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Characterization of the bacterial communities associated with the bald sea urchin disease of the echinoid *Paracentrotus lividus*

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Abstract

The microbial communities involved in the bald sea urchin disease of the echinoid *Paracentrotus lividus* are investigated using culture-independent techniques. Lesions of diseased specimens from two locations in France, La Ciotat (Mediterranean Sea) and Morgat (Atlantic Ocean), are examined by Scanning Electron Microscopy (SEM) and the diversity of their microbiota is analysed by Denaturing Gradient Gel Electrophoresis (DGGE) and 16S rRNA gene clones libraries construction. Microscopic observations demonstrated that only the central area of the lesions is invaded by bacteria but not the peripheral zone and the surrounding healthy tissues. Molecular analysis identified at least 24 bacterial genomospecies in bald sea urchin lesions: 5 are *Alphaproteobacteria*, 10 are *Gammaproteobacteria*, 8 are CFB bacteria and 1 is a *Fusobacteria*. Out of them, 4 are observed in both locations while 10 occur only in the Atlantic Ocean and 10 only in the Mediterranean Sea. *Gammaproteobacteria* are the most represented in clones libraries from both locations, with respectively 65% and 43% of the total clones. CFB and *Alphaproteobacteria* accounted for the majority of the remaining clones and were detected by DGGE in virtually all samples from both stations. Our results demonstrate that bacterial communities observed on diseased individuals of the same echinoid species but originating from distinct locations are not similar and thus support the hypothesis that bacteria involved in the worldwide echinoid disease commonly called the bald sea urchin disease are opportunistic and not specific.

Keywords: Bald sea urchin disease; *Paracentrotus lividus*; Bacterial community; DGGE; Cloning; Mediterranean; Atlantic

1. Introduction

Bacterial diseases involving sea urchins have been described in most oceans, especially in the Northern hemisphere where they caused dramatic mass mortalities. To our knowledge, 19 echinoid species, coming not only from the wild environment but also from aquacultures and laboratories aquaria, are known to be affected by bacterial infections (Table 1). The latter are characterized in most cases by body wall lesions where spines, podia, pedicellaria and epidermis are lost. Typical lesions, ranging from a few millimetres to a third of the body surface, show a green to black central area where the calcareous skeleton is exposed to the external medium and a surrounding peripheral belt of necrotic epidermal tissues. Occasionally, the perforation of the test occurs, leading to the death of the individuals (Jangoux, 1990). These symptoms, referred to the "bald sea urchin disease", were first described by Johnson (1971) in *Strongylocentrotus franciscanus* in 1970 along the US Californian coast. In the late 1970s, reports were made of severe outbreaks affecting several species of regular echinoids in the Mediterranean Sea (Höbaus et al., 1981) but also, at lower incidence, along the French coasts of the Atlantic Ocean and the English Channel (Maes and Jangoux, 1984a). More recently, Tajima et al. (1997a) studied a similar disease in reared *Strongylocentrotus intermedius* in Japan and named it the spotting disease.

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Table 1
Bacterial diseases affecting sea urchins

Sea urchin	Geographical area	Associated bacteria	Method of identification	References
Allocentrotus fragilis	NE Pacific (California), laboratory conditions	Unknown		Boolootian et al. (1959), Giese (1961)
Arbacia lixula	Mediterranean Sea	Unknown		Höbaus et al. (1981), Maes and Jangoux (1984a)
Archaeopneustes hystrix	Caribbean (Bahamas), laboratory conditions	*Vibrio alginolyticus*	Bacterial cultures and phenotypic tests	Bauer and Young (2000)
Cidaris cidaris	Mediterranean Sea	Unknown		Jangoux (1990)
Diadema antillarum	Caribbean	Unknown waterborne, host-specific agent, perhaps *Clostridium perfringens*[a] and *C. sordelli*[a]	Bacterial cultures and phenotypic tests	Lessios et al. (1984), Bauer and Agerter (1987)
Echinometra mathaei	Indian Ocean (Madagascar)	Unknown		Vaïtilingon et al. (2004)
Echinus esculentus	NE Atlantic (Brittany, France)	Unknown		Maes and Jangoux (1984a)
Hemicentrotus pulcherrinus	NW Pacific (Japan), sea urchin farms	Unknown		Kanai (1993)
Meoma ventricosa	Caribbean (Curaçao)	*Pseudoalteromonas* sp.	Not stated	Nagelkerken et al. (1999)
Paleopneustes cristatus	Caribbean (Bahamas), laboratory conditions	*Vibrio alginolyticus*	Bacterial cultures and phenotypic tests	Bauer and Young (2000)
Paracentrotus lividus	NE Atlantic (Brittany, France) and Mediterranean Sea	CFB, *Alpha-* and *Gammaproteobacteria* including *Vibrio* sp. and *Colwellia* sp.	16S rRNA gene sequencing	Maes and Jangoux (1984a), Maes and Jangoux (1984b), present study
Psammechinus miliaris	English Channel (Normandy, France)	Unknown		Maes and Jangoux (1984a)
Pseudocentrotus depressus	NW Pacific (Japan), sea urchin farms	Unknown		Kanai (1993)
Sphaerechinus granularis	NE Atlantic (Brittany, France) and Mediterranean Sea	Unknown		Höbaus et al. (1981), Maes and Jangoux (1984a)
Strongylocentrotus droebachiensis	NW Atlantic (Nova Scotia)	*Acinetobacter* sp., *Alcaligenes* sp.	Bacterial cultures and phenotypic tests	Roberts-Regan et al. (1988)
Strongylocentrotus franciscanus	NE Pacific (California)	Unknown		Johnson (1971), Pearse et al. (1977)
Strongylocentrotus intermedius	NW Pacific (Japan), sea urchin farms	*Flexibacter* sp., *Vibrio* sp.[a]	Bacterial cultures, phenotypic tests and DNA–DNA homology	Tajima et al. (1997a), Tajima et al. (1997b), Takeuchi et al. (1999)
Strongylocentrotus purpuratus	NE Pacific (California)	*Aeromonas salmonicida*, *Flavobacterium* spp., *Pseudomonas* sp., *Vibrio anguillarum*	Bacterial cultures and phenotypic tests	Johnson (1971), Gilles and Pearse (1986)
Tripneustes gratilla	Indian Ocean (Madagascar)	CFB, *Exiguobacterium* sp., *Alpha-* and *Gammaproteobacteria* including *Vibrio* spp.	Bacterial cultures and 16S rRNA gene sequencing	Becker et al. (2007)

[a] Bacteria associated with systemic diseases, with spines loss but without obvious body wall lesions.

Etiological agents of these diseases are bacteria that need a mechanical damage of the body wall to induce the infection (Jangoux, 1990). In a study based on bacterial cultures, Gilles and Pearse (1986) isolated 14 strains from infected Californian echinoids. All strains belonged to the *Aeromonas*, *Flavobacterium*, *Pseudomonas* or *Vibrio* genera but only *Aeromonas salmonicida* and *Vibrio anguillarum* were pathogenic. In Japanese farms, a *Flexibacter* bacterium has been identified as the causative agent of the spotting disease (Tajima et al., 1997b). In Europe, although bald sea urchins are still encountered, no study was performed to identify the microorganisms that infect the lesions.

In the present work, we investigated the microbial communities that colonize bald sea urchin lesions of *Paracentrotus lividus* populations of the French Mediterranean and Atlantic coasts. We used culture-independent techniques in order to obtain a more complete identification of these communities compared to bacterial cultures-based analyses performed in previous studies.

2. Materials and methods

2.1. Sampling and morphological analyses

Diseased *Paracentrotus lividus* (Lamarck, 1816) with lesions similar to those described by Maes and Jangoux (1984a) were collected by hand at low tide in Morgat (Atlantic coast, Brittany, France) and by diving near La Ciotat (Mediterranean coast, France) in February 2006. They were found in low incidences of about 1 per 100 and 1 per 1000 individuals in Morgat and La Ciotat, respectively. For each station, lesions from 5 individuals

were directly dissected and fixed in non-acetic Bouin's fluid for SEM while 3 other samples were immersed in absolute ethanol for molecular analysis.

Some fresh lesions were observed and photographed with a Leica MZ8 binocular equipped with a Nikon Coolpix 990 camera. For SEM, fixed samples 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 electron microscope.

2.2. DGGE

Phylotypes (*i.e.*, nucleotide sequences obtained from an environmental sample and having phylogenetic affiliations with sequences from known species; Muyzer and de Waal, 1994) occurring in samples were detected by DGGE. Tissues from healthy sea urchins and lesions from diseased echinoids collected in Morgat and La Ciotat were taken as samples and treated in the same way during all the procedure. For DNA extraction, 5–10 mg of tissues were removed with a sterile scalpel and placed in a sterile microcentrifuge tube. Total DNA was then extracted from the samples with an Invisorb extraction kit (Invitek) following the manufacturer's instructions.

A 550 bp-long 16S rRNA gene fragment was amplified by PCR using 1 μl of extraction product as DNA template and the bacterial primers GM5F-GC clamp and DS907R (Teske et al., 1996). Touchdown-PCR amplifications were performed with the PuReTaq Ready-To-Go PCR beads kit (GE Healthcare) 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 was 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 of 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. At least one band per position and per station has been sequenced. The acrylamide with the DNA was crushed in microcentrifuge tubes containing 300 μl of Tris 10 mM–EDTA 1 mM. After one 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 previously but with 3 μl of DNA template. The amplified products were purified with a Quantum Prep kit (Bio-Rad).

2.3. Cloning

DNA from lesion samples was extracted as described above and the complete 16S rRNA gene was amplified with the bacterial primers 8F and 1492R (Buchholz-Cleven et al., 1997) using 3 μl of DNA template. PCR consisted of 30 cycles with denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 1 min. For the last elongation step, the tubes were incubated at 72 °C for 10 min. The PCR products were then purified with QIAquick columns (Qiagen) and cloned into TOP10 chemically competent *Escherichia coli* cells using the TOPO TA Cloning kit (Invitrogen) to form one clone library for each of the echinoid population investigated. Clones containing the complete 16S rRNA gene, as revealed by PCR with primer M13, were selected for plasmid isolation with the QIAprep miniprep kit (Qiagen). Coverage value of the libraries were calculated according to Good (1953) with 97% of sequence similarity used as the criterion for sequence uniqueness.

2.4. Sequence analyses

Partial sequences of DGGE phylotypes and clones (sequences length ranging from 278 to 510 bases) were obtained with the BigDye Terminator v3.1 Cycle Sequencing kit (ABI) in a Prism 3100 Genetic Analyser (ABI) using primer GM5F. The cycle sequencing reaction consisted in 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 sequences were compared with those in the GenBank database with the basic local alignment search tool (BLAST) in order to find related species names (Altschul et al., 1990). Each sequence was also checked for chimera formation using Chimera Check v2.7 program (Cole et al., 2003).

Sequences were aligned using ClustalX (Thompson et al., 1994) and neighbour-joining (NJ) analyses were performed with Paup* (Swofford, 1998) using the Jukes and Cantor distance (Jukes and Cantor, 1969). Reliability of various inferred phylogenetic nodes was estimated by bootstrapping (1000 replicates) (Felsenstein, 1985). In agreement with the International Committee on Systematics of Prokaryotes (ICSP) and according to Stackebrandt and Goebel (1994), the concept of genomospecies was used

(see also Stackebrandt et al., 2002). A genomospecies is different from another one when their 16S rDNA sequences share less than 97% of similarity. In Section 3, we assume that sequences that have 97% of similarity or more belong to the same genomospecies meaning that the number of genomospecies is minimized as sequences displaying more than 97% of identity can belong to different species. The sequences determined for this study have been deposited at the EMBL database under Accession Nos. AM850923 to AM851001.

3. Results

3.1. Morphological observations

Bald sea urchins collected displayed lesions ranging from 1 cm^2 to large areas corresponding to about a tenth of the body surface (Fig. 1A). Appendages were lost in lesions that consisted in a central green zone with bare test surrounded by a slightly pinkish peripheral zone where the epidermis still covered the mineral skeleton (Fig. 1B). At

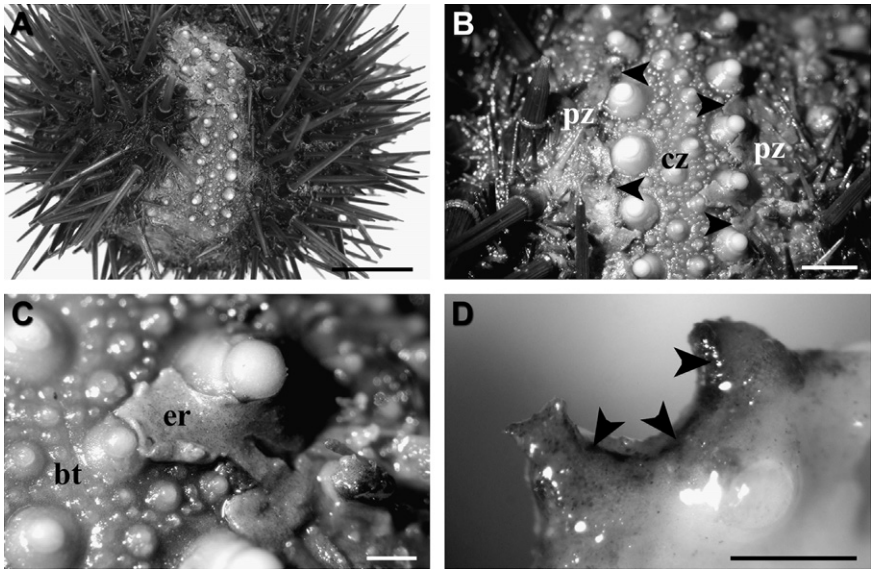

Fig. 1. Bald sea urchin lesions. (A) Diseased echinoid displaying a large lesion. (B) Close-up view showing the different parts of a lesion: the central zone with denuded test and the peripheral zone with epidermis covering the skeleton (the arrows highlight the limit between these zones). (C) Detail of an epidermis remnant at the junction between the central and the peripheral zones. (D) Black border line (arrows) surrounding a skeletal fragment from the central zone. bt, bare test; cz, central zone; er, epidermis remnant; pz, peripheral zone. Scale bars = 1 cm for (A), 2 mm for (B) and 500 μm for (C and D).

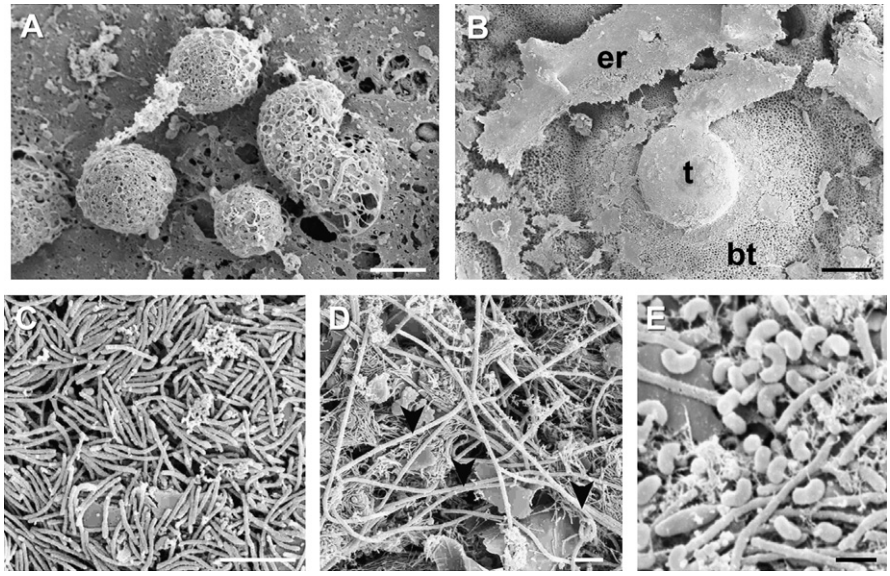

Fig. 2. Bald sea urchin lesions. SEM observations. (A) Spherical cells standing on the peripheral zone. (B) General view of the central zone showing epidermis remnants and areas with calcareous skeleton exposed to the external medium. (C–E) Main bacterial morphotypes infecting the lesions: rod-shaped bacteria (C), filamentous bacteria (D, arrows) and curved bacteria (E). bt, bare test; er, epidermis remnant; t, tubercle. Scale bars = 1 μm for (D), 5 μm for (A, C and E) and 250 μm for (B).

the junction of these two zones were necrotic epidermis remnants that could be removed without difficulties with forceps (Fig. 1C). The test of the central zone was fragile and broke easily, leaving pieces of skeleton with a black borderline (Fig. 1D).

SEM showed that the healthy tissues surrounding the lesions were free of microorganism. The peripheral zone of the lesions was mainly covered by spherical cells of 5–10 μm in diameter (Fig. 2A) while bacteria were virtually absent. These cells formed an almost continuous belt around the central zone. The latter lacked the epidermal tissues, except small remnants, and the skeleton was thus exposed to the external medium (Fig. 2B). Within this zone, lesions raised two distinct aspects: (1) areas with few microorganisms where the calcareous meshwork was clearly observable (Fig. 2B) and (2) areas with numerous bacteria and degraded tissues totally concealing the test (Fig. 2C–E). In the first areas, the skeleton appeared intact and displayed no sign of erosion. However, the connective tissue was eliminated as the spaces lined by mineral trabeculae were empty (Fig. 2B). In the second areas, several different bacterial morphotypes were observed. In both La Ciotat and Morgat samples, rod-shaped bacteria (Fig. 2C) and filamentous bacteria (Fig. 2D) were the most abundant morphotypes. Rod-shaped bacteria were 3–4 μm long and formed dense covers of up to 12 cells per 10 μm^2. Filamentous bacteria were 750 nm in width and 35–60 μm in length and reached a density of about 150 cells per square millimeter. Curved bacteria (Fig 2E) were also observed but exclusively in echinoids from Brittany. They were 1 μm long and were reported in groups of a few tens of cells. Some ciliate protozoans also occurred in all samples.

3.2. DGGE

Fig. 3 illustrates a DGGE gel obtained from six lesions. Lanes A–C correspond to lesions from diseased sea urchins collected in La Ciotat while lanes D–F correspond to those from Morgat. No bacterial DNA amplified from healthy tissues. Two replicate gels were performed and their pattern was identical. A total of seven phylotypes were revealed on the gels. Five phylotypes (bands 2, 3, 4, 6 and 7) were present in lesions from both stations amongst which two (bands 6 and 7) were revealed in all samples. Two other phylotypes (bands 1 and 5) were only present in lesions from Morgat. According to a BLAST search (Table 2), phylotypes 1–5 belonged to CFB (*Cytophaga–Flavobacter–Bacteroides*) while phylotypes 6 and 7 were two *Alphaproteobacteria*.

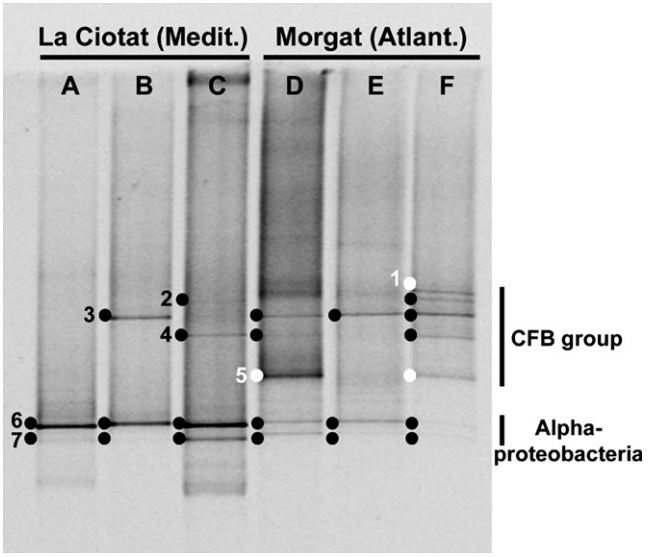

Fig. 3. DGGE gel profile of the 16S rDNA fragments from bacteria infecting bald sea urchin lesions. Lanes (A–C) lesions from echinoids collected in La Ciotat (Mediterranean coast). Lanes (D to F) lesions from sea urchins sampled in Morgat (Atlantic coast). Black spots indicate phylotypes present in lesions from both stations while white spots correspond to phylotypes occurring only in lesions from Morgat. No phylotype specific to lesions from La Ciotat was detected.

3.3. Cloning

Two clone libraries were obtained from lesions collected in Morgat (52 clones, 71% of coverage value) and La Ciotat (46 clones, 67% of coverage value). Three samples

Table 2
16S rDNA sequence identities of phylotypes from DGGE performed on bald sea urchin lesions of *Paracentrotus lividus*[a]

	No. of bases sequenced	Best-matched organism	Accession no.	ID%	Division	Location of phylotype[b]	
						AO	MS
Band 1	502	Uncultured *Flexibacteraceae* bacterium	AB240710	99	CFB	X	
Band 2	368	Uncultured *Bacteroidetes* bacterium clone Flo-32	AY684351	99	CFB	X	X
Band 3	278	Uncultured *Cytophagales* bacterium clone SL-6a	AY337037	98	CFB	X	X
Band 4	502	Uncultured bacterium clone s34	AY171371	97	CFB	X	X
Band 5	297	Uncultured bacterium clone 2164	AY172260	100	CFB	X	
Band 6	487	Unknown marine *Alphaproteobacterium* JP7.1	AY007679	99	α-Proteobacteria	X	X
Band 7	325	Uncultured *Roseobacter* sp. isolate DGGE band Mes9	EF441556	98	α-Proteobacteria	X	X

[a] Listed are the numbers of bases sequenced and the accession numbers, percent identities (ID%) and divisions of the best-matched organisms in GenBank.

[b] X signs indicate whether the phylotypes occurred in samples from Morgat, Atlantic Ocean (AO) or in La Ciotat, Mediterranean Sea (MS).

Table 3
16S rDNA sequence identities of clones associated with bald sea urchin lesions of *Paracentrotus lividus*[a]

Clone OC[b] (no. of clones)	No. of base sequenced	Best-matched organism	Accession no.	ID%	Division	Location of clone[c]	
						AO	MS
1 (1)	373	Uncultured bacterium clone 4-Org2-4	AF143828	95	α-Proteobacteria	X	
2 (11)	354–484	Unknown marine *Alphaproteobacterium* JP7.1	AY007679	99–100	α-Proteobacteria	X	X
3 (1)	370	Uncultured *Rhodobacterales* bacterium clone GL2-04	EF215729	99	α-Proteobacteria	X	
4 (1)	508	Uncultured *Gammaproteobacterium* clone PM1-22	EF215794	98	γ-Proteobacteria	X	
5 (1)	502	Uncultured *Gammaproteobacterium* clone PM1-22	EF215794	99	γ-Proteobacteria	X	
6 (2)	484	Uncultured *Gammaproteobacterium* clone PM1-22	EF215794	99	γ-Proteobacteria	X	
7 (1)	463	Uncultured *Gammaproteobacterium* clone PM1-22	EF215794	99	γ-Proteobacteria	X	
8 (1)	508	Uncultured *Gammaproteobacterium* clone PM1-22	EF215794	99	γ-Proteobacteria	X	
9 (1)	490	Uncultured *Gammaproteobacterium* clone PM1-22	EF215794	98	γ-Proteobacteria	X	
10 (1)	474	Uncultured *Gammaproteobacterium* clone PM1-22	EF215794	99	γ-Proteobacteria	X	
11 (1)	449	Uncultured *Gammaproteobacterium* clone PM1-22	EF215794	98	γ-Proteobacteria	X	
12 (1)	438	Uncultured *Gammaproteobacterium* clone PM1-22	EF215794	99	γ-Proteobacteria	X	
13 (1)	498	Uncultured *Gammaproteobacterium* clone Arctic95B-7	AF353238	98	γ-Proteobacteria	X	
14 (1)	496	Uncultured *Colwellia* sp. clone ESP450-K6III-27	DQ810716	98	γ-Proteobacteria	X	
15 (1)	426	Uncultured *Colwellia* sp. clone ESP450-K6III-27	DQ810716	99	γ-Proteobacteria	X	
16 (2)	469–479	Uncultured *Colwellia* sp. clone ESP450-K6III-27	DQ810716	98	γ-Proteobacteria	X	
17 (1)	467	Uncultured *Colwellia* sp. clone ESP450-K6III-27	DQ810716	98	γ-Proteobacteria	X	
18 (1)	509	Uncultured *Colwellia* sp. clone ESP450-K6III-27	DQ810716	97	γ-Proteobacteria	X	
19 (1)	443	Uncultured *Colwellia* sp. clone ESP450-K6III-27	DQ810716	97	γ-Proteobacteria	X	
20 (2)	452–496	*Colwellia piezophila* clone SE15	AY771749	99	γ-Proteobacteria	X	
21 (3)	377–509	*Colwellia piezophila* clone SE15	AY771749	98–99	γ-Proteobacteria	X	
22 (1)	469	*Colwellia piezophila* clone SE15	AY771749	98	γ-Proteobacteria	X	
23 (1)	420	*Colwellia piezophila* clone SE15	AY771749	98	γ-Proteobacteria	X	
24 (1)	473	*Colwellia piezophila* clone SE15	AY771749	99	γ-Proteobacteria	X	
25 (1)	457	*Colwellia piezophila* clone SE15	AY771749	98	γ-Proteobacteria	X	
26 (1)	446	*Colwellia piezophila* clone SE15	AY771749	98	γ-Proteobacteria	X	
27 (2)	473–476	*Colwellia* sp. IE7-5	AY829231	97–98	γ-Proteobacteria	X	
28 (1)	397	*Colwellia* sp. IE7-5	AY829231	99	γ-Proteobacteria	X	
29 (1)	509	*Vibrio* sp. EXT4	AB274736	100	γ-Proteobacteria	X	
30 (1)	369	*Gammaproteobacterium* L193	AY371439	93	γ-Proteobacteria	X	
31 (1)	433	Uncultured bacterium clone ZA3235c	AF382112	95	γ-Proteobacteria	X	
32 (1)	484	Uncultured bacterium clone 2164	AY172260	99	CFB	X	
33 (4)	474–501	Uncultured bacterium clone 2164	AY172260	100	CFB	X	
34 (2)	500	Uncultured *Flexibacteraceae* bacterium	AB240710	99	CFB	X	
35 (2)	394–490	Uncultured *Cytophagales* bacterium clone SL-6a	AY337037	98	CFB	X	
36 (1)	500	Uncultured *Bacteroidetes* bacterium clone Flo-32	AY684351	99	CFB	X	
37 (1)	363	Uncultured bacterium clone Milano-WF1B-12	AY592855	94	CFB	X	
38 (1)	499	*Tenacibaculum* sp. MGP-74/AN6	AF530150	94	CFB	X	
40 (1)	426	Unidentified marine eubacterium Hstpl4	U41090	99	Unknown	X	

(*continued on next page*)

Table 3 (*continued*)

Clone OC[b] (no. of clones)	No. of base sequenced	Best-matched organism	Accession no.	ID%	Division	Location of clone[c]	
						AO	MS
41 (1)	426	Unidentified marine eubacterium Hstpl4	U41090	97	Unknown	X	
42 (1)	382	Unidentified marine eubacterium Hstpl4	U41090	98	Unknown	X	
43 (1)	438	*Stappia alba* strain 5OM30	AJ889010	99	α-Proteobacteria		X
44 (1)	445	Unknown marine *Alphaproteobacterium* JP7.1	AY007679	98	α-Proteobacteria		X
45 (1)	472	Unknown marine *Alphaproteobacterium* JP7.1	AY007679	98	α-Proteobacteria		X
46 (1)	354	*Phaeobacter* sp. MED165	DQ681146	100	α-Proteobacteria		X
47 (1)	477	Uncultured marine bacterium clone AG-1	DQ787776	99	α-Proteobacteria		X
48 (2)	465–484	*Alphaproteobacterium* CL-GR35	DQ394095	99–100	α-Proteobacteria		X
49 (1)	498	*Vibrio* sp. SYS6-01	AJ414132	99	γ-Proteobacteria		X
50 (1)	491	*Vibrio* sp. SYS6-01	AJ414132	100	γ-Proteobacteria		X
51 (1)	388	*Vibrio* sp. MED181	DQ681148	98	γ-Proteobacteria		X
52 (1)	510	*Vibrio* sp. PH1	AF513461	99	γ-Proteobacteria		X
53 (1)	490	*Vibrio penaeicida* strain DSM 14398T	AJ421444	99	γ-Proteobacteria		X
54 (1)	490	*Vibrio penaeicida* strain DSM 14398T	AJ421444	98	γ-Proteobacteria		X
55 (1)	402	*Vibrio penaeicida* strain LMG 19663T	AJ437191	99	γ-Proteobacteria		X
56 (1)	464	*Vibrio penaeicida* strain LMG 19663T	AJ437191	99	γ-Proteobacteria		X
57 (1)	359	Uncultured *Gammaproteobacterium* clone DPC007	DQ269072	100	γ-Proteobacteria		X
58 (3)	470–509	Uncultured *Alteromonadales* bacterium clone ESP60-K23I-52	DQ810447	94	γ-Proteobacteria		X
59 (1)	508	Uncultured *Alteromonadales* bacterium clone ESP60-K23I-52	DQ810447	93	γ-Proteobacteria		X
60 (1)	463	Uncultured *Alteromonadales* bacterium clone ESP60-K23I-52	DQ810447	94	γ-Proteobacteria		X
61 (1)	465	Uncultured *Alteromonadales* bacterium clone ESP60-K23I-52	DQ810447	95	γ-Proteobacteria		X
62 (1)	418	Uncultured organism clone ctg NISA145	DQ396288	94	γ-Proteobacteria		X
63 (1)	497	Marine *Gammaproteobacterium* HTCC2143	AY386333	94	γ-Proteobacteria		X
64 (1)	495	*Thiotrichales* bacterium CML28	AB176554	94	γ-Proteobacteria		X
65 (1)	359	Uncultured *Gammaproteobacterium* clone IS-94	DQ223925	98	γ-Proteobacteria		X
66 (1)	451	Uncultured bacterium clone BN_84	AY550765	99	γ-Proteobacteria		X
67 (3)	383–491	Uncultured bacterium SB-11/16-Cd	AJ319831	100	CFB		X
68 (1)	411	Uncultured bacterium SB-11/16-Cd	AJ319831	96	CFB		X
69 (1)	499	*Tenacibaculum aestuarii* strain SMK-4	DQ314760	99	CFB		X
70 (1)	427	Uncultured *Bacteroidetes* bacterium clone PI_4b12a	AY580701	88	CFB		X
71 (1)	435	Uncultured *Fusobacteria* bacterium from sea urchin *Paracentrotus lividus* clone PlS49	AY770718	98	Fusobacteria		X
72 (1)	464	Uncultured bacterium	AB294297	95	Unknown		X
73 (1)	367	Marine snow associated bacterium Adriatic87	AF030773	98	Unknown		X

[a] Listed are the numbers of identical clones, the numbers of bases sequenced and the accession numbers, percent identities (ID%) and divisions of the best-matched organisms in GenBank.

[b] OC stands for "Oursin Chauve" which means "Bald sea urchin" in French.

[c] X signs indicate whether the clones occurred in samples from Morgat, Atlantic Ocean (AO) or in La Ciotat, Mediterranean Sea (MS).

(those used for DGGE) were pooled for construction of each library. Sequencing showed that the *Alphaproteobacteria*, the *Gammaproteobacteria* and the CFB dominated in echinoid lesions of both the Atlantic Ocean and the Mediterranean Sea (Table 3). In both libraries, these groups accounted for more than 90% of the total clones (Fig. 4). Proportions of these groups however varied: the *Gammaproteobacteria* were the most abundant in both regions, then CFB in the Atlantic Ocean (23%) and *Alphaproteobacteria* in the Mediterranean Sea (37%) (Fig. 4).

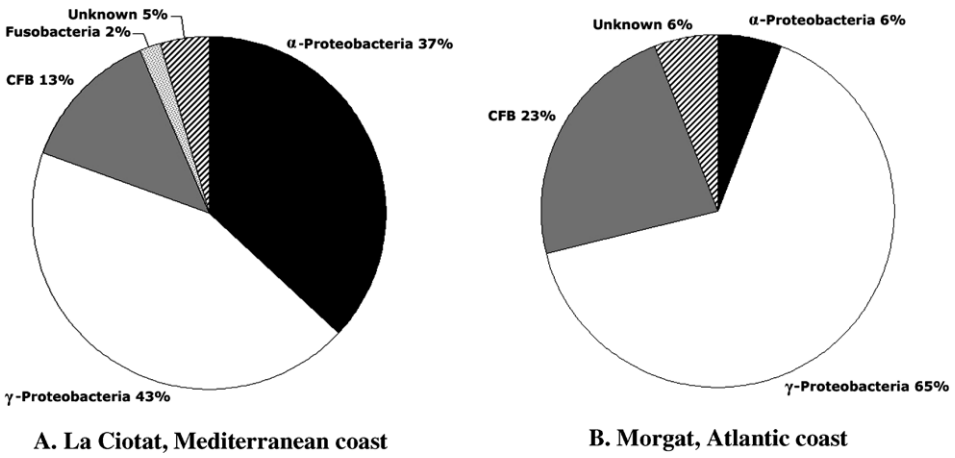

Fig. 4. Pie diagram illustrating the diversity of the partial 16S rDNA bacterial sequencing comprising the clone libraries associated with bald sea urchin lesions of *Paracentrotus lividus* from La Ciotat, Mediterranean coast, on the left (46 clones) and Morgat, Atlantic coast, on the right (52 clones).

Moreover, only one cloned sequence is shared between Atlantic and Mediterranean libraries (Table 3).

3.4. Sequence analyses

Fig. 5 shows the phylogram of the *Alphaproteobacteria* sequences obtained from DGGE and cloning. Five genomospecies were put in evidence (see Section 2). BLAST searches indicated that one genomospecies is a *Rhodobacterales*, another belonged to the *Stappia* genus and the three others were unidentified. One of the unidentified genomospecies included DGGE phylotypes 6 and 7 (detected in samples from both stations) with four clones from La Ciotat and one observed in both localities. This clade was well supported with 97% of bootstrap value (BV). The four other genomospecies were each represented by one clone, two observed in Morgat, the two other in La Ciotat. From the five genomospecies, only one was thus observed in the Mediterranean and the Atlantic.

Ten genomospecies were observed in the *Gammaproteobacteria* (Fig. 6): eight were unidentified, one was a *Vibrio* and the last belonged to the *Colwellia* genus. *Colwellia* sp. included 15 clones, all coming from Morgat, that clustered together with 100% of BV. *Vibrio* sp. included 10 clones, 1 Atlantic and 9 Mediterranean, that also clustered together with 100% of BV. The eight unidentified *Gammaproteobacteria* included from 1 to 10 clones. Each species either came from Morgat or La Ciotat but never from both locations.

Eight genomospecies belonged to the CFB group (Fig. 7) from which four were represented by a single clone (two from each station). DGGE phylotype 5 detected in samples from Morgat and two clones from the Morgat library formed an unidentified CFB genomospecies. They clustered in a clade supported by 100% of BV. DGGE phylotype 1 and one clone, both from Morgat samples, formed a *Flexibacteraceae* genomospecies (100% of BV). DGGE phylotypes 2 and 4 detected in both stations and a clone from Morgat formed a *Bacteroidetes* genomospecies (100% of BV). DGGE phylotype 3 and clones from Morgat and La Ciotat libraries formed a *Cytophagales* genomospecies (56% of BV). These last two genomospecies were thus present in samples from both stations while from the six other CFB genomospecies identified, four infected lesions of echinoids from Morgat and two from La Ciotat.

At all, 24 bacterial genomospecies were observed in bald sea urchin lesions: 5 *Alphaprotobacteria*, 10 *Gammaprotobacteria*, 8 CFB and 1 *Fusobacteria*. Out of them, four were observed in the Atlantic and the Mediterranean, 10 in the Atlantic only and 10 in the Mediterranean only.

4. Discussion

Microscopic observations showed that bacteria infecting body wall lesions of *Paracentrotus lividus* were only present in the central zone while the peripheral zone was invaded by echinodermial cells and the surrounding tissues remained healthy. The cell belt around the central zone seemed thus to act as a barrier to the extension of the infection as suggested by Maes et al. (1986) in a study based on transmission electron microscopy observations. The absence of symbiotic subcuticular bacteria in *Paracentrotus lividus* (McKenzie et al., 1998) and the absence of bacteria on healthy tissues explain the impossibility to amplify bacterial DNA from healthy tissues during the molecular procedure.

On the other hand, bacteria associated with the lesions were numerous and showed a great diversity as several genomospecies, most of them belonging to the *Alphaproteobacteria*, the *Gammaproteobacteria* and the CFB, have been identified. This diversity has been revealed using culture-independent techniques, giving the first exhaustive description of the microbiota colonizing bald sea urchin lesions. The present molecular analysis also revealed that individuals of a same bald sea urchin-diseased echinoid species originating from distinct locations show different bacterial populations in their lesions. Indeed if four genomospecies (an unidentified *Alphaproteobacterium*, a *Vibrio*, a *Bacteroidetes* and a *Cytophagales*) were observed in lesions of echinoids from the Mediterranean

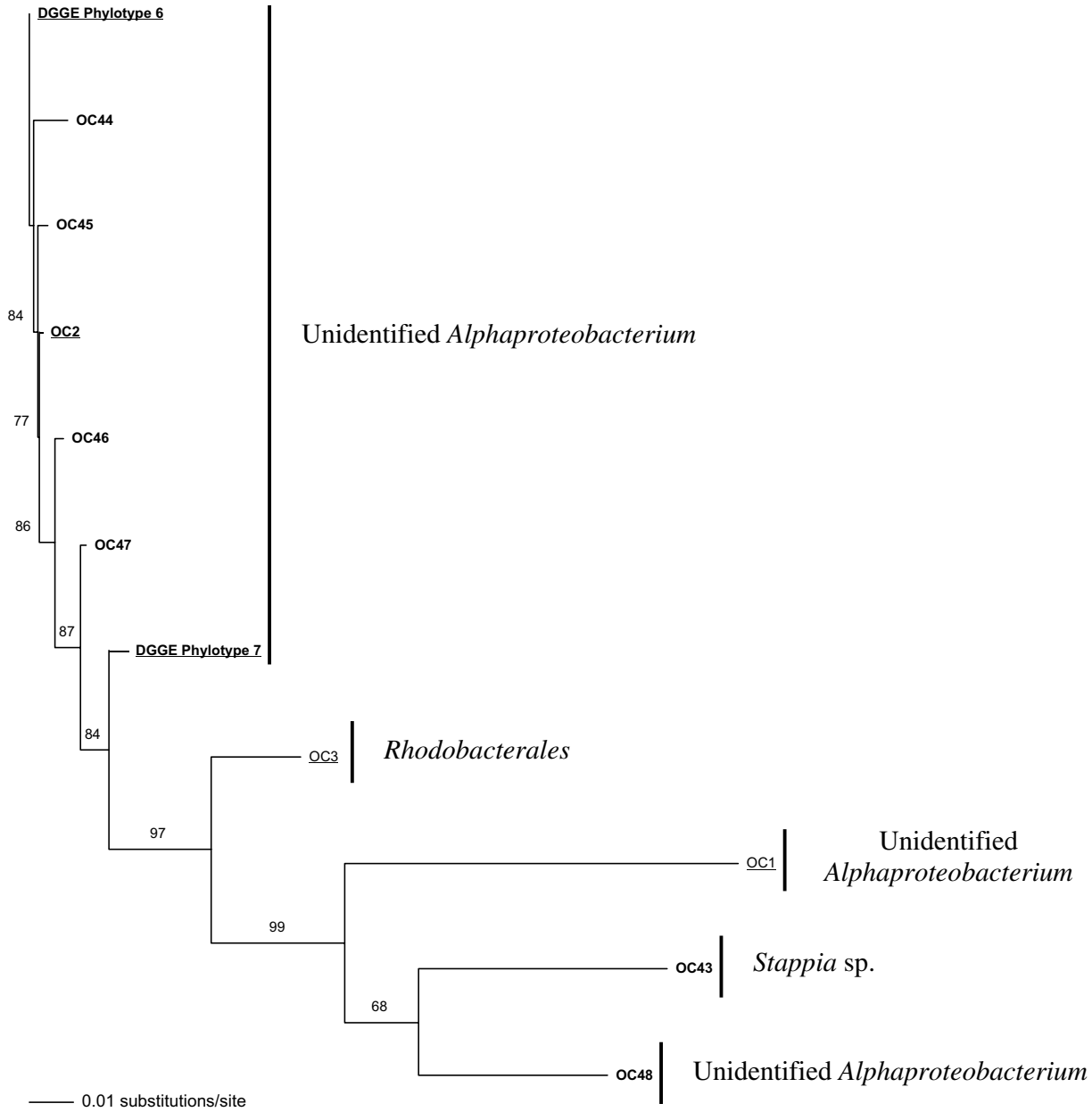

Fig. 5. Unrooted phylogram constructed using 16S rDNA sequences of DGGE phylotypes and clones of *Alphaproteobacteria* (sequences length ranging from 325 to 487 bases). The tree was built with the neighbour-joining method using the Jukes and Cantor distance. Values at nodes indicate bootstrap values (1000 replicates). Names in bold correspond to sequences obtained from samples from La Ciotat (Mediterranean coast), names underlined correspond to sequences obtained from samples from Morgat (Atlantic coast) and names in bold and underlined correspond to sequences obtained from samples from both stations.

and the Atlantic, twenty others were present either in the Atlantic or in the Mediterranean. According to Pommier et al. (2007), *Alphaproteobacteria*, *Gammaproteobacteria* and CFB are worldwide the most represented bacterial phyla in marine bacterioplankton. Moreover, the latter shows a high degree of endemism and includes few cosmopolitan species. The composition of the bacterial communities associated with *P. lividus* lesions follow this trend, indicating that the bald sea urchin disease is probably due to opportunistic bacteria originating from the sea water.

The microbial communities associated with lesions of *P. lividus* from Morgat and La Ciotat were dominated by *Gammaproteobacteria* that represented 43–65% of the total clones while CFB and *Alphaproteobacteria*, also detected by DGGE, accounted, respectively, for 13–23% and 6–37% of the clones libraries. Interestingly, these three bacterial groups were not only revealed in body wall

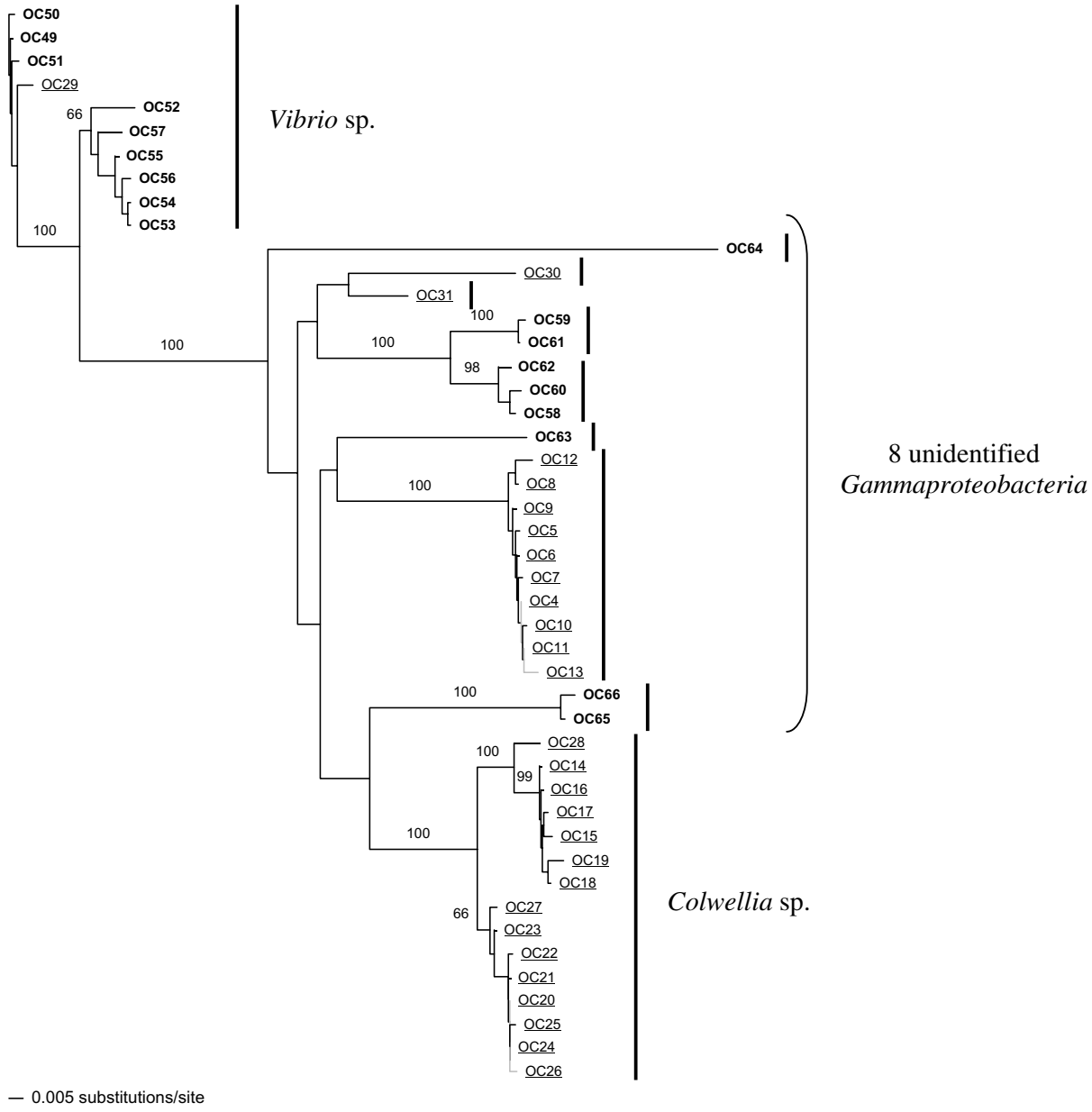

Fig. 6. Unrooted phylogram constructed using 16S rDNA sequences of clones of *Gammaproteobacteria* (sequences length ranging from 359 to 510 bases). The tree was built with the neighbour-joining method using the Jukes and Cantor distance. Values at nodes indicate bootstrap values (1000 replicates). Names in bold correspond to sequences obtained from samples from La Ciotat (Mediterranean coast) and names underlined correspond to sequences obtained from samples from Morgat (Atlantic coast).

lesions of the echinoid *Tripneustes gratilla* (Becker et al., 2007) but also in skin ulcerations of the sea cucumber *Holothuria scabra* (Becker et al., 2004) using bacterial cultures and DGGE. Moreover, *Gammaproteobacteria* and CFB have been isolated from tegumental lesions of the following echinoids: *Archaeopneustes hystrix* and *Paleopneustes cristatus* with *Vibrio alginolyticus* (Bauer and Young, 2000), *Meoma ventricosa* with *Pseudoalteromonas* sp. (Nagelkerken et al., 1999), *Strongylocentrotus droebachiensis* with *Acinetobacter* sp. (Roberts-Regan et al., 1988), *S. intermedius* with *Flexibacter* sp. (Tajima et al., 1997b) and *S. purpuratus* with *Aeromonas salmonicida*, *Flavobacterium* sp., *Pseudomonas* sp. and *Vibrio anguillarum* (Gilles and Pearse, 1986). Accordingly, *Vibrio*, which is ubiquitous in aquatic environments, is the most represented bacterial genus in skin lesions of echinoids, as it has been detected in five species worldwide: *A. hystrix* and *P. cristatus* in the Caribbean, *P. lividus* in France, *S. purpuratus* in California and *T. gratilla* in Madagascar. Consequently, although bacterial species associated with body wall lesions vary with the location of the echinoid population, the same high rank bacterial taxa are generally observed. Investigating skin lesions in other sea urchins (and other echinoderms) species using culture-

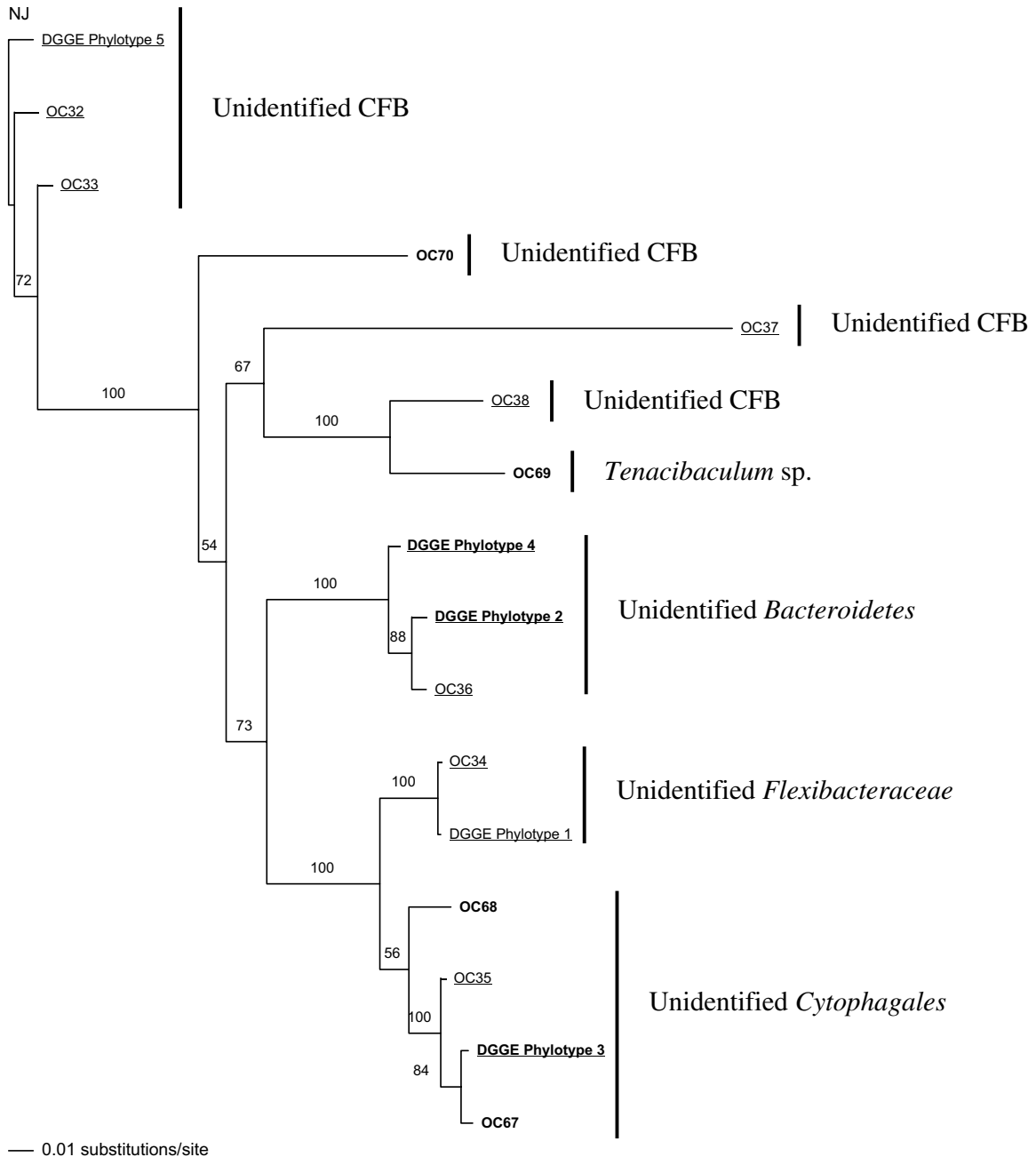

Fig. 7. Unrooted phylogram constructed using 16S rDNA sequences of DGGE phylotypes and clones of CFB bacteria (sequences length ranging from 278 to 502 bases). The tree was built with the neighbour-joining method using the Jukes and Cantor distance. Values at nodes indicate bootstrap values (1000 replicates). Names in bold correspond to sequences obtained from samples from La Ciotat (Mediterranean coast), names underlined correspond to sequences obtained from samples from Morgat (Atlantic coast) and names in bold and underlined correspond to sequences obtained from samples from both stations.

independent techniques would be interesting to confirm this trend.

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