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A single betaproteobacterium dominates the microbial community of the crambescidine-containing sponge *Crambe crambe*

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Crambe crambe is a marine sponge that produces high concentrations of the pharmacologically significant pentacyclic guanidine alkaloids (PGAs), Crambescines and Crambescidines. Although bio-mimetic chemical synthesis of PGAs suggests involvement of microorganisms in their biosynthesis, there are conflicting reports on whether bacteria are associated with this sponge or not. Using 16S rRNA gene pyrosequencing we show that the associated bacterial community of *C. crambe* is dominated by a single bacterial species affiliated to the Betaproteobacteria. Microscopy analysis of sponge tissue sections using a specific probe and *in situ* hybridization confirmed its dominance in the sponge mesohyl and a single microbial morphology was observed by transmission electron microscopy. If confirmed the presence of a simple bacteria community in *C. crambe* makes this association a very pertinent model to study sponge-bacteria interactions and should allow further research into the possible implication of bacteria in PGA biosynthesis.

Marine sponges (phylum Porifera) host a diverse array of associated microorganisms including unicellular algae, Cyanobacteria, heterotrophic bacteria and Archaea[1–5]. Although these associations are ubiquitous, the degree of association with microorganisms varies among host species. In bacteriosponges[6] or high microbial abundance (HMA) sponges[2] bacteria can constitute up to 40% of their biomass and generally present a relatively high diversity while low microbial abundance (LMA) sponges harbor much smaller bacterial communities with a lower bacterial diversity[3,4,7–9]. LMA sponges are usually dominated by one or two phyla, usually belonging to the *Proteobacteria* or the *Cyanobacteria*[8,9] whereas HMA sponges can harbor more than 8 phyla[7,9]. Many of the bacteria associated with sponges fall into sponge-specific clusters that have been recovered from several different sponge species but not from the surrounding seawater[5,10]. However, an in depth pyrosequencing study on bacterial diversity in 32 sponges from 8 locations revealed that the majority of bacterial OTUs (operational taxonomic units) were specific to a given sponge[4].

Sponges are also an important source of bioactive marine secondary metabolites making these organisms a target for research on compounds of pharmaceutical interest. The fact that many compounds found in sponges are complex polyketides or non-ribosomally synthesized peptides, whose biosynthesis is mostly associated with microorganisms suggests a bacterial origin for many sponge secondary metabolites[11–13]. However, this has only been unequivocally proven in a few cases. For example, a *Salinospora* strain isolated from the marine sponge *Pseudoceratina clavata* has been identified as a source of rifamycin antibiotics produced via polyketide biosynthesis[14].

In addition to polyketides and non-ribosomally synthesized peptides, other chemical classes such as pentacyclic guanidine alkaloids (PGAs) that exhibit a wide range of activities[15,16] have been extracted from the tissues of marine sponges. PGAs are mostly present in sponges of the Order *Poecilosceridae* [e.g. *Crambe* sp.[17] *Monanchora* sp.[18] *Batzella* sp.[19] *Hemimycale* sp.[15]] but also reported in *Halichondrida* sponges [e.g. *Ptilocaulis* sp.[20]]. This relatively wide phylogenetic distribution, as well as a hypothesized -albeit controversial (Olivier Thomas, personal

communication), biosynthetic pathway involving a polyketide-like precursor[21,22], points to a possible involvement of microorganisms in PGA biosynthesis. Remarkably, very few studies have been conducted to examine the microbial community associated with sponges known to produce these alkaloids, mostly using electron microscopy and focusing on the sponge *Crambe crambe.*

Crambe crambe (Schmidt, 1862) (Poecilosclerida) is a red incrusting marine sponge present in the Mediterranean Sea and reported to produce diverse PGAs, namely crambescidins 800, 816, 830, 844, as well as isocrambescidin 800[17,22]. The presence of bacteria associated with this sponge is somewhat controversial. Based on scanning electron microscopy *C. crambe* has a surface devoid of epibionts[23]. In addition Uriz and colleagues reported that cells and mesohyl of this sponge were free of microsymbionts (bacteria or cyanobacteria) when examined by transmission electron microscopy[24]. This observation contrasted to that of Sará[25] who reported sporadic occurrence of cyanobacteria in specimens from Italian shores. Although several thousand sponge-derived 16S rRNA gene sequences are now available[5], the few sequences originating from *C. crambe* are from unpublished studies. Two such partial sequences clustered together within a Betaproteobacteria cluster[5]. However, since in that study the sequences were added to a tree in a second parsimony step, their phylogenetic relationship to the other sponge-derived sequences is not clear.

In order to address these contrasting reports, we examined the diversity of 16S rRNA genes amplified from extracts from the sponge *C. crambe*, using 454-tag pyrosequencing and sequencing of 16S rRNA gene clone libraries for greater phylogenetic resolution. To demonstrate the presence of bacteria in *C. crambe*, the sponge tissue of a different specimen was examined using transmission electron microscopy (TEM), and the dominant bacterium was localized using a specific oligonucleotide probe combined with catalyzed reported deposition fluorescence hybridization (CARD-FISH).

Results

Bacterial community diversity analyses. *16S rRNA gene community analysis of Crambe crambe.* A total of 43 16S rRNA gene clones from *C. crambe* tissues were sequenced. After sequence quality and chimera checks a relatively high number of clones (11) were assigned as chimeras (26% of clones). The 32 remaining clones generated 12 OTUs (> 97% identity), which fell into 4 bacterial classes: Flavobacteriia, Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria and a few clones were assigned as chloroplasts. 17 sequences grouped into 9 OTUs were assigned as planktonic bacteria (> 98% identity). Phylogenetic analysis of the dominant non-chimeric and non planktonic OTU represented by 13 clones placed these sequences in a clade most closely related to the Betaproteobacteria, but separate from the known described betaproteobacterial orders (Figure 1; dashed line), and recently reported in a study of sponge-associated bacteria of the Mediterranean sponge *Tethya aurantium*[26].
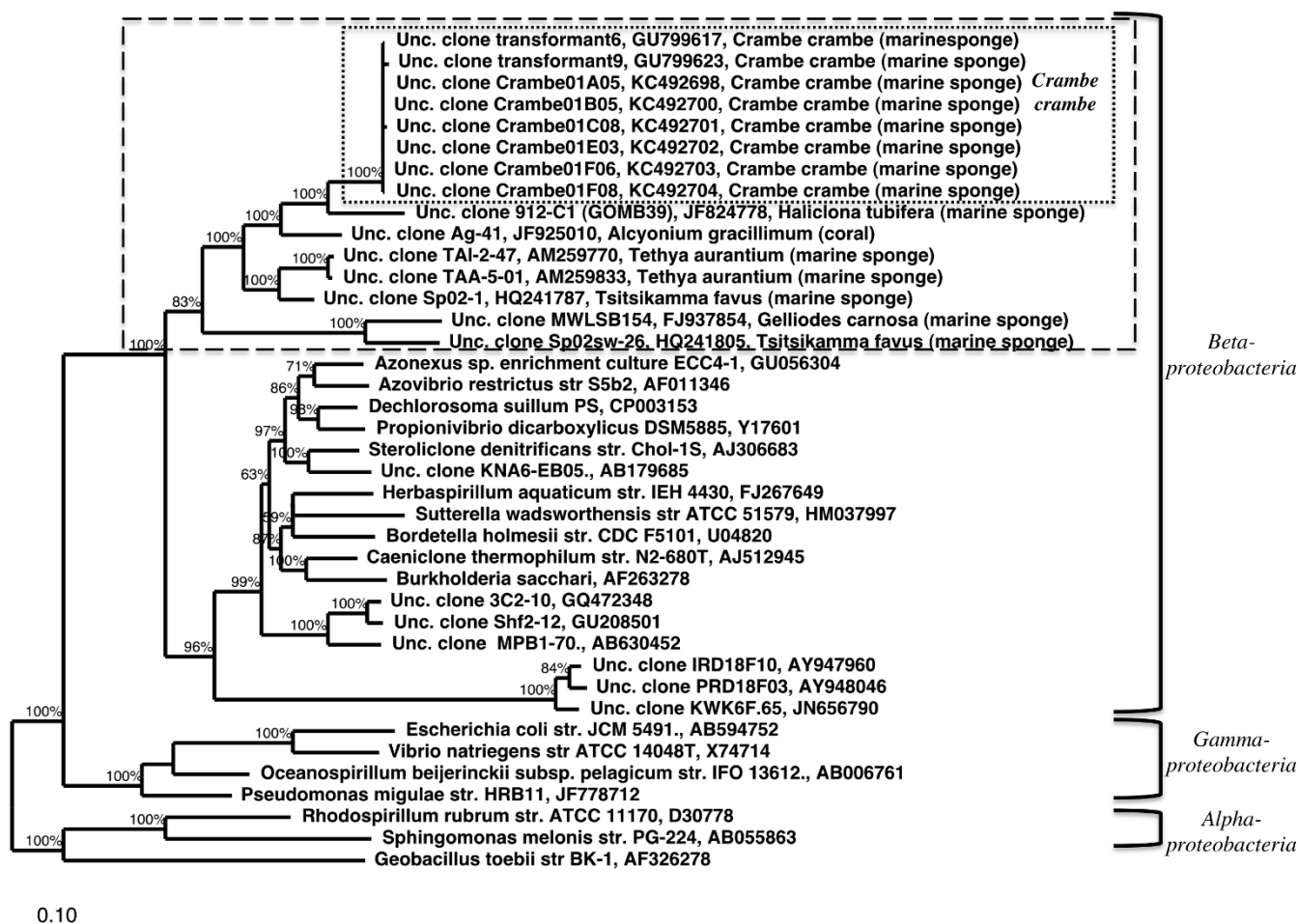

Figure 1 | Evolutionary relationship of the dominant *C. crambe* bacterium to its closest relatives inferred from almost full-length 16S rRNA gene sequences, using Bayesian phylogenetic reconstruction. Sequences obtained from the sponge *C. crambe* are shown in the smallest box. The accession numbers of the *C. crambe* sequences obtained in this study are pre-fixed by KC. A cluster of sequences associated with marine sponges and a coral is shown within the dashed lines. Percentage confidences for the Bayesian analysis are indicated at each node.

454- 16S rRNA Pyrosequencing analyses. From an initial 5973 raw bacterial 16S rRNA tag sequences obtained from *C. crambe*, 4421 sequences remained after denoising by *AmpliconNoise*. Subsequent stringent chimera detection removed a significant number of chimeric sequences, many of which were singletons, which would have otherwise significantly overinflated the total bacterial diversity (213 chimeric sequences representing 70 OTUs). Rarefaction analysis of non-chimeric sequences shows a low level of diversity with rarefaction curve reaching a plateau at about 115 OTUs (Figure S1). This low diversity was also reflected by a Chao1 value of 107.7 and a small total number of OTUs recovered (86 OTUs). Furthermore, among these OTUs, a single OTU, very similar ($> 99\%$ identity) in the V1-V3 region to the dominant clone in the 16S rRNA library, represented 74% of the total sequences (3271 sequences). Among the remaining 85 OTUs, 42 (representing 22% of total sequences) were classified as planktonic. Therefore, considering only non-planktonic bacteria, likely transient in the sponge, this dominant betaproteobacterium accounted for a remarkable 95% of the total sequences.

Catalyzed reporter deposition fluorescence in situ hybridization analyses (CARD-FISH). After optimization, CARD-FISH with the general bacterial probe confirmed the presence of bacteria in the sponge tissue. Initial hybridization experiments were performed with the general bacterial probe EUB338-I and the negative control probe NON338 according to a previously published protocol for worm symbionts[27] except that a lysozyme permeabilization step was necessary to obtain a good fluorescent signal. Strong signals were observed with the EUB338-I probe (Figure 2a) demonstrating the presence of sparsely distributed bacteria within the sponge tissue corresponding to smaller cells labeled with DAPI (Figure 2b).

The DAPI staining showed two types of cells with some variation in size (Figures 2b–d). Based on an overlap between DAPI staining and the bacterial probe hybridization, we identified the smaller cells as bacteria and the larger spherical cells (Figure 2b; arrow) as the nuclei of sponge cells. Observation of different tissue sections showed the same results indicating that bacteria were sparsely distributed in all tissues of the sponge. Negative controls including the anti-sense probe NON338 and a no-probe control yielded no signals indicating the stringency of the hybridization and a lack of endogenous peroxidases in the sponge tissue.

The same tissue sections were also stained with DAPI and hybridized with the specific BET 467 probe and its helper probes (sequences shown in Table 1). Strong signals were observed with this specific probe (Figure 2c). Probe BET467 hybridized to the overwhelming majority of the small cells stained with DAPI and identified as bacteria (Figure 2c–d; circles) demonstrating a dominant presence in tissues of *C. crambe* of the betaproteobacterial clade found in the clone library and pyrosequencing. Not all the bacteria present (EUB 338-I) in the sponge mesohyl were hybridized with the specific BET467 probe (Figure 2c–d; square) in accordance with the clone library and 454-pyrosequencing results that indicated that other bacteria were also associated with the sponge. Tissue sections were also hybridized with probe BET42a targeting the Betaproteobacteria class (along with a competitor unlabeled probe targeting
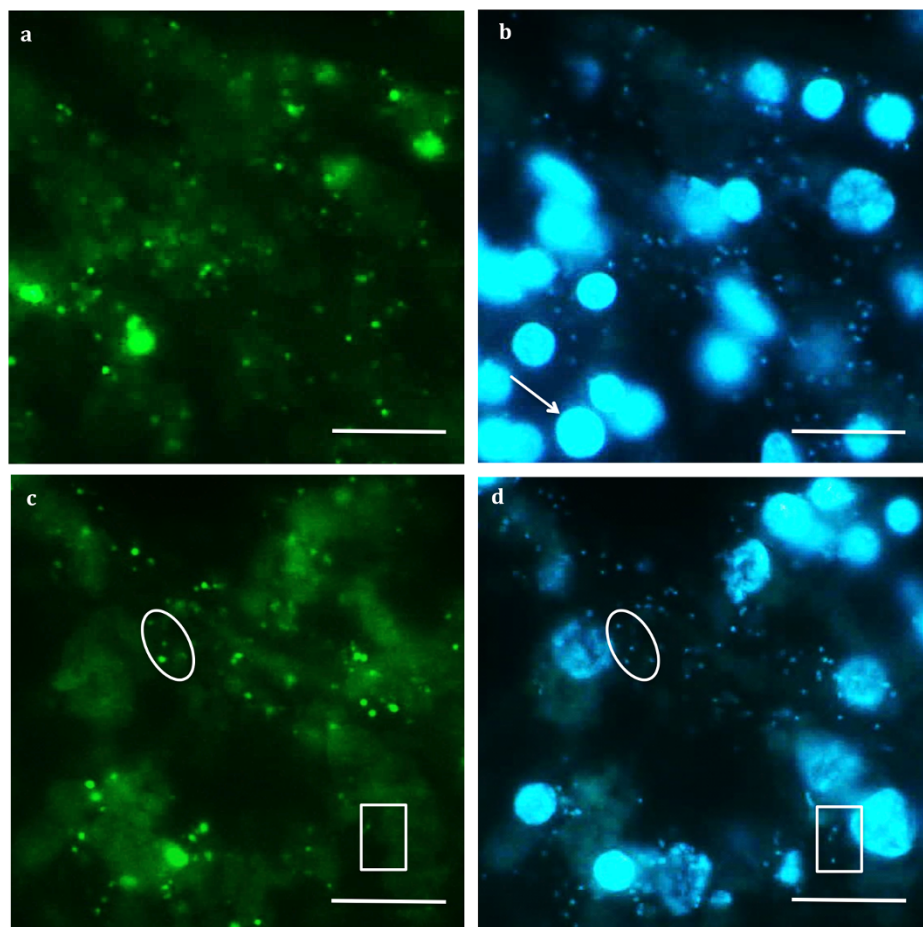

Figure 2 | Detection of bacteria in the mesohyl of *Crambe crambe* by CARD-FISH on sponge-tissue sections. Tissue section hybridized with the EUB 338 probe targeting the domain Bacteria, (a) and the corresponding DAPI-stained image, (b). Tissue section hybridized with the BET467 probe targeting the specific Betaproteobacteria, (c) and the corresponding DAPI-stained image, (d). Arrow: sponge cell nucleus. Oval: cells labeled with both DAPI and the BET467 probe. Rectangle: some cells labeled with DAPI do not hybridize with the BET467 probe.

3

Table 1 | Details of the specific horse-radish peroxidase (HRP) labeled oligonucleotide probes used in this study, together with the unlabeled competitor and helper (H-) probes

Probe	Sequence (5′–3′)	Specificity	E.coli target position	Label	Reference
EUB338-I	GCTGCCTCCCGTAGGAGT	Eubacteria	16S rRNA 338–355	HRP	62
NON-EUB338-I	ACTCCTACGGGAGGCAGC	Eubacteria Negative control	16S rRNA 338–355	HRP	63
BET42a	GCCTTCCCACTTCGTTT	Betaproteobacteria	23S rRNA 1027–1043	HRP	64
BET42a competitor	GCCTTCCCACATCGTTT	Gammaproteobacteria	23S rRNA 1027–1043	–	64
ARCH915	GTGCTCCCCCGCCAATTCCT	Archaea	16S rRNA 915–934	HRP	65
BET467	CGTCATGGACGCGGCCTG	*C. crambe* Betaproteobacteria	16S rRNA 467–484	HRP	This study
H-485	GGCGCTTCTTCTCAGGGTAC	Helper	16S rRNA 485–504	–	This study
H-446	TTCGCCCGCGTTTTTCTTTC	Helper	16S rRNA 446–466	–	This study

Gammaproteobacteria). Surprisingly no signal was detected. Finally, no signal was detected in hybridizations with the ARCH915 probe suggesting the absence of Archaea in *C. crambe*. Even though FISH targeting eukaryotes was not performed, only sporadically red/orange fluorescence was observed under green excitation, thus it is unlikely that photosynthetic eukaryotes or cyanobacteria are important members of the microbial community associated with *C. crambe*.

Although we did not use a confocal microscope, and thus did not accurately quantify bacterial cells, an analysis of 20 different 20 μm² areas showed an average of 6 bacterial cells/sponge cell (5 Betaproteobacteria/sponge cell), resulting roughly in 3000 bacterial cells (2500 Betaproteobacteria cells) per cm² of thin section. Since these micrographs were not randomly visualized, these numbers are likely upper estimates.

Transmission electron microscopy of thin sections of *Crambe crambe* tissue. Ultra thin sections were prepared from one specimen of *C. crambe* and inspected by transmission electron microscopy to observe the localization and the morphotypes of bacteria associated with this sponge. The results revealed a low density of morphologically uniform intercellular bacteria scattered in the mesohyl compartment of *C. crambe*. Bacteria were found embedded in the collagen matrix (Figure 3a–b) in the mesohyl, surrounding sponge cells remarkably including spherulous cells (Figure 3b–c). The rod-shaped bacteria observed of approximately 0.25 μm width and 0.5 μm length present dense chromatin condensation and an undulating surface. Bacteria in a potential state of cell division were also found in the mesohyl of the sponge, suggesting that the betaproteobacterium may be actively growing in the sponge tissue

(Figure 3d). Ultra thin sections from the 10 subsamples cut in different orientations were observed under the transmission electron microscope and similar results were found.

Discussion

To date, all investigations of microorganisms associated with sponges in the genus *Crambe*, were performed by electron microscopy and microbial cultivation. Early studies[1,25], reported low densities and diversity of microorganisms associated with these sponges. Later studies suggested that in fact those sponges were virtually devoid of epibionts and mesohyl-associated bacteria and this absence was suggested to be linked to the high toxicity associated of extracts of the sponge[24].

Our results demonstrate, through a combination of molecular techniques and microscopic studies, that bacteria can be associated with the tissues of the PGA containing sponge *C. crambe*, and that in our samples these bacteria overwhelmingly belong to a specific clade affiliated to the Betaproteobacteria (95% of the total non planktonic sequences generated with 454 pyrosequencing) in agreement with a recent report that specific bacteria (particularly within the phylum Proteobacteria) were found as dominant members of microbial communities associated with LMA sponges[8]. We believe that confirmation of a single dominant OTU was possible because we used a stringent denoising and chimera detection pipelines, as well as our bioinformatic approach to "filter" out primarily planktonic organisms. This dominant bacterial OTU was affiliated to a specific uncultured betaproteobacterial clade, which was not identified amongst the planktonic bacteria compiled from the literature, and a large 16S rRNA cloning effort[29]. Attempts to cultivate this microorganism in
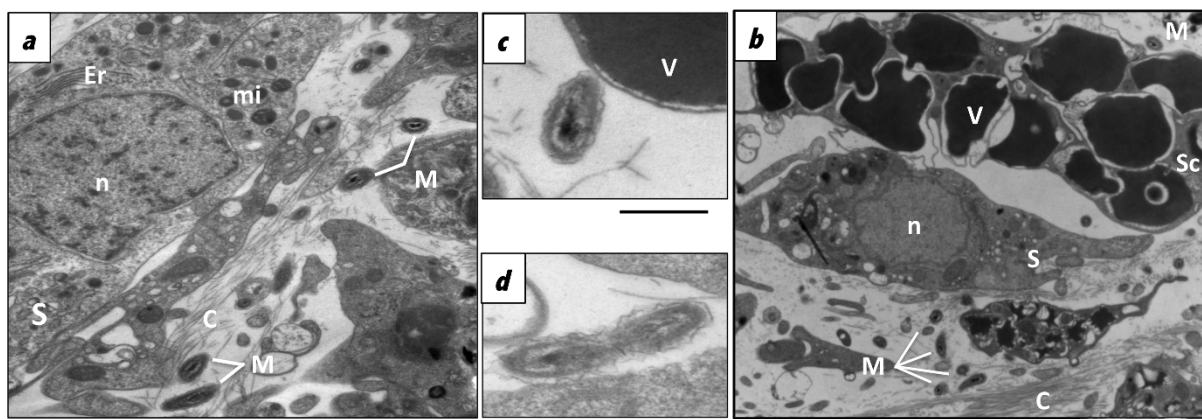

Figure 3 | Transmission electron microscopy of thin sections of *C. crambe* tissues. Microorganisms in the mesohyl of *C. crambe* embedded in the collagen matrix of the sponge, (a). Proximity between microorganisms and spherulous cells, (b–c). Bacteria in division, (d). C: collagen; Er: endoplasmic reticulum; mi: mitochondrion; M: Microorganisms; n: nucleus; S:sponge cell; Sc: Spherulous cell whose vesicles (V) contain an electron dense material.

rich and diluted R2A broth, as well as in media targeting ammonium-oxidizing bacteria were unsuccessful (data not presented).

The phylogenetic analyses of full-length 16S rRNA genes demonstrated that the clade including the *C. crambe* Betaproteobacteria branches separately from the clade containing the remainder of previously described and cultured Betaproteobacteria (dashed box in Figure 1). This clade contains exclusively sequences of bacteria associated with marine invertebrates including sequences of clones in an unpublished clone library of bacteria retrieved from *C. crambe* (D. Sipkema personal communication) that was sampled from a different site in the Mediterranean Sea, clones retrieved from the sponge *Haliclona tubifera* and clones from another LMA sponge *Tethya aurantium*, also from the Mediterranean Sea[26]. It is important to remark that in the available Silva 108 taxonomy this clade was assigned as Nitrosomonadales (thus in the main Betaproteobacteria branch), but our refined phylogenetic placement supports the basal placement of the clade within the Betaproteobacteria as reported by Thiel and collaborators[26]. This clade placement could be 1) real or 2) an artifact caused by a higher evolutionary rate known to occur in symbiotic microorganisms[30,31]. Case 1) above is supported by the observation that the probe Bet42a for the class Betaproteobacteria (targeting the 23S rRNA) did not yield a signal in CARD-FISH experiments targeting the *C. crambe* symbiont, indicating that this clade is distant from the remainder of the Betaproteobacteria. Metagenomic analyses in progress should allow us to clarify the real phylogenetic placement of this clade by the analysis of additional conserved genes.

Interestingly, several attempts to amplify a large fragment of the rRNA operon as previously described by Suzuki and coworkers[32] failed, despite the use of several combinations of forward primers targeting the 16S rRNA gene and reverse primers targeting the 23S rRNA, strongly suggesting that the 16S rRNA gene is unlinked to the 23S rRNA gene in the *C. crambe* associated bacterium (data not shown). Both unlinked 16S-23S rRNA genes and large insertions in the rRNA operon are common in endosymbiotic bacteria and obligate intracellular parasites[33,34], thus supporting Case 2) above and if proven would suggest a stronger and perhaps obligatory symbiosis between this betaproteobacterium and *C. crambe*. If this were the case, it would be interesting to determine how the dominant *C. crambe* associated bacterium is acquired. This betaproteobacterium could be part of the "rare biosphere" which would be filtered by sponge and whose growth is favored in the sponge tissue. Alternatively there could be a vertical transfer of the bacteria via their larvae since bacteria were observed in the mesohyl, the surface and in the posterior region of *C. crambe* larvae[28]. This form of transmission is common in sponges and insects when the bacteria are obligate symbionts[35,36]. Interestingly, the reproductive stages of LMA sponges were generally reported as bacteria-free[37]. Regardless of their origin, the maintenance of the desired symbionts and exclusion of other bacteria by the host sponge may be mediated through the mechanism of antibiosis[38]. This mechanism is based on the ability of few bacteria to adapt to the presence of antibiotics produced by the host. *C. crambe* is known to produce a wide variety of toxic compounds with high antimicrobial activity[23], which could help explain the selection of a single symbiont.

To confirm the localization of bacteria within the tissues of *C. crambe*, two complementary microscopy techniques were employed. The TEM pictures revealed a low density of morphologically uniform intercellular bacteria scattered in the mesohyl of *C. crambe*, already noted by Vacelet and Donadey in 1977 for *Crambe sp.*[1] and in agreement with previous observations reporting that microorganisms associated with LMA sponges, are sparse and free living in the sponge mesohyl[1]. Interestingly the dominant bacterial morphotype was also observed surrounding the spherulous cells (Figure 3) that are specialized cells that seem to be responsible for the storage of secondary metabolites[24,39,40]. The proximity between bacteria and these

specialized cells could indicate their possible resistance to the highly-toxic compounds isolated from *C. crambe*, and further support the symbiosis between these organisms. The observation of several bacteria in the process of cell division suggests that these bacteria are active. In the LMA sponge *Polymastia* sp., the dominant Alphaproteobacteria phylotypes were represented in 16S rRNA clone libraries constructed from both DNA and RNA also suggesting that the bacteria in these LMA sponges were active[7].

The application of a specific oligonucleotide probe in conjunction with CARD-FISH allowed us to microscopically demonstrate the presence of the dominant betaproteobacterium within the *C. crambe* tissues, which to our knowledge has not been shown for other single associations between Proteobacteria and LMA sponges. The majority of the bacteria (Figure 2d) were also labeled with the specific probe (Figure 2c) in agreement with the dominance of this bacterial OTU in the 16S rRNA gene clone library and in the pyrosequencing data.

In our study, two sponge specimens collected one year apart were used, and using alternative techniques showed agreeing results. Sequencing of 16S rRNA genes in the 2011 sample and CARD-FISH with the 2012 sample, show the dominance of the same specific bacterium in these two specimens suggesting that the association between the betaproteobacterium and *C. crambe* is stable, although sampling through an annual cycle with replicate sponge specimens should be done to confirm this. Such a study was carried out on the LMA sponge *Tethya stolonifera*. In that sponge, the dominant bacterial OTU was also affiliated to a sponge-specific Betaproteobacteria cluster and the community remained very stable over a two-year period[9].

A comprehensive study of sponge symbionts revealed 26 different bacterial phyla with most of the diversity occurring within the Proteobacteria[4]. The bacterial sequences retrieved from tropical sponges were more closely related to each other than from the subtropical ones, indicating that bacterial community composition could be influenced more by temperature than their localization in the same water mass[4]. This latitudinal separation of bacterial communities was also shown for five LMA sponges where the temperate sponge *Raspailia topsenti* was dominated by Betaproteobacteria whereas the tropical LMA sponges were dominated by Alphaproteobacteria, Gammaproteobacteria or Cyanobacteria[8]. Betaproteobacteria sequences also represented the dominant OTU in the temperate LMA sponges *Tethya stolonifera*[9], and in *Scopolina* sp. and *Tedania anhelans*[41]. These independent studies and our own indicate that Betaproteobacteria-LMA sponge associations in temperate waters may be a global feature but that within this clade, each sponge appears to harbor a phylogenetically distinct betaproteobacterial OTU.

Our study adds further weight to others, which have shown the low diversity within LMA sponges, making them simpler models to study the roles and molecular basis of these symbioses. From a practical standpoint in contrast to HMA sponges which harbor dense and diverse microbial communities[2,6], metagenomic or single-cell genomic techniques[42] would be simpler to conduct with simpler models such as LMA sponges harboring a single dominant OTU, as would be techniques such as transcriptomics and proteomics to study the expression of specific bacterial genes. Furthermore, a possible role of bacteria in the synthesis of secondary metabolites extracted from highly active sponges would be easier to evaluate in LMA sponges. In the specific case of *C. crambe*, and its secondary metabolites, earlier results reporting the lack of associated microflora[23,24], as well as the known high concentrations of guanidine alkaloids (about 1% of dry mass) strongly supported the idea that the sponge cells were solely responsible for the biosynthesis of these highly bioactive metabolites. However, biomimetic studies indicate that crambescidin-like molecules are produced via a condensation of guanidine to a polyketide-like molecule[21], and since polyketides are primarily produced by microorganisms, this biosynthesis model suggests a microbial

implication in PGA biosynthesis. Our report of the dominance of a single OTU in the tissues of *C. crambe*, means that the implication of these bacteria in the biosynthesis of PGAs cannot be completely ruled out.

Methods

Sample collection. The *Crambe crambe* specimen (one sponge individual growing in a rocky substrate) for 16S rRNA gene analysis were collected in the Bay of Banyuls-sur-Mer (Western Mediterranean, France; 42° 28.828' N −3° 08'666' E) at a depth of approximately 10 meters on 5 January 2011. For CARD-FISH and TEM, one sponge individual (meaning a sponge associated to both shells of the bivalve *Arca noae*) was collected at the same location on 4 January 2012. For both collections samples were placed in plastic bags underwater to avoid contact with air and immediately transported to the laboratory. The sponge specimens were rinsed 3 times in calcium magnesium free - artificial seawater (CMF-ASW) to remove exogenous particles and loosely attached bacteria present in the sponge's aquiferous channels. Sponge tissue was processed 30 minutes after sampling to avoid possible modifications of the microbial community associated with the sponge during processing. The washed specimen collected in 2011 was frozen at −80°C for subsequent DNA extraction whereas the 2012 specimen was fixed immediately for CARD-FISH and TEM analysis (see below).

DNA extraction. Cleaned sponge fragments (2011 sample) were pooled (35 g total) and homogenized in two volumes of CMF-ASW using a Waring blender with 3 cycles of: 15 s at 22000 rpm, 30 s pause, 15 s again at 22000 rpm, with a 60 s pause in between each cycle. The homogenate was centrifuged for 5 min at 3000 rpm to pellet larger particles and sponge cells. The remaining sponge cells and bacteria in the supernatant were pelleted by centrifuging for 30 min at 8000 rpm. The pellet was washed twice by resuspending in 35 ml of CMF-ASW and centrifuging at 8000 rpm for 30 min. The final pellet was resuspended in 35 ml of CMF-ASW, filtered though GF/A glass fiber filters (1.6 μm nominal retention size) to remove remaining sponge cells and centrifuged once more at 8000 rpm for 30 min. This pellet was frozen in 35% glycerol and stored at −80°C until further analysis.

To extract DNA, cells present in the pellet were disrupted by bead beating (3 pulses of 30 s at 25 Hz) using a model MM301 cell disruptor (Retsch, Germany) and 200 μm low-protein binding zirconium beads (OPS Diagnostics, Bridgewater, NJ, USA). The lysate was then gently mixed with an equal volume of guanidinium thiocyanate buffer[43]. Nucleic acids were recovered using a standard phenol and chloroform extraction, and washed and concentrated in TE buffer using Centricon 30 microconcentrators (Amicon, Danvers MA).

16S rRNA gene library analysis. Extracted DNA was used as template for 16S rRNA gene amplification using modified universal primers targeting Bacteria, 27Fmod (5' AGRGTTTGATCMTGGCTCAG 3') and 1492Rmod (5' TACGGYTACCTTGT-TAYGACTT 3') as previously described[44]. Amplification was performed in a 40 μL total-reaction volume with 1X Platinum *Taq* DNA polymerase buffer, 500 nM of each primer, 0.8 mM dNTPs, 2 mM MgCl$_2$, 2 μg/ml BSA, 0.025 U/μl Platinum *Taq* DNA polymerase, and 200 ng of extracted DNA. PCR cycling was as follows: 94°C for 3 min, 27 cycles of 94°C for 1 min, 50°C for 1 min 72°C for 2 min followed by 72°C for 10 min.

PCR products were run in a modified TAE agarose gel and purified with Ultrafree DNA columns (Millipore, Billerica, MA) using the manufacturer's protocol. Purified PCR products were cloned using the TOPO TA Cloning® Kit for Sequencing using One Shot TOP 10® Electrocompetent Cells (Life Technologies) according to manufacturers instructions and plasmid purification was performed with the Montage Plasmid Miniprep 96 Kit (Millipore). Plasmid inserts were sequenced using the dideoxy-terminator reaction using the 907 reverse primer [5' CCG TCA ATT CCT TTG AGT TT 3'][45] and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Cycle sequencing products were cleaned using the Agencourt® CleanSeq® Dye-Terminator Removal kit (Beckman Coulter, Brea, CA) and run in an AB3130xl Genetic Analyzer (Life Technologies).

Partial, raw sequences were assembled using 20 nucleotides minimum overlap and 99% maximum mismatches and visually edited using the gap4 program of the Staden package[46]. Edited contigs were exported in fasta format including the number of reads

Table 2	Summary of CARD-FISH protocol optimized for the detection of bacteria in marine sponge tissue sections. RT, room temperature		
Stage	**Step N°**	**Description**
Fixation	1	Incubate sponge tissue in 4% buffered paraformaldehyde (4°C, 4 h)
	2	Wash 3 times in same phosphate buffer (RT)
	3	Dehydrate in 70% ethanol (4°C, 24 h)
Tissue Sectioning	4	Embed tissue samples in paraffin
	5	Cut 10 μm thick sections with a microtome onto polysilane-coated microscope slides, leave to dry 3 h at 35°C
Deparaffinization	6	Incubate slides twice in xylene (RT, 10 min each)
	7	Rehydrate in an ethanol series (95%, 80%, 70%; 10 min each)
Inactivation of endogenous peroxidases	8	Incubate in 0.2 M HCl (RT, 12 min)
	9	Incubate in 20 mM Tris.HCl (RT, 10 min)
Permeabilization 1	10	Cover tissue sections with proteinase K, incubate in humid chamber (37°C, 5 min)
	11	Wash slides in 20 mM Tris.HCl (RT, 10 min), air dry
Permeabilization 2	12	Dip slides in 0.1% low melting point agarose and air dry (10 min)
	13	Cover tissue sections with lysozyme, incubate in humid chamber (37°C, 1 h)
	14	Wash slides in milliQ water (1 min)
	15	Dehydrate in 96% ethanol (1 min) and air dry
Hybridization	16	Cover tissue sections with hybridization buffer containing probe at 1/20 dilution of working solution (1 probe per slide)
	17	Insert slides into prepared humid chambers
	18	Incubate at 35°C for 3 h
	19	Wash slides in prewarmed wash buffer (37°C, 15 min)
CARD	20	Incubate slides in 1X PBS (RT, 15 min)
	21	Dab around tissue sections to remove excess buffer but do not let sections dry
	22	Incubate tissue sections with substrate mix (1/200 dilution of tyramide in amplification buffer) in humid chamber in the dark (37°C, 20 min)
	23	Wash slides in 1X PBS (RT, 15 min)
	24	Wash slides in milliQ (1 min) and air dry
	25	Counterstain with DAPI using mounting medium supplemented with DAPI

RT, Room temperature.

per contig as weights, and chimeric sequences were eliminated by using a stringent chimera checking pipeline consisting of uchime[47], Bellerophon[48] and by querying the sequences against a curated version of the Silva database SSURef_111_SILVA_NR_98_26_07 (http://www.arb-silva.de/) using blastn (see Supplementary Information for detailed parameters used). Non-chimeric sequences resulting from this pipeline were filtered for clones of planktonic origin by blastn queries against a local database including 1493 OTUs from 37, 860 16S rRNA PCR library sequences from the Global Ocean Sampling project plus 8,471 marine bacterial 16S rRNA sequences obtained from the ribosomal database project dataset, and graciously provided by S. Yooseph.

The 16S rRNA sequences of the clones used in this study have been deposited in the GenBank database under accession numbers KC492587-KC492611 and KC492698-KC492704.

Phylogenetic analysis. Clone sequences classified as non-chimeric and non-planktonic were fully sequenced using primers 907R and 785F (5' GGATTAGATACCCTGGTAGT 3'), individually assembled using gap4, and exported. These sequences were aligned using the *mothur*[49] and imported into the SSURef_111_SILVA_18_07_12 database of the Silva project[50] using the ARB software[51]. Phylogenetic trees were constructed by neighbor-joining using the phylip package V3.6[52] and by Bayesian analysis using Mr Bayes v3.2.1×64[53]. For both methods, the aligned clone sequences and reference sequences from the Betaproteobacteria, Gammaproteobacteria and Alphaproteobacteria were filtered using an 1112 bp majority filter.

454-pyrosequencing analyses. The PCR products used to construct the clone library were used as template for 454 pyrosequencing by the Research and Testing Laboratory (Lubbock, TX) according to a standard multiple protocol of this sequencing facility and described by Dowd and coworkers[54]. Briefly a PCR step (30 cycles) with a HotStart HiFidelity Polymerase and primers 28F (5' TTTGATCNTGGCTCAG 3') and 519R (5' GTNTTACNGCGGCKGCTG 3') was performed in order to amplify the hypervariable V1-V3 region of the 16S rRNA gene. Tag-encoded FLX amplicons were sequenced on the Roche 454 FLX sequencer using Titanium (Roche) reagents. Multiplex raw sff files were analyzed using a hybrid analysis pipeline. In brief, denoising was done by AmpliconNoise V1.25[55] implemented in Qiime V1.5[56] and rather than using chimera analysis by Perseus, *de novo* chimera detection and removal was done by using the uchime[47] module of Usearch V5.0[57] followed by the same analyses as for the clone library (details of parameters and commands in Supplementary Information). Non-chimeric were unweighted and grouped into OTUs using uclust with 97% sequence identity. OTU representatives were selected based on abundance. OTUs of planktonic origin were identified by blastn searches as above.

Pyrosequencing sequence reads representing the 86 OTUs have been deposited in the GenBank database under accession numbers KC492612-KC492697.

Oligonucleotide probe design. The specific oligonucleotide probe BET467 targeting the prevalent betaproteobacterial sequences in the clone library was designed using the probe design tool of the ARB software package[51]. The specificity of the probe sequence was confirmed by the probe_match tool of ARB, the probe match tool of Ribosomal Database Project (http://rdp.cme.msu.edu/) and blastn searches. Only 3 non-target sequences in the probe_match tool of ARB database had less than 3 mismatches with this specific probe. As the 16S rRNA target region was potentially inaccessible[58], two unlabeled helper oligonucleotides H-485 and H-446 were also designed. Oligonucleotide sequences of probes used, labeling and details are shown in Table 1. All probes were synthesized by ThermoFisher Scientific (Germany).

Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH). A summary of all the steps performed for CARD-FISH is given in Table 2. To determine the distribution of the bacteria in the sponge, 10 tissue samples of *C. crambe*, from different anatomic locations of two sponges specimens (about 1 cm³) were fixed as in steps 1–3 (Table 2), embedded in paraffin (at different orientations), cut into 10 µm thick sections with a microtome (MICROM HM340E; Thermo Scientific) and mounted on polysilane-coated slides (steps 4 and 5[59]). Subsequent sample treatment and CARD-FISH was carried out according to a previously described protocol[27] with some modifications. After drying, slides were deparaffined (steps 6 and 7). A step to inactivate endogenous peroxidases was performed, followed by a permeabilization step where a solution of Proteinase K (0.5 µg/ml) was added to tissue sections. A lysozyme permeabilization step was not included in the protocol from Blazejak and coworkers, but since this step has been shown to improve significantly CARD-FISH signals[60] our tissue sections were subject to lysozyme permeabilization steps [10 mg ml⁻¹ lysozyme (Fluka) in 0.05 M EDTA, 0.1 M Tris-HCl (pH8.0) (step 12–15)].

Hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH7.5], 10% dextran sulfate, 0.02% sodium dodecyl sulfate [SDS], 55% formamide [Sigma] and 1% Blocking Reagent [Roche]) - probe mixtures were added to the tissue sections on slides, and incubated in hybridization chambers prepared with a 55% formamide-water mixture at 35°C for 3 h. Due to the absence of a cultured representative of the targeted betaproteobacterium, the optimum formamide concentration to be used for the specific probe BET467 was determined by carrying out hybridizations with the sponge tissue sections, varying formamide concentrations (50%, 55%, 60%), and selecting the highest formamide concentration before a reduction in fluorescence signal was observed. This was determined to be 55%. Hybridizations for the BET467 probe were carried out as above and also included the helper probes H-485 and H-446

at a concentration of 2.5 ng/µl. Following hybridization, the slides were prepared as in steps 20–25 (Table 2). Washing buffer contained 5 mM EDTA [pH 8], 20 mM Tris–HCl [pH 8], 0.01% [w/v] sodium dodecyl sulfate and 13 mM NaCl). The CARD substrate mix was prepared by mixing amplification buffer (10% [w/v] dextran sulfate, 2 M NaCl, 0.1% [w/v] blocking reagent, in 1X PBS [pH 7.6]) with a freshly prepared H_2O_2 solution (0.15% in 1X PBS) at a ratio of 100 : 1. Mounting medium contained Citifluor and Vectashield at a 4 : 1 ratio with DAPI at 0.5 µg/ml.

Slides were observed under a model BX61 microscope (Olympus) equipped with DAPI (U-MNU2) and FITC (U-N41001 HQIF) filter sets. Images analyses were performed using a model Olympus DP72 (Olympus) camera system and Cell^A (Olympus) imaging software. To obtain a rough estimate of bacterial cell numbers in the sponge tissue, 20 square zones of 20 µm² were selected. In each of these zones, the Betaproteobacteria signals were counted from the specific probe signals, and total bacterial cells and sponge nuclei were counted from the corresponding DAPI image.

Transmission electron microscopy. TEM was performed according to Uriz and collaborators[61] with minor modifications. Briefly, small samples of sponge tissue (3–4 mm³), were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 (osmolarity adjusted to 980 mOsM with sucrose), washed in the same buffer at room temperature, and post-fixed for 1 hour in 1% osmium tetroxide. The samples were dehydrated in an ethanol-graded series (70%; 95%; 100%) and embedded in Epon 812 (EMS). Ten sub samples of *C. crambe* tissue (two sponges specimens) were prepared and embedded with different orientation. Contrasted ultra thin sections (produced using an ultramicrotome (Ultracut R Leica)) were observed at 80 kV on a transmission electron microscope (Hitachi, H7500, Japan).

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7

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8