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Cytotoxicity of the Ascidian *Cystodytes dellechiajei* Against Tumor Cells and Study of the Involvement of Associated Microbiota in the Production of Cytotoxic Compounds

Manuel Martínez-García [1,*], Marta Díaz-Valdés [2], Alfonso Ramos-Esplá [2], Nélida Salvador [3], Patricia Lopez [1], Eduardo Larriba [1] and Josefa Antón [1]

[1] División de Microbiología, Universidad de Alicante, 03080 Alicante, Spain
[2] Departamento de Ciencias del Mar y Biología Aplicada, Universidad de Alicante, 03080 Alicante, Spain
[3] Instituto Biomar, S. A. Pol. Ind. Edificio CEI 2.02, 24231 Onzonilla, León, Spain

* Author to whom correspondence should be addressed; E-mail: m.martinez@ua.es

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Abstract: Many cytotoxic compounds of therapeutic interest have been isolated from marine invertebrates, and some of them have been reported to be of microbial origin. Pyridoacridine alkaloids are the main compounds extracted from the ascidian *Cystodytes dellechiajei*. Here we describe the *in vitro* antiproliferative activity against different tumor cell lines of the ascidian extracts and provide some insights on the role of the microbial community associated with the tunicate in the production of these compounds. *C. dellechiajei* extracts showed remarkably high antiproliferative activity (IC_{50} ≤5 µg/mL) in human lung carcinoma A-549, colon adenocarcinoma H-116, pancreatic adenocarcinoma PSN-1 and breast carcinoma SKBR3 cell lines. Moreover, we found that the maximum activity was located in the tunic tissue of the colony, which harbours a microbial community. In order to ascertain the involvement of this community in the synthesis of the bioactive compounds different approachs that included culture and culture independent methods were carried out. We undertook a screening for antiproliferative activities of the bacterial isolates from the ascidian, as well as a comprative analysis of the cytotoxic activities and the microbial communities from two color morphs of the ascidian, green and blue. In addition, the changes of the antiproliferative activities and the composition of the microbial communities were studied from ascidians kept in aquaria and treated with antibiotics for one month. Our

data obtained from the different experiments did not point out to bacteria as the source of the cytotoxic compounds, suggesting thus an ascidian origin.

Keywords: Ascidian, cytotoxicity, tumor, *Cystodytes*, bacteria

1. Introduction

The number of natural products isolated from marine organisms increases rapidly, and now exceeds 18,000 [1], with hundreds of new compounds being discovered every year [2, 3]. A large proportion of these natural compounds have been extracted from marine invertebrates, especially sponges, ascidians, bryozoans and molluscs, and some of them are currently in clinical trials [4]. Most marine invertebrates are sessile soft bodies that inhabit benthic rock environments. In the sea, rock substrate is limited, and benthic organisms have to compete for the space to live and develop. Invertebrate organisms have evolved defence strategies based on the synthesis of cytotoxic compounds in order to avoid predation and epibiosis [5-7]. Many invertebrate animals, like sponges, tunicates, bryozoans, molluscs and oligochaetes are symbiotically associated with microorganisms belonging to the *Bacteria* and *Archaea* domains [8-15]. In some cases, the source of the cytotoxic compounds isolated from marine invertebrates are the symbiont bacteria. For instance, the tunicate *Lissoclinum patella* is simbiotically associated with the cyanobacteria *Prochloron* sp. [13], which produces the cytotoxic compounds patellamides A and C, each with clinical potential [16, 17]. Davidson *et al*. [18] provided evidence in the bryozoan *Bugula neritina* that its symbiont "*Candidatus* Endobugula sertula" is the source of bryostatins, which show excellent potential as therapeutic agents against leukemias, lymphomas, melanomas and solid tumors [19].

The colonian ascidian *Cystodytes dellechiajei* (Della Valle, 1877) (Aplousobranchiata, Polycitoridae) inhabits benthonic rock environments in tropical and temperate waters in the Atlantic, Pacific and Indian oceans, and in the Mediterranean sea. Its life cycle has two phases, as an adult sessile colony and as free-living larva. Larvae exhibit all the characteristic chordate features: a notocord, a dorsal, hollow nerve cord, pharyngal gill slits and a muscular post-anal tail. Colony and larva are surrounded by a protective tunic, which is analogous to a mesenchymal tissue, formed by a matrix of acidic mucopolysaccharides and diverse eukaryotic cell lines [20, 21]. *C. dellechiajei* stores acid substances in the vacuoles of the bladder cells of tunic tissue. These cells break upon aggression and release the vacuolar content into the tunic, transiently lowering the local pH down to 1-2 [20]. In addition, the tunic contains calcium carbonate spicules that protect the zooids of the colony and accumulate different cytotoxic compounds: mainly pyridoacridine alkaloids [20, 22], as well as diterpenes [23], sphingosines and ceramides [24]; some of which have antileukemic properties [25-29]. These cytotoxicity and acidity mechanisms of the tunic of *C. dellechiajei* are defence strategies to deter predators and competitors [21, 30-32].

The aim of the present study was, first, to analyze the antiproliferative activity against different tumor cell lines of tissue extracts from the two colour morphs (blue and green) of *C. dellechiajei* that inhabit the southeastern Mediterranean sea. In addition, since this tunicate harbours a microbial community associated with the tunic tissues [33], our second aim was to analyze the involvement of

these bacteria in the synthesis of bioactive compounds, by both culture of bacterial isolates and culture independent methods based on denaturing gradient gel electrophoresis of 16S rRNA genes, a widely used molecular approach for the description of microbial community composition [34].

2. Results and Discussion

2.1. In vitro antitumor activity from natural samples

The organic crude extracts from the 11 blue colonies analyzed showed high inhibitory activity against breast SKBR3, colorectal H-116, lung A-549, and pancreas PSN-1 cancer cell lines and displayed essentially no antiproliferative activity against the glioblastoma T98G cancer cell line (Table 1). Previous studies proved that the ascidian showed cytotoxicity against HL-60 and P338 leukemic cells, and the MCF7 breast cancer cell line [25, 27, 35, 36]. Morover, when the blue ascidians were kept in aquarium for up to 75 days, the antiproliferative activities were conserved with high levels, (IC_{50} <5 μg/ml in A-549, H-116, PSN-1 and SKBR3 cancer lines).

Table 1. *In vitro* antitumor activity from organic crude extract obtained from *C. dellechiajei.*

	IC_{50} for cancer cell line (μg/ml)				
Samples	**[a]A-549**	**[b]H-116**	**[c]PSN-1**	**[d]SKBR3**	**[e]T98G**
Blue colony 1	5	<5	5	10	25
Blue colony 2	5	<5	5	5	10
Blue colony 3	5	<5	5	5	25
Blue colony 4	5	<5	<5	5	10
Blue colony 5	5	<5	<5	5	25
Blue colony 6	5	<5	5	5	25
Blue colony 7	5	<5	5	5	25
Blue colony 8	5	<5	<5	5	25
Blue colony 9	5	<5	5	5	25
Blue colony 10	5	<5	5	5	25
Blue colony 11	<2.5	2.5	5	5	10
Green colony 1	25	5	-	-	-
Green colony 2	25	25	-	-	-
Green colony 3	25	25	-	-	-
Blue colony tunic	2.5	2.5	-	-	-
Blue colony zooids	12.5	12.5	-	-	-
Larva	12.5	25	-	-	-

[a] Human lung carcinoma

[b] Colon adenocarcinoma

[c] Pancreatic adenocarcinoma

[d] Breast carcinoma

[e] Caucasian glioblastoma

In good agreement with toxicity data from the larvae reported by Tarjuelo *et al*. [31], we found that larva crude extracts did not display antiproliferative activity against human lung carcinoma A549 or colon adenocarcinoma H116. The lack of antitumor activity in larva extracts could be due to the absence of cytotoxic compounds in the tunic tissue of the larvae. Adults and larvae of a given species may use distinct chemical and physical defence strategies [31, 37-41] and unpalatable larvae do not always come from unpalatable adults [39]. Larval defence could thus be associated with the reproductive strategy of the species rather than the chemical and physical defence mechanisms [37, 38, 40, 42].

On the other hand, our results showed a significative difference between antiproliferative activities between green and blue ascidian colonies. The cytotoxic activity detected in the green colonies was very low, $IC_{50} \approx$ 25 μg/mL, and only the blue pigmented colonies showed high values. Within the population of *C. dellechiajei* in the sampling area, the blue pigmented colonies were much more abundant (around 3 fold) than the green colonies. It could thus be possible that the high cytotoxicty of the blue colonies provide them with an adaptative advantage compared to green colonies.

Although we have not analyzed the chemical nature of the active compounds, it has been widely reported that the main compounds extracted from *C. dellechiajei* are pyridoacridine alkaloids. These compounds are ascididemins, 11-hydroxyascididemin, cystodytins A-I, shermilamine B, kuanoniamine D, and sebastianines A and B [20, 22, 25, 28, 32, 35, 43-45], some of which showed antitumor properties [25-29]. However, only ascididemin, 11-hydroxyascididemin and sebastianines A and B have been detected in the blue colonies [20, 21, 29]. Therefore, the cytotoxic activities we have observed could be due to these products. In other hand, Rottmayr *et al*. [20] and Turon *et al*. [21] reported that the pyridoacridine compounds were located in the pigmented cells of the tunic tissue in purple color colony, and, in addition, López-Legentil *et al*. [46] showed seasonal variations in the production of these compounds, with minimum values in summer, attributable to sexual exhaustion and seasonally varying biotic interaction or abiotic parameters. Therefore, according to the data from the location of these compounds inside the ascidian tissues [20, 21], our results provided from the different parts of the ascidian analyzed, zooid, tunic and larva, suggest that the cytotoxic compounds could be also located inside the pigmented cells of the tunic tissue.

2.2. Antibiotic treatment of the C. dellechiajei colonies

Antibiotic treatment did not produce any apparent detrimental effect on the ascidian growth, as no morphology and/or pigmentation differences were observed between treated and untreated colonies. *In vitro* antitumor activities were very similar in treated and control colonies in triplicate samples. For both control and treated colonies, the antitumor activity remained constant during the experiment (Figure 1), showing a range of IC_{50} values from 4 to 6 μg/mL, except for the control sample after 2 weeks, which showed a higher value, $IC_{50} \approx$ 8 μg/mL against A-549 cell line, since one specimen of the three replicates samples at that time displayed a low antiproliferative activity. Although, as shown in Figure 1, treated samples during two weeks showed the IC_{50} value lower than the control samples.

The data provided by DGGE analysis showed that bacterial communities associated with sample controls (sample C0, before starting the experiment, and samples C1 and C4, cultured during one and

four weeks respectively) were similar (above 95% similarity, Figure 2), indicating that the community underwent few changes when the tunicate was maintained out of its natural environment.

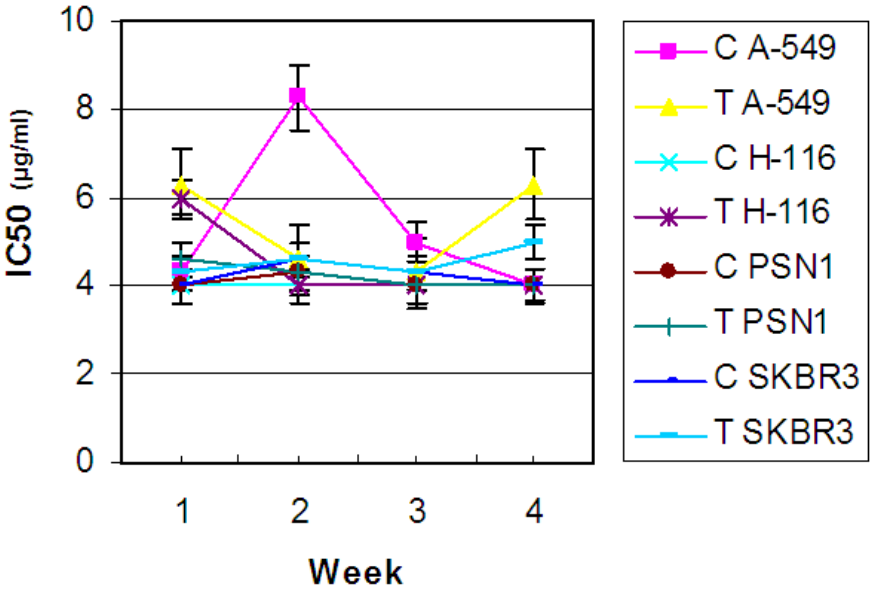

Figure 1. *In vitro* antitumor activity against lung A-549, colorectal H-116, pancreas PSN-1 and breast SKBR3 cancer cell lines of extracts from ascidians treated with antibiotics. Results are expressed in IC_{50} (µg/mL), the concentration required to inhibit growth by 50%, obtained from crude extracts from control and treated colonies in triplicate samples during four weeks. IC_{50} (µg/mL) values for control colonies (C) and treated colonies (T) are shown for each cancer cell line.

After 4 weeks of antibiotic treatment (T4), the bacterial community in the tunicate had changed remarkably, and displayed 75% similarity (Figure 2b) to the rest of the samples, which were all above 95% similar to each other. T4 DGGE pattern displayed changes in the intensity of some bands present in the rest of the samples (2, 3, 4 and 5 in Figure 2a) as well as some new bands (bands 1, 6 and 7).

These bands were reamplified and sequenced, and they were found to be related to the bacterial 16S rRNA gene sequences shown in Table 2. The uppermost gel bands belong to 16S rRNA sequences of chloroplast of photosynthetic epibionts of *C. dellechiajei*, like diatoms and rhodophyte algae [33]. The rest of bands corresponded to 16S rRNA sequences of bacteria. Most of the DGGE bands were present in all samples without significative variation, representing bacteria that were either resistant or inaccesible to gentamicine and bacitracine at the assayed doses. However, the treatment favoured the growth of new bacteria related to the alpha-proteobacterium *Stappia* sp. and beta-proteobacterium *Janthinobacterium agaricidamnosus* (DGGE bands bands 1, 6 and 7 in Figure 2a) and other alpha-proteobacteria that were previuolsy present in the ascidian tissues (bands 2 and 3). Finally, only bacteria related to *Mesorhizobium* sp. (bands 4 and 5 in Figure 2a) were directly sensitive to the antibiotic treatment. Since treated colonies had stable and high antitumor activity, those bacteria related to *Mesorhizobium* sp. (bands 4 and 5), *Stappia* sp. (bands 6 and 7) and *Janthinobacterium agaricidamnosus* (band 1) were most likely not involved in the synthesis of cytotoxic compounds.

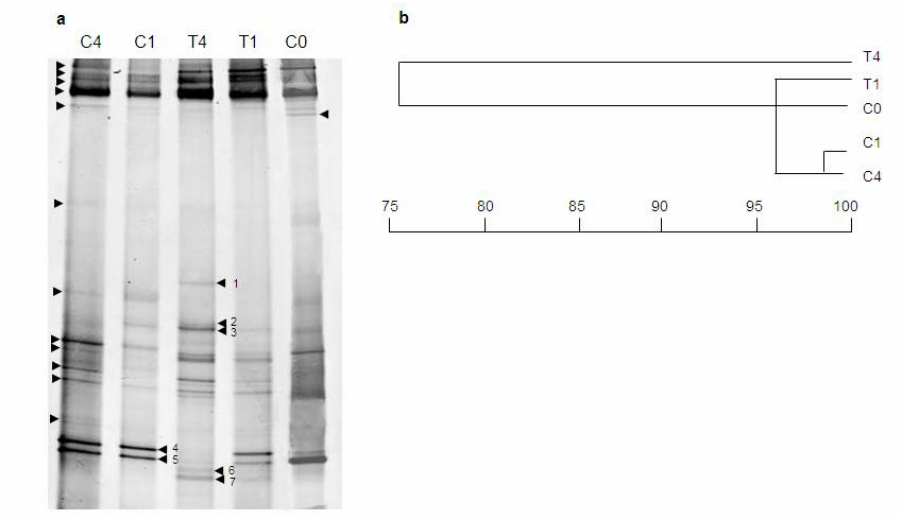

Figure 2. Antibiotic treatment of ascidia kept in aquaria; (a) DGGE gel patterns representing the bacterial communities in different samples: control sample before starting the experiment (C0), sample treated for 1 week (T1), control sample without treatment cultured for 1 week (C1), sample treated for 4 weeks (T4) and control sample without treatment cultured for 4 weeks (C4). Arrows point to bands included in the similarity analysis. Numbered arrows point to bands excised, reamplified and sequenced that showed different pattern among the samples. (b) Dendrogram based on the Jaccard index calculated from band patterns in panel (a) showing the relationship among the microbial communities of different samples.

Table 2. 16S rRNA sequence identities of bands exicised from DGGE gel (520 pb)

DGGE bands	Accession no. (GenBank)	Closest taxon (% similarity BLASTn)	Division
1	EF028016	98% *Janthinobacterium agaricidamnosus* (AY167838)	Beta-proteobacteria
2	EF028017	99% Uncultured alpha-proteobacterium NJ1-1-1 (AY626827)	Alpha-proteobacteria
3	EF028018	97% Uncultured alpha-proteobacterium NJ1-1-1 (AY626827)	Alpha-proteobacteria
4	EF028019	98% *Mesorhizobium* sp. (AY690680)	Alpha-proteobacteria
5	EF028020	99% *Mesorhizobium* sp. (AY690680)	Alpha-proteobacteria
6	EF028021	94% *Stappia* sp. (AY307927)	Alpha-proteobacteria
7	EF028022	94% *Stappia* sp. (AY307927)	Alpha-proteobacteria

On the other hand, as expected, the antibiotic treatment did not affect to archaeal community associated to *C. dellechiajei*. As shown in Figure 3, treated and control samples displayed the same DGGE pattern.

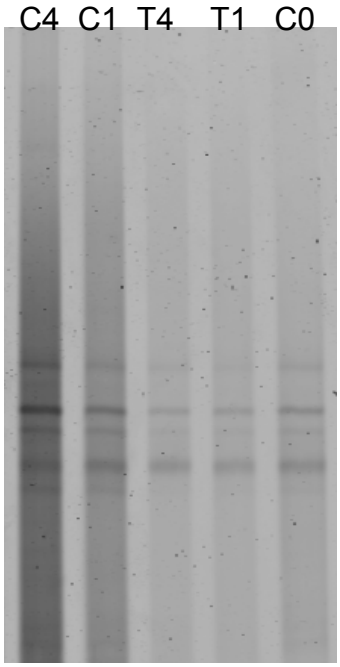

Figure 3. Antibiotic treatment of ascidian kept in aquaria. DGGE patterns representing the archaeal communities in different samples: control sample before starting the experiment (C0), sample treated for 1 week (T1), control sample without treatment cultured for 1 week (C1), sample treated for 4 weeks (T4) and control sample without treatment cultured for 4 weeks (C4).

2.3. Culture of ascidia-associated bacteria and in vitro antitumor activity of the isolates

A total of ninety-four isolates were obtained, and twenty-seven of them were randomly selected and analyzed by 16S rRNA gene PCR amplification and sequencing as described above. All the isolates were Gram negative. A predominant (85% of the selected colonies) colonial morphotype (number 1, see Table 3) was observed.

Table 3. Identification of bacteria isolated from the ascidian *C. dellechiajei*

Strain	[a]Morphotype	No. of isolates	[b]Culture media	Closest taxon (% similarity BLASTn)
Dell4	1	7	2,3	99.3% Alpha-proteobacterium NW001 (AF295099)
Dell5	1	6	2,3	99.3% Alpha-proteobacterium NW001 (AF295099)
Dell6	1	4	2,3	96.1% Alpha-proteobacterium NW001 (AF295099)
Dell7	1	5	2,3	91% Alpha-proteobacterium NW001 (AF295099)
Dell8	1	1	2,3	98.2% Alpha-proteobacterium Sb89 (AF218241)
Dell9	2	1	1	93.5% *Erythrobacter luteolus* (AY5739662)
Dell1	3	1	2	97% Gamma-proteobacterium C1 (AJ620879)
Dell2	4	1	3	97.6% *Halomonas salina* (X87217)
Dell3	5	1	3	98.9% *Alteromonas macleodi* (X82145)

[a] Colony morphotype according to pigmentation, size and shape
[b] See Experimental

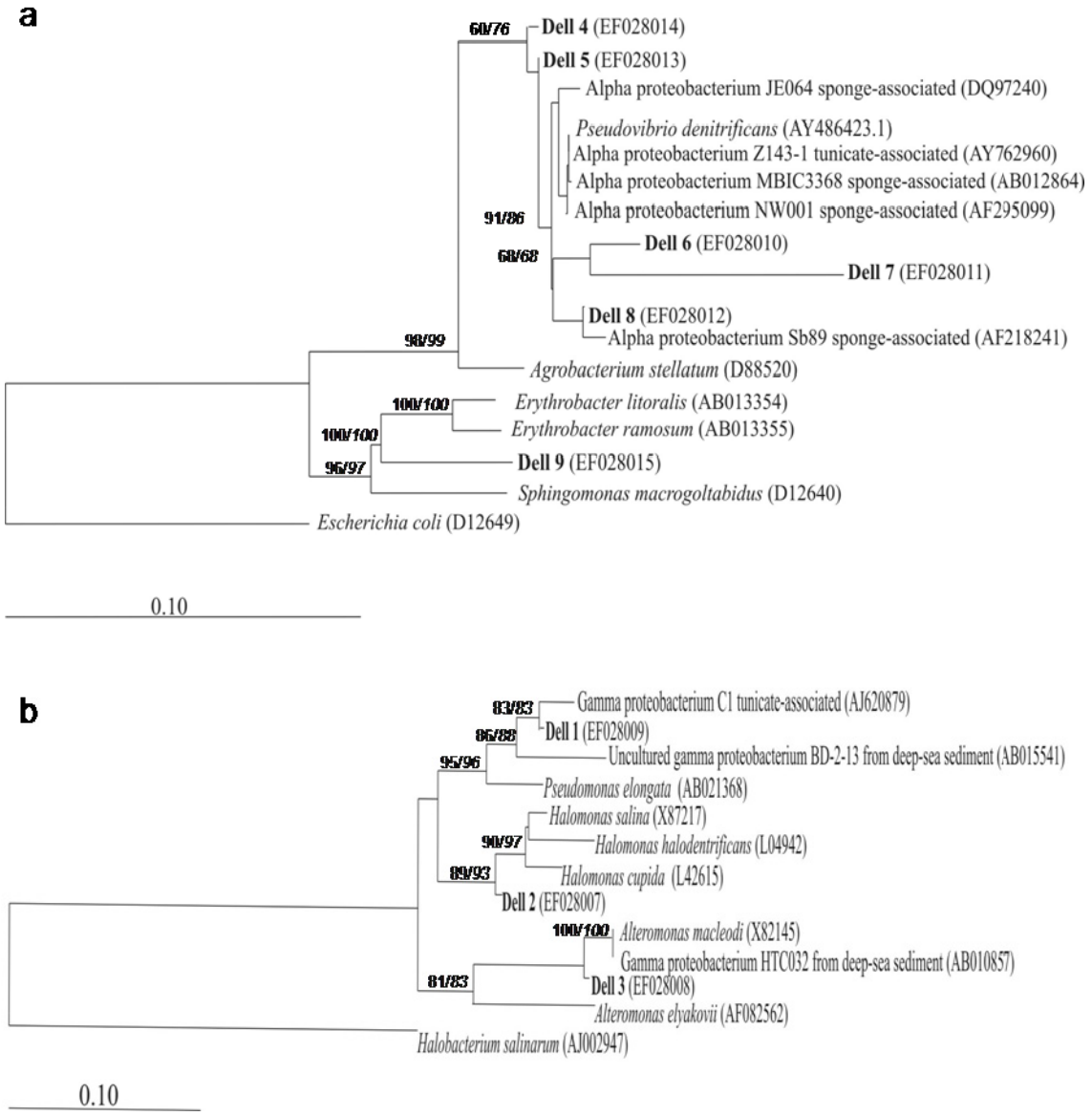

Figure 4. Phylogenetic relationships among the bacteria isolates from the ascidian *C. dellechiajei* and their relatives. (a) Neighbour-joining tree using Jukes-Cantor correction based on 16S rRNA gene partial sequences (430 bp) from strains Dell4-Dell8 and complete sequence (1435 bp) from strain Dell9 related to alpha-proteobacteria. Numbers at the branch nodes are quartet puzzling support values (normal face) and bootstrap values (1000 replicates) (italic face). The bacterium *Escherichia coli* was used as the outgroup. The horizontal scale bar represents 0.1 substitution per nucleotide position. (b) Neighbour-joining tree using Jukes-Cantor correction based on partial sequences (430 bp) of 16S rRNA gene from strains Dell1-Dell3 related to gamma-proteobacteria. Numbers at the branch nodes are quartet puzzling support values (normal face) and bootstrap values (1000 replicates) (italic face). The archaeon *Halobacterium salinarum* was used as the outgroup. The horizontal scale bar represents 0.1 substitution per nucleotide position.

These bacteria formed irregular highly mucous colonies with white-yellow pigmentation, and were easily distinguishable from the other colonies. However, inside this morphotype, five different ARDRA patterns were found. 16S rRNA gene sequences analysis revealed that the isolates Dell4, Dell5, Dell6, Dell7, and Dell8 (representatives of each ARDRA pattern) were related to alpha-proteobacteria associated with invertebrate marine organisms (Figure 4a), like strain NW001 associated with the sponge *Rhopaloeides odorabile* [47], common throughout the Great Barrier Reef, strain SB89 isolated from the Mediterranean sponge *Aplisina aerophoba* [48], strains MBIC3368 and JE064 isolated from sponges [49, 50] and strain Z143-1 isolated from a Philippine tunicate [51].

Strains Dell 4, Dell 5 and Dell 8 displayed >98.2% partial rRNA gene sequence similarity to their closest cultured relatives, and Dell 6 and Dell 7 showed 96.1 and 91% respectively. The second morphotype was represented by Dell 9, which formed irregular colonies without pigmentation and was related to *Erythrobacter litoralis* (93.5% complete 16S rRNA sequence similarity), an aerobic anoxygenic phototroph, previously cultured from open surface waters [52]. The rest of the three strains isolated, Dell 1, Dell 2 and Dell 3, were related to gamma-proteobacteria (Figure 4b). Strain Dell 1, which showed pleomorphic forms in pure culture as well as cocci and rods, was related to the gamma-proteobacterium strain C1, isolated from *C. dellechiajei* [53, 54]. Strain Dell 2 was clustered with *Halomonas* sp. group (97.6% similarity with the closest specie, *H. salina*). Dell 2 colonies were circular, convex and sligthly opaque with smooth edges. Strain Dell 3, which formed circular, smooth, raised and cream-colored colonies, was related to *Alteromonas macleodi* (98.9% similarity). 16S rRNA gene clone sequences related to strains Dell1 (97.3% similarity) and Dell9 (99% similarity) have been found in the bacterial community associated with tunic tissues of *C. dellechiajei* using a culture independent approach [33].

All the ninety-four isolates were screened for antitumor compounds. None of the strains showed *in vitro* antitumor activity against breast SKBR3, colorectal H-116, pancreas PSN-1, glioblastoma T98G or lung A-549 cancer cell lines. Interestingly, phylogenetic analysis (Figure 4) showed that the isolates Dell 4, 5, 6, and 8 were closely related to the strains alpha-proteobacteria SB89 and Z143-1 associated with sponges and tunicates that produce secondary metabolites with antimicrobial activity against *Staphylococcus aureus* and Gram negative and positive reference strains [51]. Although antimicrobial activities were not analysed, since this was out of the scope of this study, others authors described cytotoxic compounds with antimicrobial properties extracted from *C. dellechiajei* [36, 55]. According to the close relationship between isolates Dell 4, 5, 6 and 8 with alpha-proteobacteria that produce antimicrobial products, these antibacterial activities detected in the ascidian extracts could be due to secondary metabolites produced by the associated bacteria.

2.4. Is the microbial community involved in the synthesis of cytotoxic compounds?

Turon *et al*. [21] and Rottmayr *et al*. [20] showed that in *C. dellechiajei* cytotoxic pyridoacridine alkaloids involved in chemical defense were stored in pigmented cells of the tunic, with maximum abundance in upper tunic zones. In addition, Turon *et al*. [21], detected signals corresponding to the pyridoacridine alkaloids shermilamine B and kuanoniamine D and their deacetylated from the compartments of the ascidian tissues, mainly pigmented cells, but no signals from the associated bacteria, by X-ray spectra. Therefore, these authors pointed out that the host produces these cytotoxic

compounds, and not the associated bacteria. In order to clarify this point, we compared the microbial communities associated to the blue and green colonies by DGGE analysis in order to study the differences in the composition of the communities and correlate these data with antiproliferative results against tumor cells obtained from the two colour morphs extracts. As shown in figure 5, the DGGE patterns between the blue and green colonies was very similar. However, the green colonies did not show antiproliferative activity against tumor cells (see Table 1). Since the microbial communities associated to green and blue colonies are very similar and only the blue colonies show cytotoxicity, it does not seem probably that the associated bacteria are the source of the cytotoxic compounds nor they have an essential role in the production. These data, together with the lack of the activity in the isolated bacteria extracts and the results obtained from the antibiotic treatments of the ascidian, and data from Rottmayr *et al*. [20] and Turon *et al*. [21] do not indicate a microbial origin for the compounds.

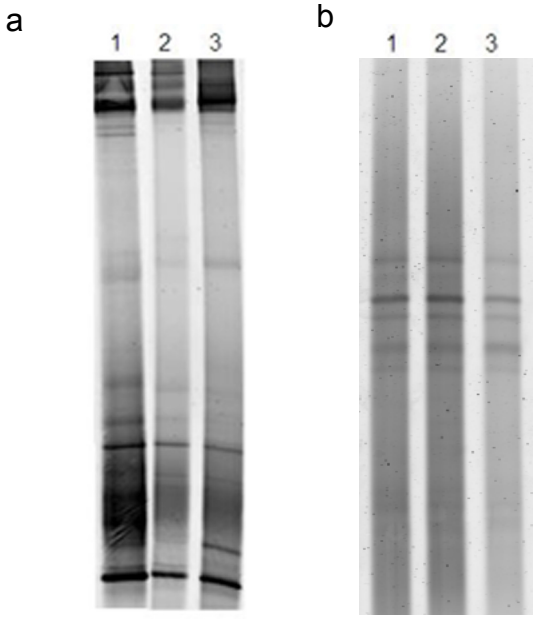

Figure 5. DGGE patterns representing the bacterial (a) and archaeal (b) communities associated to two blue colonies, sample C0 used in the antibiotic treatment (lane 1), one sample harvested in April 2005 (lane 2), and one green colony harvested in the same date (lane 3).

3. Conclusions

In summary, the current data provided from this and other studies [20, 21, 33, 46] that used complementary techniques, suggest an ascidian origin for the cytotoxic compounds, although it cannot be excluded that symbionts associated with *C. dellechiajei* synthesize cytotoxic compounds. It would be possible that the compounds were produced by the symbionts, immediately exuded to the ascidian tunic and finally stored in pigmented cells, as was the case with the ascidian *L. patella* and its symbiotic *Prochloron* sp. Cytotoxic patellamides A-C were isolated from the tunicate [56, 57], and were found distributed throughout the ascidian tunic. Salomon *et al*. [58] proposed that the location of these compounds indicated that they were synthesized by the tunicate. Lately, two independent studies

[16, 17] identified the biosynthetic pathway of patellamides in the cyanobacteria *Prochloron* sp. by using a metagenomic approach and confirmed their function by heterologous expression in *Escherichia coli*. This work constitutes the first approach to study the involvement of the microbial community associated with the ascidian *C. dellechiajei* in the production of cytotoxic compounds, and could be used as a starting point to study in depth the source of cytotoxic metabolites in the tunicate by using a metagenomic approach [59].

4. Experimental Section

4.1. Sample collection

Specimens of the two varieties, blue and green, of the ascidian *C. dellechiajei* were collected by scuba diving to depths of 2-5 m in the Mediterranean sea (Cape Palos, Murcia, Spain). Samples for *in vitro* antitumor activity assays were collected in May 2002 and March 2003. Colonies for antibiotic treatment were harvested in October 2003. Colonies and seawater were transferred directly to a container with an autonomous aeration pump, and immediately brougth back to the laboratory. Samples were processed immediately for antiproliferative assays or cultured in aquarium for antibiotic treatment. Larvae were obtained from ripe colonies under aseptic conditions, rinsed three times in sterile seawater and processed for antiproliferative assays.

4.2. Maintenance of C. dellechiajei in aquarium and antibiotic treatment

Twenty four adult colonies were collected and maintained in a seawater aquarium for one month. Half of the colonies were treated every day with gentamicine sulfate (Sigma, 100 mg) and bacitracine (Sigma, 100 mg) per litre of seawater, and the other half were used as controls without antibiotic treatment. Antibiotics active against *Bacteria* that did not cause detrimental effects against eukaryotic (i.e. ascidian) cells were chosen, according to their action spectra [60]. Antibiotic concentration was adjusted according to Davidson *et al*. [18]. The aquarium water was changed every day. At the end of each week, triplicates of treated and control colonies were collected from the aquarium and used for antiproliferative activity measurements and DGGE analysis. Activity was measured for each of the three replicates while DGGE was carried out with only one of them. Prior to the experiment of the antibiotic treatment, five adult colonies were maintained in a seawater aquarium for seventy-five days, then collected and screened for antiproliferative activity in order to study the effect of the artificial maintenance of the colonies on the production of cytotoxic compounds.

4.3. DNA extraction, PCR and DGGE

Colonies from the antibiotic treatment experiment were dissected into tunic and zooids under aseptic conditions and rinsed three times in sterile seawater. Prior to DNA extraction, tunic tissues (1.3 g) were frozen and homogenized in liquid N_2 with a sterile mortar. Pulverised tissue was suspended in 25 ml TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8) with 0.5% SDS. DNA was purified by phenol-chloroform-isoamyl alcohol 25:24:1 (all Sigma) and ethanol precipitated essentially as

described by Sambrook *et al*. [61]. The DNA pellet was air-dried at room temperature, resuspended in ultrapure sterile water (200 µL) and stored at -80 ºC. PCR amplification of partial 16s rRNA gene for *Bacteria* was performed using primers 341-GC and 907R [34], and 344-GC and 907R primers for *Archaea* [62]. The PCR conditions for *Bacteria* were as follows: 5 min at 94 ºC, 1 min at 65ºC, 3 min at 72ºC, 10 cycles of 1 min at 94ºC, 1 min at 64ºC, 3 min at 72ºC, 20 cycles of 1 min at 94ºC, 1 min at 55ºC, 3 min at 72ºC, with a final extension step of 72ºC for 10 min in a PTC-100 (MJ Instruments). The PCR conditions for *Archaea* were as follows: 94^{o}C for 5 min (initial denaturation), and 30 cycles of 94^{o}C for 30 s, 56 ^{o}C for 45 s, 72 ^{o}C for 2 min. The length of extension step was increased to 10 min in the last cycle.

Each 50 µL reaction sample contained 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 200 µM of each dNTP, 1 U *Taq* I DNA polymerase (Invitrogen), 0.25 µM of each primer and 100 ng of DNA. The PCR products were quantified by comparison with a Low DNA Mass Ladder (Invitrogen) using agarose gel electrophoresis. DGGE analysis was carried out using a DCODE system^{TM} (Universal mutation detection system, Bio-Rad) with a denaturing gradient of 45-60% for *Bacteria* and 40-55% for *Archaea* (100% was defined as 7 M urea and 40% deionized formamide) in a 0.75 mm-thick 6% polyacrylamide gel. Approximately 600-800 ng of PCR product were applied to each lane in the gel. Electrophoresis was performed at 5 V/cm at 60ºC for 16 h in 1X TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4). DGGE gels were stained with SYBR Gold (Molecular Probes), 100 µL/L in 0.5X TAE buffer, for 15 min, rinsed with 1X TAE buffer for 15 min, visualized and photographed with a Typhoon 9410 image analysis system (Amersham Biosciences). Selected DGGE bands were excised with a sterile blade and incubated overnight at 4ºC in ultrapure sterile water (20 µL). The eluent was used as template DNA for reamplification with the primers and conditions described above. PCR products were sequenced using primer 907R in a genetic analyzer ABI PRISM 310 (Applied Biosystem).

4.4. DGGE data analysis

A similarity dendrogram of the DGGE patterns from the antibiotic treatment experiment was computed using SPSS® 12.0 software (SPPS, Inc., Chicago, IL) with the Jaccard index (shared characters/total characters).

4.5. Bacterial isolation and 16S rRNA gene analysis

All culture media contained an ascidian organic extract (AOE) prepared as follows: a sample of the the colony (2 g) was homogenized using a mortar and boiled in filtered sterile seawater (35 mL) for 15 min. The homogenized colony was centrifuged at low-speed (2,000 rpm at room temperature, Labofuge 400R, Heraeus) for 1 min and the resulting supernatant constituted the AOE. Three different solid (2% agar) media were prepared with 12% v/v of AOE in filtered seawater collected at the sampling area. Medium 1 did not contain additional components, medium 2 was supplemented with 0.001% (p/v) yeast extract, and medium 3, with 0.001% (p/v) yeast extract and 1% (p/v) sucrose. The pH of the media was adjusted to 8 and they were sterilized at 121 ºC for 15 min. The plates were inoculated by either spreading a small piece of tunic (1-2 mm^3) or homogenized tunic (100 µL, 2 g of

tunic in 1 mL of filtered and sterile seawater). Cultures were incubated at room temperature under aerobic conditions for at least two weeks, and representatives of each bacterial colonial morphotype were serially streak-plated until pure cultures were obtained. Bacterial colonies were then suspended in TE buffer (400 µL, 10 mM Tris-HCl and 1 mM EDTA, pH 8) with 0.5 % SDS. DNA extraction and purification were carried out as described previously. PCR amplification of bacterial 16S rRNA genes was performed using primers 27f and 1492r [63]. Cycling conditions were as follows: 3 min at 94ºC, 30 cycles of 15 sec at 94ºC, 30 sec at 55ºC, 2 min at 72ºC and a final extension of 10 min at 72ºC. PCR products of bacterial 16S rRNA were analyzed using ARDRA [64] with the enzymes HinfI and MboI (Invitrogen) according to the manufacture´s protocol. The digestion products were analyzed in 2% agarose gel (LE, FMC Bioproducts) in 0.5X Tris-boric acid-EDTA buffer. 16S rRNA genes from each restriction pattern were sequenced using primers B1055, 16S5r, 338f and 785f [65].

4.6. Phylogenetic analysis

16S rRNA gene sequences were initially compared with the reference sequences at NCBI (http://www.ncbi.nlm.nih.gov) using BLASTn [66]. The correlation between primary sequence and secondary structure was analyzed using the ARB sequence editor. Complete and partial sequences obtained in this study were added to an alignment of over 50.000 primary aligned gene structures available at http://db-central.arb-home.de/. Sequences not included in the ARB database were obtained from the GenBank database. Phylogenetic analysis was performed by using the three algorithms implemented in the ARB package: maximum-likelihood, neighbour joining using Jukes-Cantor correction and maximum parsimony. Phylogenetic trees calculated by neighbour-joining were evaluated after 1.000 bootstrap resampling of the data. In addition, the TREE-PUZZLE program of the ARB package was used to reconstruct phylogenetic trees by maximum-likelihood with quartet puzzling support values for each internal branch [67, 68].

4.7. Preparation of crude extracts from C. dellechiajei and microbial cultures for in vitro antitumor assays

For *in vitro* antitumor assays, samples of whole colonies (2 g), tunic tissue (1.8 g), zooids (100 mg) and larvae (50 mg) were homogenyzed separately in methanol-acetone (10 mL, 1:1 v/v) with a mortar and centrifuged at 4,500 rpm (Labofuge 400R, HERAEUS) for 1 min. The resulting supernatants were dried under vacuum with a rotary evaporator (Laborata 4000, Heidolph, France) at room temperature and resuspended in ultrapure sterile water at different final concentrations (50, 25, 10, 5 and 2.5 µg/mL).

Microbial cultures were grown in liquid media designed to favour the synthesis of the secondary metabolites (C. Acebal, personal communication). Media 1, 2 and 3 were supplemented with pharmamedia® (1.5% p/v), soya flour (0.8% p/v), corn step (0.8% p/v), yeast extract (0.2 % p/v), $MgSO_4$ $7H_2O$ (5% p/v), and $CaCO_3$ (0.3% p/v). pH was adjusted at 7.1. All media were incubated at 30ºC, 220 rpm for 4 days. Once grown, each culture (5 mL) was lyophilized, resuspended and homogenized in methanol-acetone (10 mL, 1:1 v/v) and centrifuged at 4,500 rpm (Labofuge 400R,

HERAEUS) for 5 min. The resulting supernant was dried and resuspended at different final concentrations (50, 25, 10 and 5 μg/mL).

4.8. Tumor cell cultures

All the tumor cell lines were obtained from the American Type Culture Collection (ATCC). Human lung carcinoma A549, colon adenocarcinoma H116, breast carcinoma SKBR3 and pancreatic adenocarcinoma PSN1 were cultured in RPMI medium [69] containing 2 mM glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin. Media were supplemented with 5% fetal bovine serum (FBS) for A549 and H116 carcinomes, and 10% FBS for SKBR3 and PSN1 carcinomes. Caucasian glioblastoma T98G was maintained in RPMI 1640 medium [69] containing 2mM glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin, 1 mM pyruvate supplemented with 0.1 mM nonessential amino acids, and 10% FBS.

4.9. In vitro antiproliferative assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT, Sigma Chemical Co., St. Louis, MO) dye reduction assay in 96-well microplates was used, essentially as previously described [70]. Tumor cells ($4x10^3$ A-549 cells or $6x10^3$ H-116, $6x10^3$ PSN1, $6x10^3$ SKBR3 and $6x10^3$ T98G cells in a total volume of 200 μL of complete medium) were incubated in each well with different amounts of the ascidian and bacterial extracts (see above). After 2 days of incubation (37ºC, 5% CO_2 in a humid atmosphere), MTT (50 μL, 5 mg/mL in PBS) were added to each well and the plate was incubated for a further 2 h (37ºC). The resulting formazan was dissolved in DMSO (100 μL) and the absorbance read at 490 nm. All determinations were carried out in triplicate. IC_{50} was calculated as the concentration of drug yielding a 50% cell survival rate. The cytotoxicity assays were performed in two stages: first the extracts were tested for activity against human lung carcinoma A549 and colon adenocarcinoma H116. Only active extracts were further analyzed for activity against breast carcinoma SKBR3, pancreatic adenocarcinoma PSN1 and caucasian glioblastoma T98G.

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