) Uncultured bacterium from environmental growth chamber [AY172249]	88
	(3) *Cytophaga fermentans* [M58766]	87
	(4) Uncultured *Cytophaga* sp. in permanently cold marine sediments [AJ240979]	87
	(5) Uncultured bacterium [AJ535255]	87
	(6) Uncultured Bacteroidetes bacterium associated with tubes of the vent worm *Riftia pachyptila* [AF449260]	90
	(7) Uncultured Bacteroidetes bacterium associated with mucous secretions of the hydrothermal vent polychaete *Paralvinella palmiformis* [AJ441219]	88
	(8) *Cytophaga fermentans* [D12661]	90
	(9) Uncultured bacterium from marine sediment [AY171329]	88
	(10) *Bacteroides* sp. from murine microbiota [AF157056]	87
Phylotype 5	(1) *Roseobacter* sp. [AY136132]	97
	(2) Uncultured α-Proteobacterium from the Weser estuary [AY145603]	97
	(3) *Roseobacter* sp. from Hawaiian hypersaline lake [AF513439]	97
	(4) Uncultured α-Proteobacterium from the consortium associated with black band disease in coral [AF473929]	97
	(5) Id. [AF473928]	97
	(6) Id. [AF473921]	97
	(7) Id. [AF473920]	97
	(8) Uncultured Rhodobacteraceae bacterium from Hawaiian hypersaline lake [AF513935]	97
	(9) Id. [AF513930]	97
	(10) Uncultured bacterium in cultures of marine diatoms [AJ319859]	97
Cultured bacterium	(1) *Vibrio* sp. From a deep-sea hydrothermal vent [AF319769]	99
	(2) Id. [AF319768]	99
	(3) *Vibrio natriegens* [X74714]	99
	(4) *Vibrio harveyi* [X74693]	99
	(5) *Vibrio harveyi* [AF134581]	99
	(6) Luminous *Vibrio* sp. [AF094702]	99
	(7) Unculturable *Vibrio* sp. associated with excessive mortality in larval haddock [AF108137]	99
	(8) *Vibrio* sp. From marine sediment [AF064559]	99
	(9) *Vibrio alginolyticus* [X56576]	99
	(10) Marine *Vibrio* sp. [AF246980]	99

The first ten names sorted by BLAST analysis are shown for each of the three sequences obtained. The numbers in brackets are the accession numbers in GENEBANK. % similarity is the genetic similarity between the GENEBANK candidate and the sequence analysed.

short. Table 3 gives, for each of the two fragments, the names of the 10 species for which the sequences sorted during a BLAST search matched the fragments best. The first names appearing from the list for band 1 were *Cytophaga fermentans*, *Cytophaga* sp., *Bacteroidetes* bacteria and *Bacteroides* sp. indicating that it was a bacterium from the CFB group (Cytophaga–Flavobacterium–Bacteroides). Band 2 was close to a *Roseobacter* sp., an uncultured α-Proteobacteria and a Rhodobacteriaceae indicating that it was an α-Proteobacterium. The unrooted phylogram in Fig. 5 illustrates the results obtained during a NJ analysis made on the data matrix that included the sequences of the two bands and

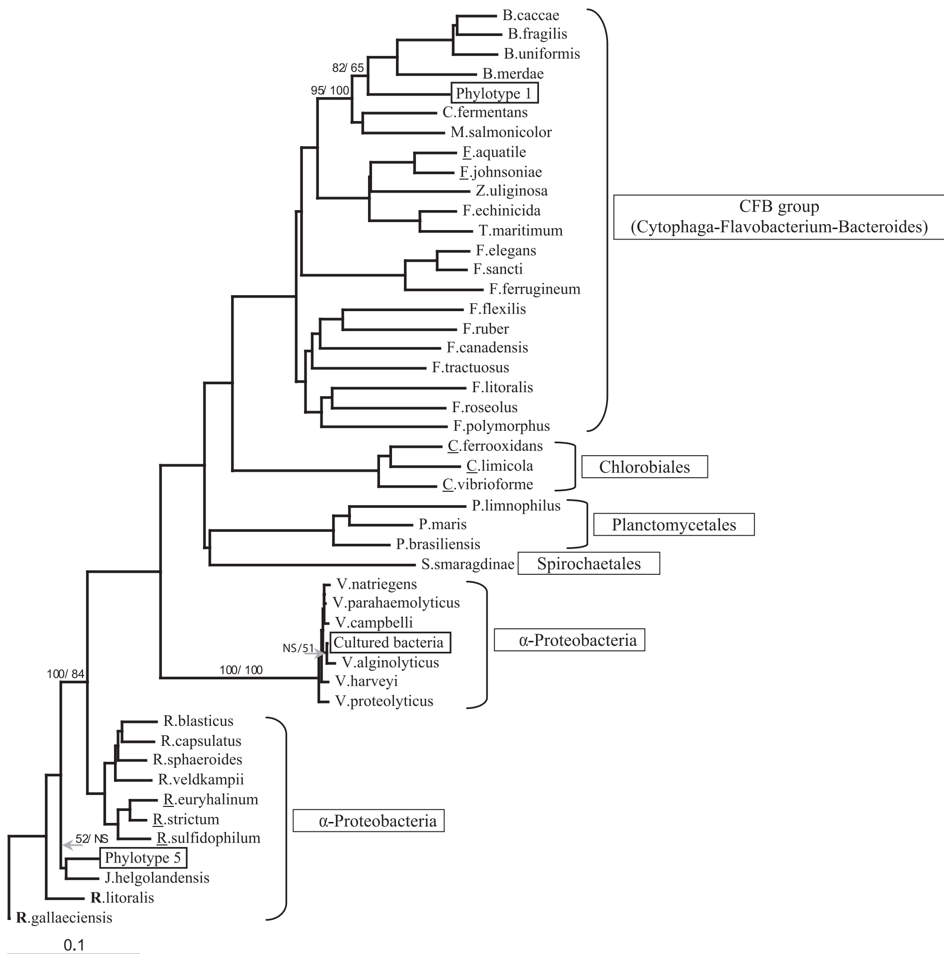

Fig. 5. Unrooted phylogram obtained during a neighbour_joining analysis on 16S rDNA fragments of the cultured bacterium, of the two DGGE phylotypes and of their closest relatives. Bootstrap support (expressed in %) obtained during MP and NJ analyses (1000 replicates) are indicated at the root of the relevant clades (MP/NJ). NS: not supported. The scale bar indicates 0.1 substitutions per nucleotide position. B: Bacteroides, (C̲): Chlorobium, C: Cytophaga, (F̲): Flavobacterium, F: Flexibacter, J: Jannaschia, M: Marinilabilia, P: Planctomyces, R: Rhodobacter, (R̲): Rhodovulum, **R**: Roseobacter, S: Spirochaeta, T: Tenacibaculum, V: Vibrio.

their closest relatives. Phylotype 1 fits at the base of a clade joining all the *Bacteroides* species. The clade and the basal position of phylotype 1 are well supported by both MP and NJ bootstrap values. Phylotype 5 clusters with *Jannaschia helgolandensis* and two *Roseobacter* species, *R. littoralis* and *R. gallaeciensis*. The clade of the four species is well supported by MP and NJ bootstrap values but the position of phylotype 5 within the clade varies. It is best supported when it clusters with *J. helgolandensis*.

3.3. Bacterial cultures and infection assays

Six cultures were obtained from the lesions of six diseased juveniles. All the bacterial populations in these cultures were Gram-negative. SEM analysis revealed that organisms from the cultures resembled the three bacteria already observed in juvenile's lesions: the rod-shaped bacteria of 2 to 3 μm long, the rough ovoid bacteria of 300 to 800 nm long and the smooth ovoid bacteria of 1 to 2 μm long (Fig. 2F). The proportion of these bacteria varied from a culture to another but on average the proportion was 1:60:500 for rod-shaped, smooth ovoid, and rough ovoid bacteria, respectively. One of the six cultures was an almost totally pure culture of rough ovoid bacterium. Each bacterial culture was sampled for sequencing. The six sequences obtained varied in length from 451 to 485 bp. Even though the sample was a mix of three species, all the sequences were those of a *Vibrio* species, in the α-proteobacteria subclass. The first names sorted from a blast search were *Vibrio* sp., *V. natriegens*, *V. harveyi* and *Vibrio alginolyticus* (Table 3). A neighbour-joining analysis clustered the cultured bacterium within the clade grouping all the *Vibrio* species with a high bootstrap support (both with MP and NJ) (Fig. 5). The position of the cultured bacterium within the group was unstable and was best supported when it clustered with *V. alginolyticus*.

None of the infection experiments done induced the skin ulceration disease. Twelve days after the beginning of the assays, all the treated juveniles were healthy except the wounded holothuroids that were painted with a fragment of a bacterial culture. These juveniles were diseased 5 days after the treatment. The disease however did not appear as a skin ulceration. The treated juveniles were much less active than those of the control. They became totally yellow and their podia stayed contracted until death occurred.

4. Discussion

The recent "Advances in Sea Cucumber Aquaculture and Management" (ASCAM) workshop held in China (14–18 October 2003) has shown that the skin ulceration disease or very closely related diseases occur worldwide and on different species: the disease has also been recorded on *Apostichopus japonicus* in China, on *Isostichopus fuscus* in Equator and on *H. scabra* in Australia and in New Caledonia. In all, the skin ulceration disease begins with the appearance of a white lesion. In juvenile *H. scabra* from Madagascar, the white lesion appears near the cloacal orifice and extends over the whole body surface. The disease is highly contagious and expands quickly in

the reared populations. It destroys first the cuticle and the epidermis, then the connective tissue and ossicles. The disease is due to a bacterial infection but the agent that initiates the skin ulcerations has not been identified yet as none of the infection assays performed in this work was successful. This suggests that either bacteria are not the etiological agent of the disease or, if they are, their action must be combined to other factors to induce it properly. In the first case, non-bacterial agents such as viruses or chemicals cannot be excluded. However, the transmission electron microscopy observations did not reveal any viral infection and a chemical does not probably initiate the disease which would have also spread over genitors of *H. scabra* that stand in the same water than juveniles. Combined events or agents, including bacteria, are probably required to induce the disease. The intervention of copepods, for example, that sometimes swarm at the surface of holothuroids, might cause appropriate wounds that would be colonized by pathogen bacteria. Whatever its origin, one agent of the disease is certainly carried by water currents because most buried holothuroids are not infected. Moreover, the appearance of the first lesion close to the cloacal opening is probably a consequence of the breathing behaviour of juveniles that, when they are buried, expose their posterior end to breathe.

The morphological techniques used suggested that at least three microorganisms are implicated in the skin ulceration disease. They are rod-shaped bacteria, rough ovoid bacteria and smooth ovoid bacteria. These morphotypes are all three present on the degrading epidermis and could thus initiate the lesions or be opportunist bacteria. After the destruction of the cuticle and the epidermis, the connective tissue seems to be invaded mainly by the smooth ovoid bacteria. These bacteria are the only organisms that have been observed on ossicles and they are probably responsible of their lysis as they lie at the level of the holes of degraded ossicles. To our knowledge, this is the first time that bacteria are observed degrading ossicles of holothuroids.

The biomolecular techniques identified three species of bacteria in the lesions: *Vibrio* sp., isolated in the cultures, and *Bacteroides* sp. with an α-Proteobacterium detected by the DGGE. *Vibrio* sp., obtained from bacterial cultures, was not put in evidence by DGGE probably because it corresponds to one of the faint bands undetected by the computer program or to the bands 2 and 3 that were not sequenced successfully. *Vibrio* sp. is close to *V. harveyi* and *V. alginolyticus*, two well-known pathogenic bacteria. *V. harveyi* is responsible of numerous infections to both vertebrates and invertebrates. It causes enteritis (Soffientino et al., 1999), ocular infections (Kraxberger-Beatty and MacGarey, 1990), exophthalmia (Alvarez et al., 1998; Iwamoto et al., 1995), melanosis and haemorrhages (Alvarez et al., 1998; Iwamoto et al., 1995) in fishes. The brown shark, *Carcharhinus plumbeus*, held in captivity, suffered of meningitis, encephalitis and vascular infection of its internal organs caused by *V. harveyi* (Grimes et al., 1984). *V. harveyi* also affects adults and larvae of several cultured crustacean species including lobsters (Diggles et al., 2000) and penaids shrimps (Robertson et al., 1998; Liu et al., 1996; Alvarez et al., 1998). Principally the hepatopancreas and the body surface which are attacked. *Vibrio alginolyticus* shows a high pathogenecity towards vertebrates, as to the sea bream, *Sparus aurata* (Balebona et al., 1998), and to invertebrates. Shrimps are again the most studied victims of this *Vibrio* that has been isolated from the hæmolymph of *Penaeus*

monodon (Lee et al., 1996a), and from the hepathopancreas of *Penaeus japonicus* (Lee et al., 1996b) and *Litopenaeus vannamei* (Esteve and Herrera, 2000). *V. alginolyticus* was identified as the etiological agent of a disease affecting two spatangoid sea urchins, *Paleopneustes cristatus* and *Archaeopneustes hystrix* (Bauer and Young, 2000). The bacterium provoked epidermal lesions, initiated by small mechanical damages, that would provide a way for infectious organisms which would enter the coelom and provoke death (Bauer and Young, 2000). Vibriosis is one of the major threats in fish and shellfish marine aquaculture. It is very tempting to identify *Vibrio* sp. as the etiological agent but the infection assays have not established a direct link between this *Vibrio* and the disease.

This is only the second time that DGGE has been performed on bacteria infecting diseased marine invertebrates. This molecular tool was previously used by Cooney et al. (2002) on the bacterial consortium of the black band disease affecting corals. DGGE analysis performed on diseased juvenile *H. scabra* revealed that three phylotypes (1, 2 and 5; 1 and 5 were identified) were exclusively present in lesions. Phylotype 1 forms the most intense band of the DGGE analysis and also appears faintly in the samples of the early lesions. Sequencing and phylogenetic analyses of the 16S rDNA show that it is a member of the CFB group (100% bootstrap value) closer to the *Bacteroides* genus than to the *Cytophaga* and the *Flavobacterium* genera. *Bacteroides* species are Gram-negative bacteria that live in the colon and faeces of several mammals including man (Moore and Holdeman, 1974). While the *Bacteroides* occupy a significant position in the normal intestinal flora, they are also opportunistic pathogens, primarily in infections of the peritoneal cavity. *Bacteroides fragilis* is the most notable pathogen: although it makes up only 12% of the normal intestinal flora, it is the *Bacteroides* species isolated from 81% of anaerobic clinical infections (Werner, 1974). *Bacteroides* species are however not known to induce diseases in non-mammalian animals and the *Bacteroides* sp. identified here probably derives from local contamination by sewage.

Phylotypes 2 and 5 form less intense bands on gels than phylotype 1. They appear only in late lesions, the corresponding bacteria are probably therefore secondary invaders rather than etiological agents. Only, the sequence of phylotype 5 has been obtained. In the phylogenetic analyses, it clusters with several species of the *Roseobacter* genus in a highly supported clade. Representatives of this genus are able to transform organic and inorganic sulfur compounds (Gonzalez et al., 1999; Zubkov et al., 2001) or to degrade aromatic compounds (Buchan et al., 2000). Others are associated with algae: *Roseobacter algicola* has been isolated from a culture of the toxin-producing dinoflagellate *Prorocentrum lima* (Lafay et al., 1995) and several species of the marine red algal genus *Prionitis* bear bacterial galls due to a *Roseobacter* parasite (Ashen and Goff, 2000). *Roseobacter* species have also been found in the accessory nidamental glands of the Sepioid *Sepia officinalis*, together with other α-Proteobacteria and Gram-positive bacteria but their symbiotic role has not been established yet (Grigioni et al., 2000). One species of *Roseobacter* was found to be a true pathogen and has been described as being the etiological agent of a juvenile oyster disease affecting *Crassostrea virginica* in a US hatchery (Boetttcher et al., 2000). The species was isolated from affected individuals and could induce the disease

to healthy oyster after experimental infection (Boettcher et al., 2000). At the opposite, a *Roseobacter* showed an inhibitor effect to *Vibrio* species on agar plate and extracts of this bacterium has a probiotic effect on scallop larvae, enhancing their survival (Ruiz-Ponte et al., 1999). Phylotype 5 clusters best with *J. helgolandensis*, a new species belonging to the Roseobacter–Sulfitobacter–Silicibacter group within the α-subclass of the Proteobacteria. It is a Gram-negative, strictly aerobic and heterotrophic bacterium isolated from sea water samples of the North Sea (Wagner-Döbler et al., 2003).

Acknowledgements

We thank Drs. E. Mara and M.W. Rabenevanana of the IH.SM of Toliara for providing facilities and Dr. M. Milinkovitch for opening the doors of his Evolutionary Genetics Laboratory to make sequencing. This work was supported by a FRFC grant and a "Projet Interuniversitaire Ciblé" (PIC) financed by the Belgian Coopération Universitaire au Développement. Contribution of the "Centre Interuniversitaire de Biologie Marine" (CIBIM).

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