

Microbiological study of the body wall lesions of the echinoid *Tripneustes gratilla*

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ABSTRACT: The microbiota of the body wall lesions of the echinoid *Tripneustes gratilla*, initiated by the grazing action of the parasitic gastropod *Vexilla vexillum*, was investigated with a pluridisciplinary approach. Parasitised sea urchins showed body wall lesions strongly infected by bacteria that progressed through the test and reached the coelomic cavity after ca. 1 mo. We report here on the bacterial community observed in lesions of echinoids collected *in situ* and on the bacteria that successively appeared during laboratory experiments. Two *Alphaproteobacteria*, a CFB (*Cytophaga-Flavobacterium-Bacteroides*) bacterium, 3 *Vibrio* species and *Exiguobacterium aestuarii* were identified in the field-collected lesions by 16S rDNA sequencing. The last 4 bacteria were cultured and each induced the disease when inoculated on scalpel-made wounds, with 100% of the individuals infected within 2 d. Scalpel-induced scarifications tended to heal within 3 wk, while gastropod-induced lesions evolved into disease, suggesting a role of *Vexilla vexillum* in the development of the infection. Denaturing gradient gel electrophoresis (DGGE) and sequencing suggest that (1) bacteria associated with healthy integument were not present in the lesions and were thus not responsible for their infection, (2) *Alphaproteobacteria* with close phylogenetic affiliation with other bacteria involved in several diseases affecting marine invertebrates were present, and (3) these *Alphaproteobacteria* were present from the beginning of the infection and appeared earlier in the infection than other bacteria such as CFB bacteria.

KEY WORDS: Echinoderm · DGGE · Bacteria · Infection · Lesion · Gastropod

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INTRODUCTION

Body wall lesions consisting of infected areas of test devoid of spines, tube feet and pedicellariae are recurrently observed in wild echinoid populations. Such lesions, described by Maes & Jangoux (1984) as the 'bald sea urchin disease', were noted for the first time in California in 1970 (Johnson 1971) and have been recorded since 1978 in the Mediterranean Sea (Höbaus et al. 1981). A similar disease, the spotting disease, was also observed in Japanese echinoid hatcheries during summer (Tajima et al. 1997). Although bacteria are thought to be responsible for these infections (Gilles & Pearse 1986, Tajima et al. 1997), it is also believed that a preliminary abrasion of the integument is required (Jangoux & Maes 1987). In the field, abrasions are usually mechanical, but can be

induced by organisms such as grazing gastropods. Echinoids are indeed hosts for many parasitic marine gastropods that act either as ecto- or endoparasites (Jangoux 1990). Most ectoparasites live attached to the echinoid's body surface by their proboscis, but some species move freely and browse the host's epidermis (Jangoux 1990). Among the latter, Kay (1979) described 2 muricid species, *Vexilla lineata* and *V. vexillum*, as echinoid epidermal grazers in Hawaii.

A recent survey on the southwest coast of Madagascar (off Toliara) revealed the presence of the parasitic gastropod *Vexilla vexillum* on 2 echinoid species, *Tripneustes gratilla* and *Echinometra mathaei* (Vaïtilingon et al. 2004). The gastropod is always found associated with these echinoids and is never observed free-living. Prevalence of the infestation reaches 25% for *T. gratilla* and 5% for *E. mathaei*. In both species, the

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gastropod grazes the body surface, which progressively turns black as the infection by microorganisms develops. Infections then progress through the test, often causing fatal perforations (Väitilingon et al. 2004). The present study examines this infection that leads to the death of *T. gratilla*. Microscopic, microbiological and molecular techniques were used to characterise and identify the microbial community associated with the lesions. The role of the gastropod in the infection is also investigated. This is the first report of a bacterial infection affecting sea urchins in the Indian Ocean.

MATERIALS AND METHODS

Sampling. *Tripneustes gratilla* (Linnaeus, 1758) and *Vexilla vexillum* (Gmelin, 1791) individuals were collected by hand at low tide on the barrier reef off Toliara, Madagascar, from January to February 2004 and in February 2005. The collection site, Ambatobe (23°25' 00" S, 43° 39' 23" E), was located on the boulder tract of the seaward slope of the reef flat. Eight lesions (named 'field-collected lesions' hereafter) were sampled on the reef, of which 4 were fixed in non-acetic Bouin's fluid for scanning electron microscopy (SEM) and 4 in absolute ethanol for molecular analyses. Other specimens were brought to the laboratory and left in aquaria with running seawater at 28°C (for aquarium system, see 'toboggan structures' in Grosjean et al. 1998).

Laboratory experiments. The course of the lesions caused by *Vexilla vexillum* was followed in the laboratory. Echinoids and gastropods were maintained separately over 3 d in aquaria before the experiments. Then, 70 *Tripneustes gratilla* (mean test diameter 53 ± 3 mm) and 70 *V. vexillum* (mean shell length 9 ± 1 mm) were chosen, and one gastropod was allocated to each echinoid to produce lesions. Each host–parasite pair was placed in a cylindrical plastic tube 20 cm long \times 10 cm in diameter. The 70 tubes were closed by sieves, tagged individually and placed in aquaria provided with running seawater. After 3 d, gastropods were removed from sea urchins. The latter were fed regularly and observed every day for 4 wk. Samplings including 5 diseased echinoids were made at Days 0 (time of gastropod removal), 2, 5, 10 and 28: 2 individuals were fixed in non-acetic Bouin's fluid for SEM and the 3 others were immersed in absolute ethanol for molecular analysis.

In order to estimate the role of *Vexilla vexillum* in the bacterial infection, lesions caused by gastropods were compared to lesions performed with scalpels. Forty-five *Tripneustes gratilla* (mean test diameter 54 ± 2 mm) were mechanically abraded with a sterile

scalpel to remove the epidermis, spines, tube feet and pedicellariae from an area of 29 ± 9 mm² (i.e. area corresponding to the surface of lesions caused by *V. vexillum*). Out of these 45 echinoids, 15 were chosen, from which 3 lesions were fixed in absolute ethanol for molecular analysis at Days 0, 2, 5, 10 and 20.

Microbiological analyses. A total of 10 infected and 5 healthy sea urchins were collected on the reef and brought to the laboratory in separate containers. Immediately upon arrival, lesions from infected individuals and random areas from healthy individuals were swabbed with a sterile cotton swab, and the swab applied to Petri dishes containing Nutrient Agar (Merck) with 3.5% NaCl. After 72 h growth at 30°C (i.e. equivalent to reef seawater temperature), bacterial colonies were characterised by the shape, colour, margin and surface texture. Each colony was then sub-cultured twice in order to obtain pure cultures. Strains were characterised by Gram staining (Madigan & Martinko 2006), fixed in absolute ethanol for sequencing (see 'Molecular analyses' below) and spread on cover glasses before being fixed in non-acetic Bouin's fluid for SEM.

Aliquots of 10^8 bacteria of each pure culture were inoculated on abraded areas of 5 healthy echinoids. Other abraded zones of healthy echinoids were inoculated with pieces of necrotic tissue taken from field-collected lesions using sterile cotton swabs. Echinoids from both groups were fed regularly, and their lesions were observed for 4 wk. Individuals of these groups were compared to a control that consisted of non-inoculated abraded sea urchins.

Scanning electron microscopy. After fixation in non-acetic Bouin's fluid, sampled lesions and bacterial cultures were dehydrated through a graded series of ethanol (50, 70, 90 and 100%), critical-point dried, mounted on stubs, coated with gold and examined with a Jeol JSM-6100 scanning electron microscope. To evaluate the abundance of the various bacterial morphotypes, photographs of 10 different areas of each lesion (4 field-collected lesions and 2 laboratory-induced lesions for each sampling day) were taken at 1000 \times magnification and the bacteria were counted.

Molecular analyses. The number of phylotypes (i.e. nucleotide sequences obtained from an environmental sample and having phylogenetic affiliations with sequences from known species; Muyzer & de Waal 1994) occurring in samples was determined by DGGE. Samples were (1) lesions of echinoids collected in the field, (2) integument of healthy echinoids, (3) gastropod-induced lesions in laboratory experiments and (4) scalpel-induced lesions. Samples were treated in the same way during all the procedures. Using a sterile scapel, 5 to 10 mg of tissue was removed from each sample, placed in a 1.5 ml microcentrifuge tube and

total DNA was extracted with a DNeasy (Qiagen) extraction kit following the manufacturer's instructions.

A 16S rRNA gene fragment was specifically amplified by PCR using 1 µl of extraction products as template DNA and the bacterial-specific primers GM5F-GC clamp and DS907R (Teske et al. 1996). Touchdown-PCR amplifications were performed with the kit Ready-To-Go PCR Beads (Amersham Pharmacia) in a Thermal iCycler (Bio-Rad). After an initial denaturation step of 4 min at 95°C, the annealing temperature was decreased from 65 to 54°C within 22 cycles (-0.5°C every cycle). The cycles consisted of a 30 s denaturing step at 95°C, a 30 s annealing step (from 65 to 54°C) and a 30 s elongation step at 72°C. After reaching the temperature of 54°C, 13 additional cycles were performed with an annealing temperature of 54°C. The amplification results were checked on a 1% agarose gel stained with ethidium bromide (0.5 mg l⁻¹) in order to verify that DNA samples produced approximately the same quantity of PCR products.

DGGE were performed with a Bio-Rad Protean II system using 15 µl of PCR products applied directly onto 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% corresponded to 7 M urea and 40% [v/v] formamide). The gradient was performed using a gradient-maker (Bio-Rad) and a Masterflex peristaltic pump (Cole-Parmer). Electrophoresis were performed for 16 h at a constant 75 V and a temperature of 60°C. After electrophoresis, the gels were incubated for 30 min in 0.5× TAE buffer containing ethidium bromide (0.5 mg l⁻¹), photographed and analysed with the Gel Doc System 1000/2000 (Bio-Rad). The number of bands per lane was determined with the Quantity One 4.1 program and checked manually.

DGGE bands were excised from the gels for sequencing and identification. The acrylamide with the DNA was crushed in 1.5 ml microcentrifuge tubes containing 300 µl of Tris-EDTA. After 1 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 and the same protocol as above. The mobility of the re-amplified bands was not checked. The amplified products were then purified with a QIAQuick Purification kit (Qiagen). Sequences were obtained with the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI) in a Prism 3100 Genetic Analyser (ABI). The cycle-sequencing reaction consisted of 25 cycles with a 10 s denaturing step at 96°C, a 5 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 AM495239 to AM495253.

The sequences obtained were checked against the GenBank database (www.ncbi.nlm.nih.gov/GenBank), using the BLAST tool, in order to find related species (Altschul et al. 1990). The 16S rDNA of these related species were then aligned with the sequences obtained in this study using ClustalX (Thompson et al. 1994). Maximum-parsimony (MP) analyses were performed with PAUP (Swofford 1998) with the tree bisection reconnection (TBR) swapping heuristic algorithm. The reliability of the inferred phylogenetic nodes was estimated by bootstrapping (10³ replicates) (Felsenstein 1985). A maximum-likelihood (ML) analysis was performed with the likelihood model selected by Modeltest v3.6 (Posada & Crandall 1998). The TrN model (Tamura & Nei 1993), with rate heterogeneity (TrN + G), was identified to best fit the observed data.

RESULTS

Morphological observations

Field-collected lesions appeared pale green to black and were devoid of epidermis and appendages, thus exposing the test to the water medium. With SEM, the test appeared damaged and infected by microorganisms. Slightly affected areas of the stereom showed small cracks and holes on the trabeculae. Cocci (1 µm in diameter) and filamentous bacteria (20 µm in length and 0.4 µm in width) were the most abundant microorganisms in these zones and stood in the stroma (Fig. 1A,B). Highly degraded parts of the test were devoid of stroma, and trabeculae of the stereom were strongly eroded, some of them having almost disappeared (Fig. 1C). The most frequent bacterium in these areas was the 1 to 2 µm long rod (Fig. 1C) that stood on the stereom. The presence of some ciliate protozoans was also noted.

In the laboratory, from the 70 *Tripneustes gratilla* put in contact with *Vexilla vexillum*, 39 displayed a lesion of 22 ± 16 mm² in surface. Lesions appeared initially reddish and were surrounded by a black line of necrotic tissue (Fig. 2A). The test was stripped of epidermis, and the appendages were eliminated from the grazed zone (Fig. 2A). The internal side of the integument (i.e. delimited by the mesothelium) also showed a red colouration on the reverse side of the lesion (Fig. 2B). Two days after, lesions took on a light-green colour, with some pink-red patches, while, after 10 d, lesions appeared dark green, almost black, and the red

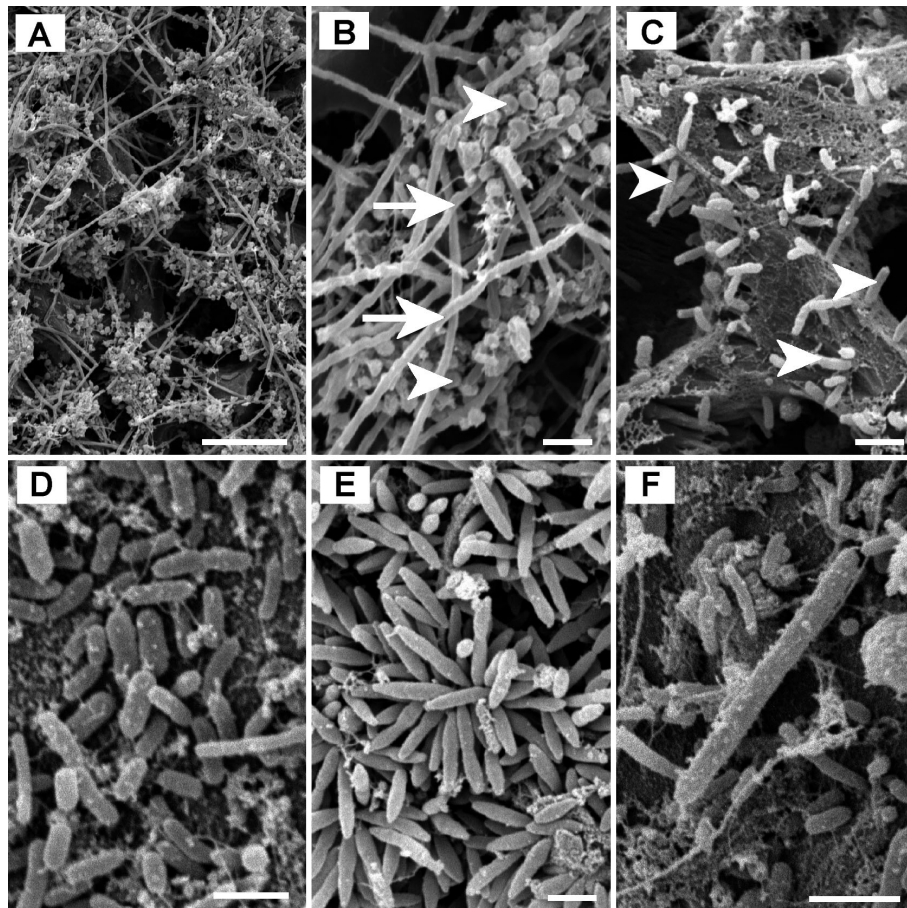


Fig. 1. Microorganisms from lesions of *Tripneustes gratilla*. (A–C) Field-collected lesions. (D–F) Laboratory-induced lesions. (A) General view of an infected area. (B) Cocci (arrowheads) and filamentous bacteria (arrows) in contact with the connective tissues. (C) Rods adhering to the mineral stereom (arrowheads). (D) Rod-shaped bacteria with rounded ends. (E) Rod-shaped bacteria with tapered ends. (F) Large rod-shaped bacterium. SEM observations. Scale bars = 10 µm (A) and 2 µm (B–F)

colouration had disappeared (Fig. 2C). Transversal view of the test showed that the infection progressed from the external to the internal side of the test (Fig. 2D). After 4 wk, the test was infected throughout its thickness, lesions being completely black on the echinoid surface as well on their reverse side (Fig. 2E,F).

Microorganisms that colonised the gastropod-induced lesions during laboratory experiments showed differences from microorganisms infecting field-collected lesions. In the laboratory, after removal of *Vexilla vexillum*, the reddish central part of the lesions was free of any organism, while the black edge displayed 3 types of bacteria: 1 to 2 µm long rod-shaped bacteria with rounded ends (Fig. 1D), 1 to 2 µm long rod-shaped bacteria with tapered ends (Fig. 1E) and large rod-shaped bacteria of 5 to 10 µm in length (Fig. 1F). These 3 bacterial morphotypes were not observed in field-collected lesions. They made up the

dense microbial community that covered the whole surface of the lesions at Day 2. The presence of some cocci, appearing either isolated or in groups of 10s to 100s of individuals, and filamentous bacteria was noticed at Day 5. Ten days after grazing, the filamentous bacteria were more numerous and completely covered the lesions.

From the 30 echinoids abraded with a scalpel (45 minus the 15 sampled, see 'Materials and methods'), 16 recovered after 28 d, while 4 sea urchins displayed a black infected lesion. The last 10 individuals died during the experiment. In contrast, all the observed lesions caused by *Vexilla vexillum* were infected. Regeneration of a lesion took about 3 wk. After removal of the epidermis and the appendages, the bare test showed a whitish aspect. The next day, the lesion displayed a red colouration that remained throughout the recovery process. The first obvious signs of regeneration were visible after 2 wk. The epi-

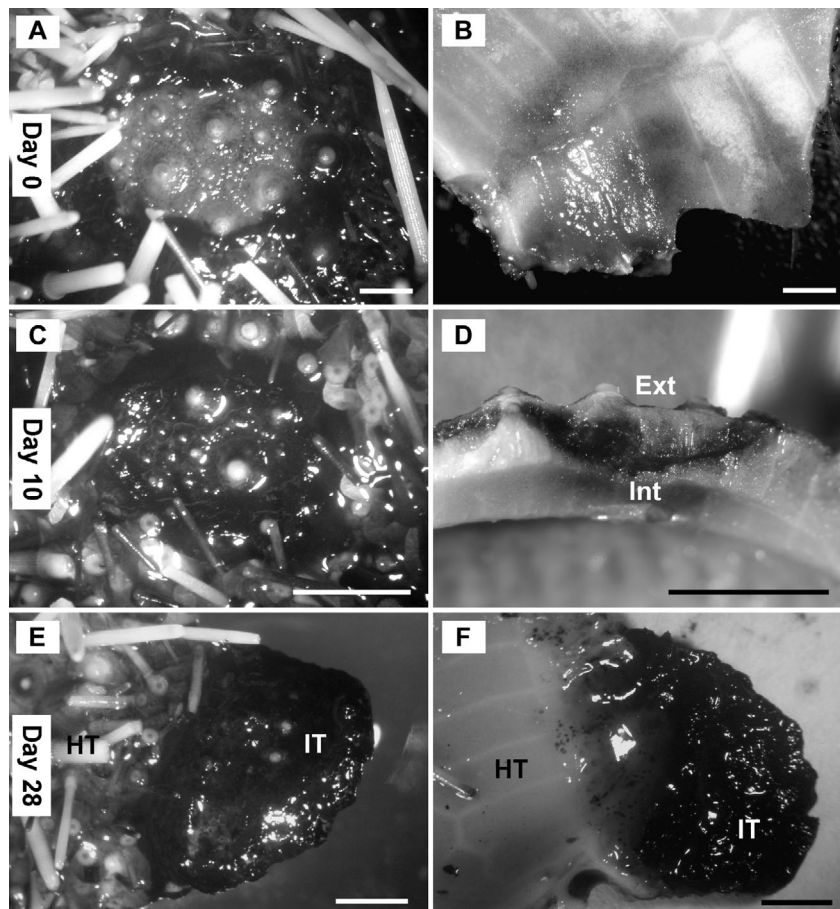


Fig. 2. Laboratory-induced lesions of *Tripneustes gratilla* (A,B) after removal of *Vexilla vexillum*, (C,D) 10 and (E,F) 28 d after grazing by the gastropod. (A,B) Lesion displays a red colouration on its surface (A) as well as on its reverse side (B). (C,D) Lesion shows a dark green colour (C) associated with an infection that progresses towards the internal side of the test (D). (E,F) Lesion turns black and is infected from its surface (E) to its reverse side (F). Scale bars = 2 mm. Ext: external side of the test; Int: internal side of the test; HT: healthy test; IT: infected test

dermis, which grew from the periphery towards the centre of the lesion, progressively covered the abraded zone; subsequently the emergence of small spines and epidermal pigmentation was noted. The lesion was completely healed 3 to 4 wk after abrasions, with a pigmented epidermis, pedicellariae, podia and large spines.

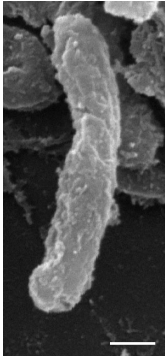
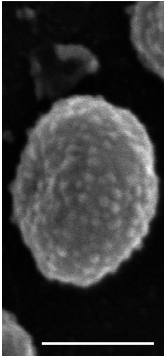
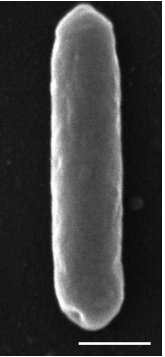
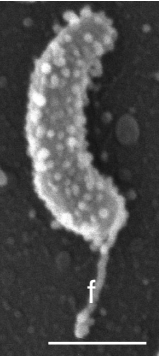
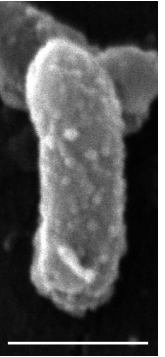
Microbiological analyses

Table 1 summarises the features of the 5 bacterial strains isolated during the microbiological procedure. The first one, H-1, was cultured from integument of healthy echinoids, while the 4 others, I-1 to I-4, were isolated from infected lesions. All strains were Gram-negative, except I-2. H-1 was a large (2 to 3 μm long), slightly curved, rod-shaped bacterium with a rough surface; I-1 was a small (0.6 to 0.9 μm long), spherical

to ovoid bacterium; I-2 was a rod-shaped bacterium 1 to 3 μm long, with a smooth surface; I-3 was a comma-shaped bacterium 1 μm in length with a single flagellum; and I-4 was a straight to curved rod 1 μm long. I-1, I-3 and I-4 were closely related to *Vibrio* species. H-1 was a *Gammaproteobacterium*, while I-2 was related 100% to *Exiguobacterium aestuarii* (Table 1). Each strain inoculated on abraded areas caused infections. None of the treated individuals (5 replicates per strain) recovered from their lesion. The infection was strong and rapid, lesions turning black 2 d after inoculation. Lesions also extended quickly, increasing 2- to 3-fold in surface after the first week. In contrast, after 1 wk, only 1 of the 30 echinoids (3%) used as a control became infected.

The fates of scalpel-made lesions inoculated with pieces of necrotic tissues and non-inoculated controls are compared in Fig. 3. The mortality in both groups of echinoids did not show significant differences (Chi-

Table 1. Characteristics of the 5 bacterial strains isolated from healthy and diseased echinoids. f: flagellum. Scale bars = 500 nm

	H-1 Healthy tissues	I-1 Lesion	I-2 Lesion	I-3 Lesion	I-4 Lesion
Morphology of the colonies	Circular, yellow, translucent, smooth surface and regular margin	Circular, yellow, translucent, smooth surface and regular margin	Rosary-shaped, white, wrinkled surface and regular margin	Circular, yellow, translucent, smooth surface and regular margin	Circular, yellow, opaque, smooth surface and regular margin
Gram staining	Negative	Negative	Positive	Negative	Negative
Morphology of the bacteria					
Sequence length obtained (16S rDNA)	531 bases	490 bases	467 bases	458 bases	492 bases
Closest relative, GenBank Accession No. (% sequence similarity)	<i>Spongiobacter nickelotolerans</i> AB205011 (94%)	<i>Vibrio parahaemolyticus</i> DQ164802 (98%)	<i>Exiguobacterium</i> sp. AY612767 (100%)	<i>Vibrio nigripulchritudo</i> X74717 (99%)	<i>Vibrio parahaemolyticus</i> DQ164802 (100%)
Taxonomic group	<i>Gamma-proteobacteria</i>	<i>Gamma-proteobacteria</i>	Firmicutes	<i>Gamma-proteobacteria</i>	<i>Gamma-proteobacteria</i>

square, $p > 0.5$). The number of healing individuals, however, was significantly smaller (Chi-square, $p < 0.05$) with inoculated lesions.

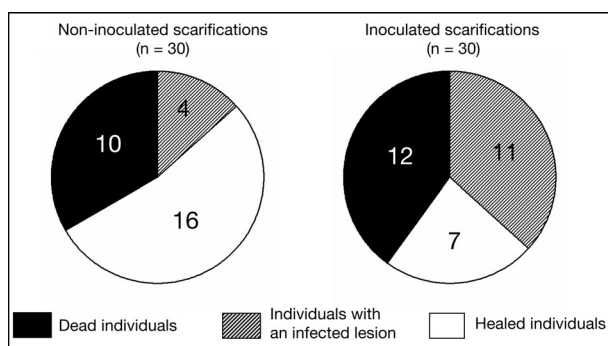


Fig. 3. Comparison, after 4 wk, between non-inoculated scarifications (on the left) and scarifications inoculated with necrotic tissues (on the right). Although mortality is not significantly different between the 2 groups (Chi-square, $p > 0.5$), the number of healed individuals is significantly higher (Chi-square, $p < 0.05$) with non-inoculated lesions

Denaturing gradient gel electrophoresis (DGGE) and sequencing

Fig. 4 illustrates a gel obtained on field-collected samples (Lanes A to C: integument of 3 healthy sea urchins; Lanes D to G: lesions of 4 infected individuals). Two other replicate gels were made, and their patterns were identical. Patterns of lanes corresponding to healthy tissues were very different from patterns of lanes assigned to lesions. None of the bands detected in Lanes A to C were present in Lanes D to G and vice versa. Three bands (Bands 1, 2 and 3) were visible in all lanes of healthy sea urchins, while Lane A displayed an additional band (named Band 4) below Band 3. Bands 8 (which was very faint) and 9 were the only bands revealed in all lesion patterns. Bands 5, 6 and 7 were only present in some of the lanes corresponding to lesions. A total of 9 bands was thus detected on the gel, but, from the sequencing of their 16S rDNA, only Bands 1, 6, 8 and 9 have been identified. Phylotype 1 belonged to Spirochaetes, Phylotype 6 was a member of the

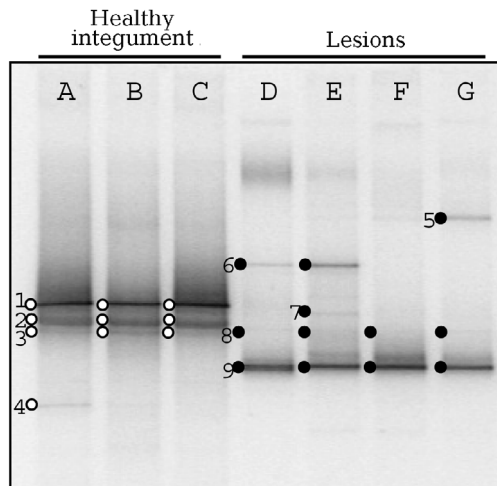


Fig. 4. DGGE (denaturing gradient gel electrophoresis) profile of the 16S rDNA fragments of bacteria present in field-collected samples. Lanes A to C: part of the integument of 3 healthy sea urchins; Lanes D to G: lesions of 4 diseased individuals. White spots highlight bands detected in patterns from healthy integuments, while black spots correspond to bands in fingerprints of lesions. At least 1 band per identified phylotype was sequenced

CFB group (*Cytophaga-Flavobacterium-Bacteroides*), and Phylotypes 8 and 9 were 2 *Alphaproteobacteria*.

Fig. 5 illustrates a gel obtained after DGGE on gastropod-induced lesions in laboratory experiments (Lanes B, D, F, H and J for Days 0 to 28) and scalpel-induced lesions (Lanes C, E, G, I and K for Days 0 to 20). Two replicate gels were also performed, and their

patterns were identical. Patterns of lanes for both types of lesions showed several similarities, as Bands 12, 13, 15, 16 and 17 were present in both types of lesions. Some differences occurred, as Bands 10 and 11 were only visible in gastropod-induced lesions, while Bands 14 and 18 were only present in scalpel-induced lesions. Two field-collected samples were added to this DGGE: the integument of a healthy echinoid (Lane A) and a field-collected lesion (Lane L). It appeared that Bands 12 and 14 from the gel in Fig. 5 corresponded, respectively, to Bands 6 (lesions) and 1 (healthy integuments) of Fig. 4. A total of 9 bands was thus detected on the DGGE in Fig. 5. All, except Band 15, have been identified from the sequencing of their 16S rDNA. Phylotypes 12 and 14, already identified in the previous DGGE, were, respectively, assigned to the CFB group and Spirochaetes. Phylotypes 10, 11 and 13 were members of the CFB group, while Phylotypes 16 and 17 belonged to the *Alphaproteobacteria*. Finally, Phylotype 18 was identified as a *Betaproteobacterium*.

Fig. 6 is the phylogram obtained during a ML search, showing the phylogenetic affiliations of the DGGE phylotypes and the cultured bacteria identified in this study. Their closest relatives obtained during a BLAST search were added to the ingroup. The Spirochaetes, Firmicutes, CFB group, *Alphaproteobacteria* and *Betaproteobacteria* were monophyletic in both MP and ML searches; *Gammaproteobacteria* were paraphyletic in the ML search but monophyletic in the MP search. Phylotypes 8, 9, 16 and 17 clustered in *Alphaproteobacteria*, Phylotype 18 in *Betaproteobacteria*, Phylotypes 6 & 12, 10, 11 and 13 in the CFB group and

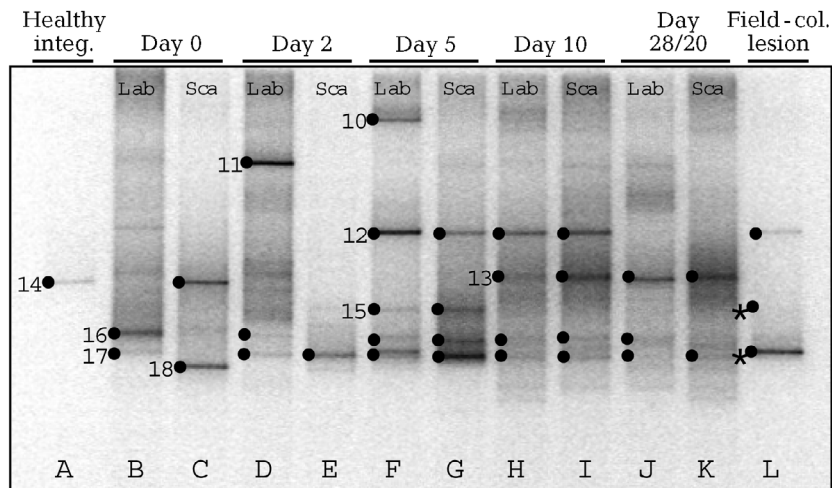


Fig. 5. DGGE profile comparing 16S rDNA fragments of bacteria present in lesions from the laboratory-induced lesions (caused by *Vexilla vexillum*) and from the scalpel lesions. Comparisons were also made with healthy integument (Lane A) and a field-collected lesion (Lane L). Lanes B, D, F, H and J correspond to laboratory-induced lesions at Days 0, 2, 5, 10 and 28, respectively, while Lanes C, E, G, I and K correspond to scalpel lesions at Days 0, 2, 5, 10 and 20, respectively. The 20 d old artificial scarifications showed signs of regeneration. *: bands corresponding to Bands 8 and 9 of the DGGE in Fig. 4; Lab and Sca: laboratory- and scalpel-induced lesions, respectively. At least 1 band per identified phylotype was sequenced

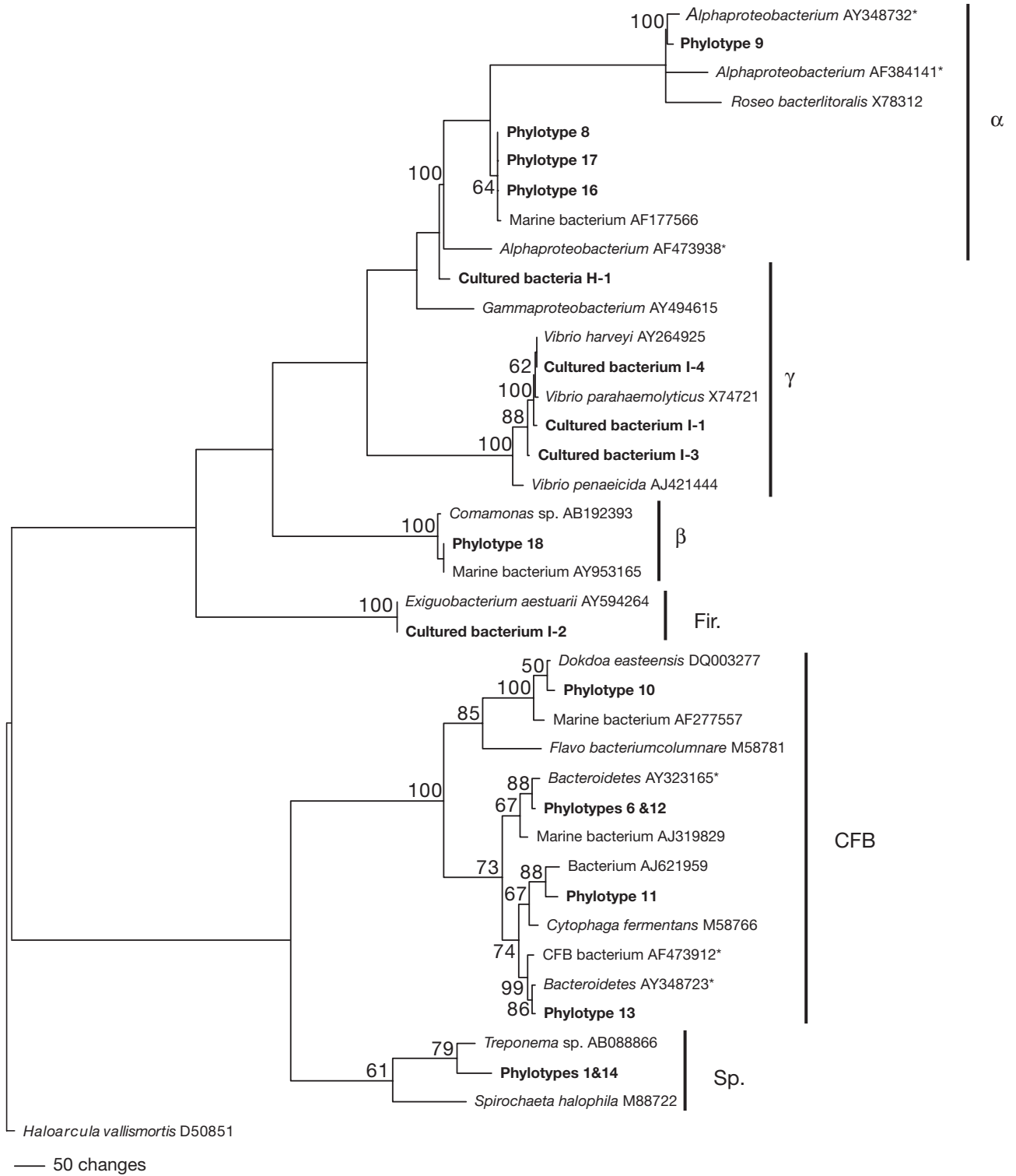


Fig. 6. Phylogram showing the maximum-likelihood tree obtained with the 16S rDNA sequences obtained in the present study (DGGE and bacterial cultures) aligned with the most similar sequences retrieved from a BLAST search. The numbers above the branches indicate the bootstrap values obtained during a maximum-parsimony search (1000 replicates). α , β , γ : subdivisions of *Proteobacteria*; Fir.: Firmicutes; Sp.: Spirochaetes; CFB: *Cytophaga-Flavobacterium-Bacteroides*; *: organisms associated with marine diseases

Phylotype 1&14 in Spirochaetes. All grouping is supported at 100% of the bootstrap value, except Phylotype 1&14, which received a bootstrap support of 79%. The cultured bacteria I-1, I-3, I-4 grouped in *Gammaproteobacteria* and the cultured bacterium I-2 in Firmicutes, with a bootstrap value of 100%. The cultured bacterium H-1 clustered in *Gammaproteobacteria*, but with no bootstrap support.

DISCUSSION

Lesions on *Tripneustes gratilla* induced by *Vexilla vexillum* showed several similarities with those of echinoids affected by the bald sea urchin disease described by Maes & Jangoux (1984). In both cases, lesions appeared, which were devoid of epidermis and appendages and displayed a dark green to black colouration. Both types of infections were transmissible and could be lethal in inducing a perforation of the test. Several bacterial morphotypes were observed in field-collected lesions amongst which filamentous bacteria, cocci and rods were particularly abundant. Although these 3 morphotypes dominated field-collected lesions, they were not the only morphotypes observed. Additionally, laboratory-induced lesions displayed a different microbiota. In this case, rod-shaped bacteria with rounded and sharp ends were the most numerous. Morphological analysis thus revealed the diversity of the bacteria infecting the lesions. Our experiments have also demonstrated that lesions induced with a sterile scalpel tended to heal, while lesions caused by *Vexilla vexillum* became infected. The gastropod could thus favour the establishment of opportunistic bacteria, but how this occurs remains to be investigated.

Five strains were isolated during the microbiological analyses and all induced heavy infections in abraded zones of the echinoid test, demonstrating that the infection was due to opportunistic bacteria. Three of these 5 strains (I-1, I-3 and I-4) were *Vibrio*. *Vibrio* species are a major threat for marine invertebrates, and many cases of vibriosis have been described, notably in clams (Allam et al. 2002), oysters (Lacoste et al. 2001) and shrimps (Aguirre Guzman & Ascencio Valle 2000). Strain I-2 showed 100% of sequence identity with *Exiguobacterium aestuarii*, which was isolated from a tidal flat of the Yellow Sea in Korea (Kim et al. 2005). Finally, Strain H-1 was an unidentified *Gamma-proteobacterium*.

DGGE was performed on 4 types of samples: healthy echinoids, field-collected lesions, gastropod-induced lesions and scalpel-induced lesions; the last 2 were made in the laboratory. Results firstly suggest that the 4 bacterial phylotypes revealed from healthy echinoids were not present in diseased echinoids and were thus not respon-

sible for the infection. They are probably subcuticular bacteria (SCB), such bacteria living under the cuticle of many echinoderms (De Ridder & Foret 2001). Although their roles have not been definitely established, it has been suggested that SCB would participate in the uptake of toxic product or would be a source of nitrogen for the hosts (De Ridder & Foret 2001).

Secondly, DGGE performed on field-collected samples revealed the diversity of the microbial community associated with the lesions, as at least 5 bacterial phylotypes were detected. Out of these bacteria, only 2 types, assigned to *Alphaproteobacteria* (Phylotypes 8 and 9), were present in all lesions; they were close to the *Alphaproteobacteria* implicated in several marine invertebrate diseases. One of them was identified as the etiological agent of an infection affecting the Great Barrier Reef sponge *Rhopaloides odorabile*. This bacterium caused the necrosis of external tissues and pinacoderm, revealing the connective fibres (Webster et al. 2002). Other close relatives were associated with the bacterial consortium responsible for black band disease that destroys tissues of several coral species (Cooney et al. 2002) and from lesions of the coral *Montipora aequituberculata* (Jones et al. 2004).

Thirdly, bacterial communities revealed in the DGGE patterns of gastropod-induced lesions at various stages of development showed that *Alphaproteobacteria* appeared earlier during the infection than CFB bacteria. This result, coupled with the close phylogenetic affiliations to marine parasites of the *Alphaproteobacteria* identified in this study, and the fact that *Alphaproteobacteria* were present in all lesions, strongly suggests that they are among the principal infectious bacteria colonising the lesions.

Finally, comparisons between the DGGE patterns of gastropod-induced lesions and scalpel-induced lesions showed major differences during the 2 first days, while, afterwards, the DGGE patterns were almost identical. It would thus be interesting to study the early stage of the infection to explain why most scalpel-induced lesions healed, while gastropod-induced ones did not.

DGGE gels and bacterial cultures revealed different microorganisms, showing the complementarities of the 2 methods, both being selective. It is indeed believed that <1% of the extant bacteria are cultivable, and, with DGGE, bacteria present in very small amounts are not detected. With the now available molecular tools, identification of infectious bacteria based only on cultures should be considered carefully: an infectious strain that may play only a secondary role can be isolated and inaccurately considered as the main etiological agent. Molecular techniques can, for their part, detect uncultivable bacteria that are suspected to play an important role in a disease.

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