RESEARCH ARTICLE

Sulphur-oxidizing extracellular bacteria in the gills of *Mytilidae* associated with wood falls

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Abstract

Six morphotypes of small mussels (*Bivalvia*: *Mytilidae*) were found attached to naturally sunken wood collected in the Bohol Sea (Philippines). These specimens are related to the large *Bathymodiolus* mussels that are found worldwide at cold seeps and hydrothermal vents. In these habitats, the mytilids harbour sulphur- and methane-oxidizing endosymbionts in their gills and depend on the energy and carbon provided by the symbionts. In this study, bacteria associated with the gills of wood-associated mussels are characterized using molecular and microscopic techniques. The existence of bacteria in the lateral zone of gill filaments in all specimens is demonstrated. Comparative analyses of 16S rRNA gene and adenosine 5′-phosphosulphate (APS) reductase gene sequences indicate that the bacteria are closely related to sulphur-oxidizing endosymbionts of *Bathymodiolus*. FISHs using specific probes confirm that sulphur oxidizers are by far the most abundant, if not the only bacteria present. Electron micrographs displayed mostly extracellular bacteria located between microvilli at the apical surface of host gill epithelial cells all along the lateral zone of each gill filament. In some specimens, occasional occurrence of intracellular bacteria with similar morphology was noted. This study provides the first molecular evidence for the presence of possible thiotrophic symbiosis in sunken wood ecosystems. With their epibiotic bacteria, wood-associated mussels display a less integrated type of interaction than described in their seep, vent and whale fall relatives.

Introduction

Mussels of the subfamily Bathymodiolinae are part of the dominant fauna occurring at hydrothermal vents and cold seeps worldwide, where they can reach high numbers and specimen lengths of up to 40 cm (Sibuet & Olu, 1998; Von Cosel & Olu, 1998; Van Dover, 2000). These mussels live in association with endosymbiotic chemosynthetic bacteria located within gill epithelial cells (EC) named bacteriocytes. The endosymbionts supply their host with carbon and energy. Smaller bathymodiolines such as *Idas washingtonia*, *Idas macdonaldi* or *Adipicola longissima* are also found on whale carcasses and sunken woods (Deming *et al*., 1997; Distel *et al*., 2000; Baco & Smith, 2003). Because of the great ecological interest in vent and seep environments, almost all data available today regarding mussel symbioses have been obtained from vent and seep species. Until recently, only sulphur- and methane-oxidizing symbionts were reported, with some species harbouring a single type of symbiont while others, from the Atlantic and its marginal basins (Gulf of Mexico, Gulf of Guinea), harbour 'dual symbioses' (Cavanaugh *et al*., 1987; Fisher *et al*., 1993; Distel *et al*., 1995; Fiala-Médioni *et al*., 2002; Duperron *et al*., 2005). Such dual symbioses show variable densities of methano- and thiotrophs depending on environmental parameters such as the availability of electron donors (Trask & Van Dover, 1999; Colaco *et al*., 2002; Duperron *et al*., 2006). Recent molecular work has shown that two seep mussel species not very closely related, namely *Bathymodiolus heckerae* from the Gulf of Mexico and *Idas* sp. from the eastern Mediterranean, harbour 4 and 6 distinct symbiont phylotypes, respectively (Duperron *et al*., 2007; Duperron

et al., 2008). These symbiont types include *Methylophaga*- and *Cytophaga/Flavobacter/Bacteroides*-related bacteria as well as an unknown gammaproteobacterium. This unexpected diversity of symbionts raises a question as to whether the common ancestor of bathymodiolines was associated with a single type of sulphur-oxidizing bacterium, or with multiple types of bacteria.

Sunken wood ecosystems are known since the end of the 19th century but remain poorly studied (Pailleret *et al*., 2007). The recent discovery of links between wood and seep/vent fauna, and the suggestion that wood ecosystems could have served as 'stepping stones' for the introduction of early bathymodiolines to vents and seeps has prompted new interest recently (Distel *et al*., 2000). The functioning of sunken wood ecosystems is far from being understood, but microbial generation of hydrogen sulphur and possibly methane as end products of wood degradation are likely, providing a niche for thiotrophic and methanotrophic symbioses (Leschine, 1995). Preliminary transmission electron microscopy (TEM) results indicate the presence of extracellular bacteria in the gill tissue of some wood-associated specimens, but no information regarding their phylogenetic relatedness or metabolism is available to date (Gros & Gaill, 2007).

In the present study, symbiotic associations are investigated in six new and distinct morphotypes of small mussels recently collected on pieces of naturally sunken wood recovered from shallow to deep waters (219–1775 m) in the Bohol sea (Philippines, Western Pacific). The aim was to assess whether these mussel morphotypes harbour extracellular bacteria, as observed previously in other morphotypes by Gros & Gaill (2007), whether single or multiple bacteria occur and their relatedness to bacteria associated with other mytilids. Bacterial diversity was characterized using 16S rRNA gene sequence analysis and FISH. The potential of the bacteria to oxidize sulphur was assessed by sequencing a fragment of their gene encoding adenosine 5′-phosphosulphate (APS) reductase, an enzyme involved in the sulphur oxidation pathway. Finally, the localization of bacteria was checked by electron microscopy.

Materials and methods

Sample collection and preparation

Pieces of sunken wood were collected using a beam-trawl at depths ranging between 219 and 1775 m during the Panglao cruise (May 2005, chief scientist: P. Bouchet) in the Bohol sea (8.3–9.6°N and 123–124°E, Fig. 1). Small mytilids, up to 3 cm in length, were found attached to the surface of small pieces of naturally submerged wood and plant debris (Table 1, Fig. 2). Very few specimens were collected from each piece of wood or coconut; thus, no replicate specimens were available for five of the six morphotypes investigated. Mussel samples were processed onboard within 1 h after collection.

Fig. 1. Sampling area in the Bohol Sea (8.3–9.6°N and 123–124°E). Mussel specimens were collected on wood fragments collected at depths between 219 and 1775 metres during the Panglao cruise in 2005 (chief scientist: P. Bouchet).

For each mussel specimen, three fragments of the gills were dissected and treated according to the following protocol. The first gill piece was stored in 100% ethanol for molecular investigation of bacterial diversity. The second gill piece was prepared for electron microscopy. The sample was prefixed in 2.5% glutaraldehyde/0.1 M caccodylate buffer (pH 7.2) adjusted to 900 mOsM with NaCl and $CaCl_2$ (1 h, 4 °C). Following a brief rinse, the piece of the gill was stored in the same caccodylate buffer at 4 °C. After returning to the lab, the pieces of gill were fixed in 1% osmium tetroxide (45 min, room temperature) and then rinsed and postfixed using 2% aqueous uranyl acetate (1 h). Tissues were then embedded in epon-araldite, sectionned and observed using a Philips 212 electron microscope (at 80 kV). The third piece of the gill was treated for *in situ* hybridization experiments. After fixation in 4% paraformaldehyde in seawater (1–3 h, 4 °C), the piece of gill was washed three times in seawater (10 min each, 4 °C) before dehydration in an increasing ethanol series. Samples were then stored in 100% ethanol at − 20 °C.

16S rRNA and APS reductase gene sequence analysis

DNA was extracted from the gill tissue from all specimens according to the protocol described by Duperron *et al*. (2005). The gene encoding bacterial 16S rRNA gene

Table 1. Coordinates and depth of collection sites, type of substrate where specimens were collected, and analyses performed on each specimen

Specimen	Sample	Collection site	Depth (m)	Substrate	16S rRNA gene	APS	FISH	TEM
BC 272	CP 2349	9°31.6′N 123°55.7′E Bohol Sea, off Pamilacan island	219–240	Coconut			+	–
BC 279	CP 2353	9°25.6′N 124°02.1′E Bohol Sea	1750–1763	Wood	12 (4)		+ (Not shown)	+
BC 288	CP 2355	9°24.3′N 124°10.7′E Bohol Sea	1764–1775	Inside fibers of a cocoleaf	14 (7)	12	+	+
BC 289	CP 2355	9°24.3′N 124°10.7′E Bohol Sea	1764–1775	Wood			+	+
BC 294	CP 2356	9°20.9′N 124°08.7′E Bohol Sea	1764	Wood	5 (1)		+ (Not shown)	+
BC 1007 (2 sp)	CP 2405	9°39.0′N 123°46.1′E Bohol Sea, Maribojoc island	387–453	Wood	6 (2)+41 (20)	8+4	+	+

Number of full 16S rRNA gene sequences are displayed in parentheses. All APS sequences are full sequences of the amplified fragment. For BC 1007, two specimens (sp) were available.

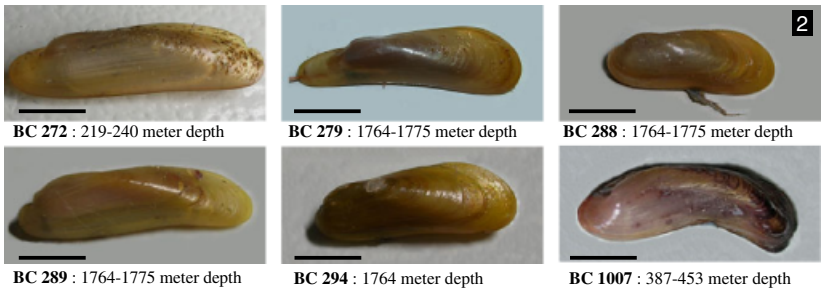


Fig. 2. Mussel morphotypes used in this study, with collection depths. The scale bar is 0.5 cm.

(~1500 bp) was amplified with primers 27F (5′-AGAGTTT GATCATGGCTCAG-3′) and 1492R (5′-GTTACCTTGT TACGACTT-3′) using the following PCR conditions: 94 °C for 4 min, then 31 cycles of denaturation at 94 °C for 1 min, primer annealing at 44 °C for 1 min and elongation at 72 °C for 1 min 30 s. A final 10-min elongation step was added. A 395-bp fragment of the gene encoding APS reductase α subunit was amplified with primers APS1-FW (5′-TGGCA GATCATGATYMAYGG-3′) and APS4-RV (5′-GCGCCAA CYGGRCCRTA-3′) using a similar program with an annealing temperature of 58 °C and 32 cycles (Blazejak *et al.*, 2006).

PCR products were purified on columns using a QIAquick kit (Qiagen, CA) and ligated into a pCR® 2.1-TOPO vector (Invitrogen, CA). The vectors were used to transform competent *Escherichia coli*. After blue-white screening, plasmids from positive clones were extracted using a DirectPrep 96 kit (Qiagen, CA) and inserts were sequenced using a vector primer. One sequencing run allowed for the recovery of full sequences of amplified APS fragments, but only partial sequences of 16S rRNA gene. To obtain full sequences, representative 16S rRNA gene clones were sequenced in both directions. Except for the two specimens of BC 1007, few positive clones were obtained (Table 1).

Sequences were compared with databases using BLAST (Altschul *et al.*, 1990). A 16S rRNA gene nucleic acid sequence dataset was built including sequences from bacteria, either symbiotic or free-living, representative of several groups including sulphur and methane oxidizers as well as best BLAST hits. An APS amino acid sequence dataset was built with best BLAST hits and APS sequences from representative sulphur-oxidizing and sulphate-reducing bacteria. Sequences were aligned using CLUSTALX and manually corrected and truncated to discard ambiguously aligned positions using BioEdit (Hall, 1997–2001). Bayesian (BA) phylogenetic analyses were performed using M.BAYES v3.1.2 (Huelsenbeck & Ronquist, 2001). For 16S rRNA gene alignments (1435 nt), a General Time Reversible model was used, along with a 'Γ+invariant' approximation of rate heterogeneities. BA analysis was run using four Monte Carlo Markov Chains (MCMC) chains for 2 000 000 generations; BA posterior probabilities were calculated from the 1650 best trees. For APS reductase amino acid sequence alignments (130 aa), a GTR model with a Γ-shaped distribution of rates was used. Four MCMC chains were run for 500 000 generations, and posterior probabilities were calculated from the 350 best trees.

In situ hybridization experiments

The authors mostly performed FISH, and only performed Catalyzed Reporter Deposition (CARD)-FISH, a more sensitive technique, when no signal was obtained with the basic FISH technique.

Gill fragments were embedded in paraffin. Transverse sections (4 μm thick) of gill filaments were cut and collected on precoated slides (Sigma, France). Paraffin was removed

Table 2. Oligonucleotide probes used in this study

Probe	Sequence (5′–3′)	Position*	Target	References
EUB338	5′-GCTGCCTCCCGTAGGAGT-3′	338	Most eubacteria	Amann *et al*. (1990)
DSS658	5′-TCCACTTCCCTCTCCCAT-3′	658	*Deltaproteobacteria*	Manz *et al*. (1998)
GAM42	5′-GCCTTCCCACATCGTTT-3′	42	*Gammaproteobacteria*	Manz *et al*. (1992)
BET42a	5′-GCCTTCCCACTTCGTTT-3′	42	*Betaproteobacteria*	Manz *et al*. (1992)
ALF968	5′-GGTAAGGTTCTGCGCGTT-3′	968	*Alphaproteobacteria*	Glöckner *et al*. (1999)
Bthio-193	5′-CGAAGATCCTCCACTTTA-3′	193	Thiotrophic mussel symbionts	Duperron *et al*. (2007)
NON338	5′-ACTCCTACGGGAGGCAGC-3′	338	Antisense EUB338	Amann *et al*. (1990)

*Position in the 16S rRNA gene of *Escherichia coli*.

prior to hybridization experiments using toluene, and sections were rehydrated in a decreasing ethanol series and then distilled water. Basic FISH was performed using the protocol described in Dubilier *et al*. (1995). After bacterial cell membranes were permeabilized using HCl (0.2 M, RT), Tris-HCl (20 mM, RT) and proteinase K (0.5 μg mL^{-1}, 37 °C), hybridizations were performed using 20% formamide. CARD-FISH experiments were performed following the protocol described by Schönhuber *et al*. (1997). After cell membrane permeabilization, peroxidases present in the tissue were inhibited using HCl (0.01 M, RT). Hybridizations were performed using 50% formamide, and signals were amplified using a buffer containing Carboxyfluorescein (FITC). For both FISH techniques, slides were mounted with cytomation fluorescent mounting medium (DAKO, France) and visualized under an epi 80i epifluorescence microscope (Nikon, France).

Six general oligonucleotide probes including the negative control NON338 were used in basic FISH experiments to test for the presence of different bacterial groups (see Table 2); an additional probe targeting only sulphur-oxidizing symbionts of mussels, Bthio-193 (Duperron *et al*., 2007), was used to test for the presence of sulphur-oxidizing bacteria similar to those found in other bathymodiolines such as *Bathymodiolus* spp. or *Idas* sp. (Table 2). All probes were labelled with Cyanine Cy3. Only probes EUB338 and Bthio-193 were used in CARD-FISH experiments.

Results

Mytilids sampled

Small mytilid morphotypes (< 3 cm long) were found associated with wood pieces, fibres of cocoleaves and coconuts that were collected using as beam trawl between 219 and 1775 m depth. Six morphotypes were analysed: BC 279, BC 288, BC 294, BC 1007, BC 272 and BC 289 (Table 1, Fig. 2), of which only BC 279 was identified as *Adipicola longissima* (J. Lorion, pers. commun.). The others corresponded to species not yet described. Very few specimens were recovered; thus, replicates were not available, except for BC 1007 (two specimens).

Phylogenetic characterization of bacterial symbionts

Bacterial 16S rRNA-encoding gene sequences were recovered from five of the seven mussel specimens (BC 279, BC 288, BC 294 and both specimens of BC 1007). No amplification was obtained from specimens BC 272 and BC 289, possibly due to the state of degradation of the extracted DNA. BLAST-based similarity analyses indicate that all sequences were of gammaproteobacterial origin and highly similar to sequences from sulphur-oxidizing bacterial endosymbionts of cold seep and hydrothermal vent mussels of the genera *Bathymodiolus* and *Idas* (> 97% similarity). A single phylotype was recovered from each specimen, except for a possible second phylotype (partial sequence 2) from BC 294, which displayed 21 differences among 828 bp (2.54%) compared with the dominant sequence. This second phylotype could either represent a second symbiont or a second operon. Morphotypes BC 1007 (two specimens) and BC 294 (full sequence 1) harboured very similar 16S rRNA gene phylotypes, differing from one another by only two to five nucleotides out of 1452, a variation that is within the error ranges of Taq polymerase and sequencing reactions (0.14–0.28%). This phylotype differed by 1.44–2.90% from sequences recovered from BC 288, BC 279 and the second sequence from BC 294 (partial sequence 2). Phylogenetic trees based on Bayesian analyses confirm that sequences from mussels obtained from sunken wood cluster within the group of mussel-associated thiotrophs (Fig. 3). Sequences from BC 1007 (two specimens) and BC 294 (full sequence 1) were most closely related to symbiont sequences from a bathymodioline from New Zealand (DQ321718) and from the thyasirid clam *Conchocele disjuncta* (probability: 0.79) (Imhoff *et al*., 2003). The sequence from BC 288 clustered together with partial sequence 2 from BC 294 (probability: 0.84). Both groups discussed above as well as the sequence from BC 279 branched within the large multifurcation that formed the base of the bivalve-associated thiotroph group.

A single APS phylotype was amplified from both specimens of BC 1007, and another sequence was obtained from specimen BC 288 displaying only 0.8% amino acid (one over 120) but 10.8% nucleotide sequence divergence to

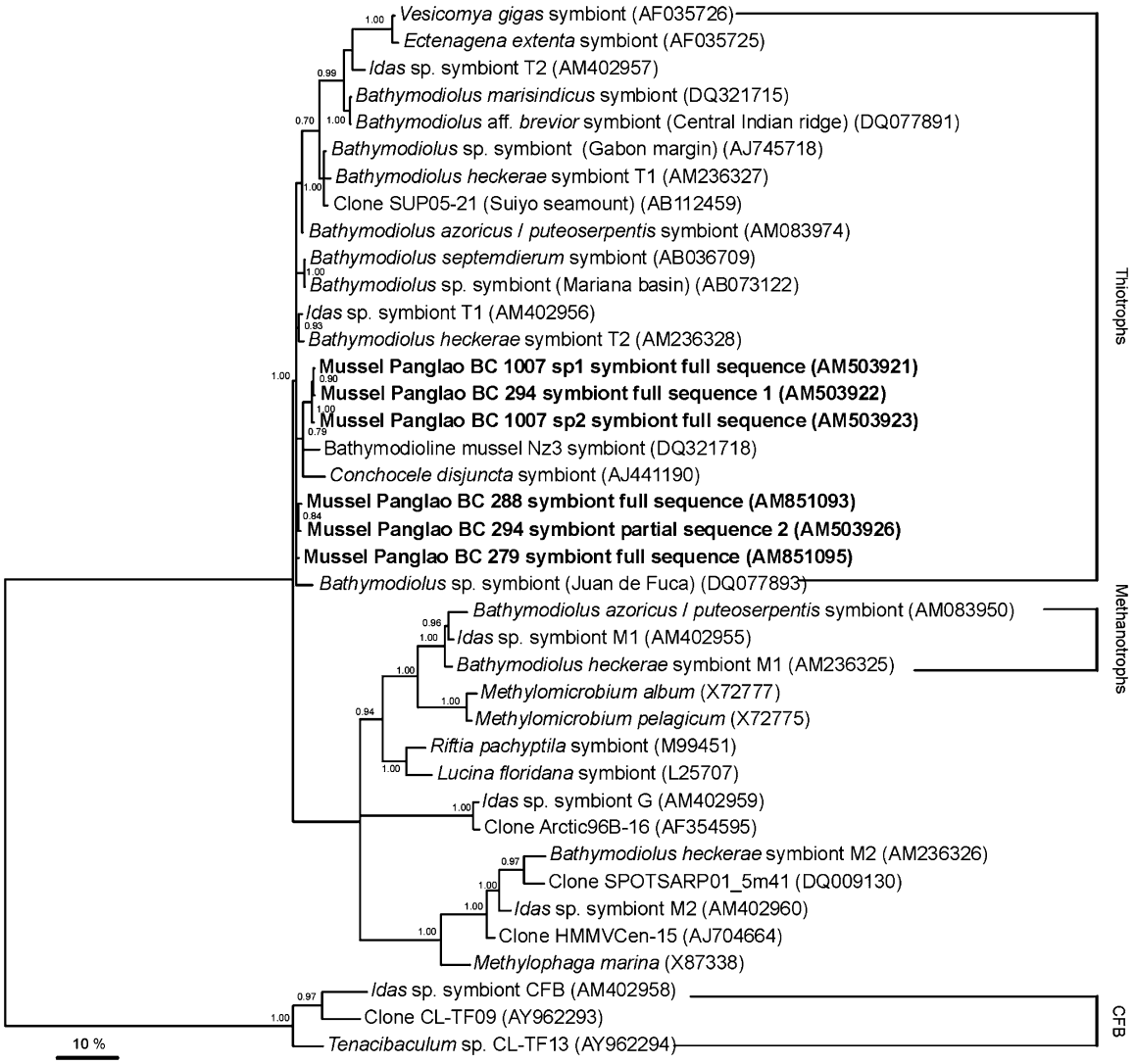


Fig. 3. Tree displaying the phylogenetic position of bacterial symbionts associated with the different morphotypes of bathymodioline mussels collected in the Philippine area based on their 16S rRNA gene sequence. All sequences from this study (in bold) cluster within the monophyletic group of thiotrophic, bivalve-associated, gammaproteobacterial symbionts. *Cytophaga/Flavobacter/Bacteroides* (CFB) are used as an outgroup. This tree is a consensus from the 1650 best trees obtained using Bayesian analysis, a GTR+Γ model of evolution, four Monte Carlo Markov Chains and 2 000 000 generations. Posterior probabilities are displayed at nodes (only > 0.70 shown). The scale bar represents 10% estimated nucleotide sequence divergence.

the sequence from BC 1007. No amplification was obtained from other specimens. These three sequences cluster together with sequences from thiotrophic bacterial symbionts of *Idas* sp. from the eastern Mediterranean (Duperron *et al.*, 2008) (Fig. 4). No amplification could be obtained for *pmoA*, a gene encoding the alpha subunit of particulate methane monooxygenase in methane-oxidizing bacteria.

Hybridization experiments

Hybridization with the universal probe EUB338 confirmed the presence of eubacteria in gill filaments of all mytilid specimens investigated (Fig. 5, BC 272). No signal was detected using the probe NON338 (not shown), indicating that the fluorescence obtained with EUB338 resulted from true positive hybridization. Among group-specific probes (DSS658, GAM42, BET42a and ALF968), only GAM42 did yield a signal, indicating that eubacteria associated with the gill filaments were likely *Gammaproteobacteria*. Their distribution was identical to that observed with EUB338. Bacteria are located in the lateral zone of each gill filament in all six specimens investigated (BC 272, BC 279, BC 288, BC 289, BC 294 and BC 1007), meaning that bacteria are only associated with this region of the gills.

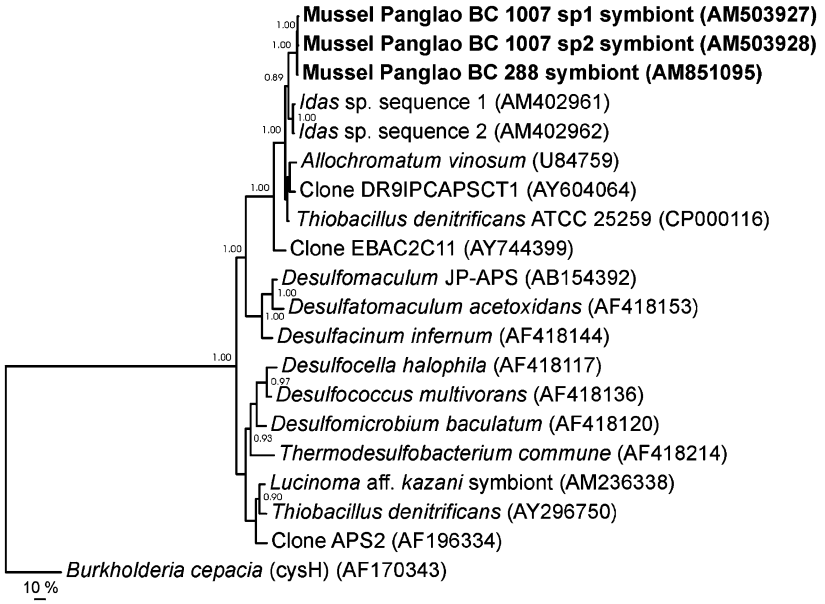


Fig. 4. Tree displaying the phylogenetic position of fragments of APS sequences recovered from bacterial symbionts associated with mussels from this study (in bold; sp: specimen). A sequence of the gene CysH from *Burkholderia cepacia* is used as an outgroup. This tree is a consensus from the 350 best trees obtained using Bayesian analysis, a GTR model of evolution, 4 Monte Carlo Markov Chains and 500 000 generations. Posterior probabilities are displayed at nodes (only > 0.80 shown). The scale bar represents 10% estimated amino acid sequence divergence.

Hybridization experiments using probe Bthio-193, specific for some sulphur-oxidizing symbionts of mussels (Duperron *et al.*, 2007), were positive in all six specimens. Distribution and abundance patterns were identical to those displayed by EUB338- and GAM42-hybridized sections, indicating that Bthio-193 probably hybridized with most of the bacteria (Figs 6–8). Dual hybridizations using EUB338 and Bthio-193 simultaneously did not display any bacteria hybridized only with EUB338, indicating that thiotroph-related bacteria were by far the most abundant, if not the only bacteria present.

TEM analysis

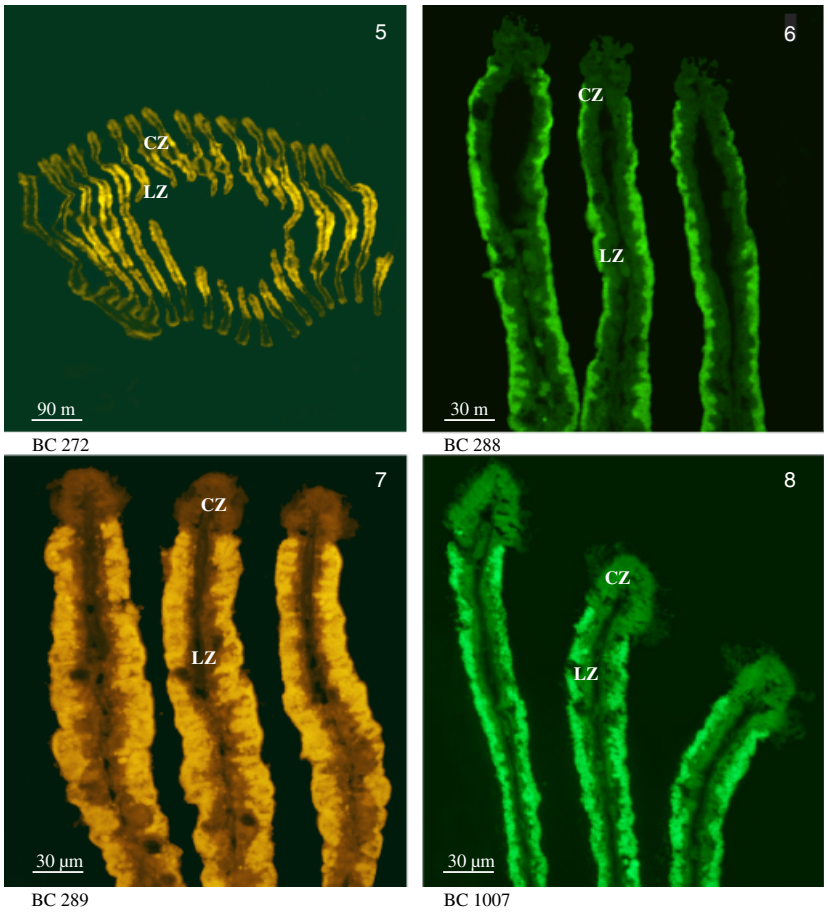
The overall structure of gill filaments in all specimens was comparable to that described previously in other mussel morphotypes (Gros & Gaill, 2007), with a lateral zone composed of a dominant type of EC with extracellular bacteria located outside their apical pole (i.e. in the area that was in contact with seawater), between microvilli differentiated by host cells (Figs 9–14). The thickness of the bacterial layer ranged, depending on the specimen, from a few (Figs 9 and 10) to 20 μm (Fig. 12). Gill ECs were large and relatively thin compared with bacteriocytes from large bathymodiolines (Fiala-Médioni *et al.*, 1986). They displayed a basal nucleus, few mitochondria and numerous lysosome-like structures (Figs 9–14). In specimens BC 1007 and BC 289, large phagosome-like structures enclosed numerous bacteria (Figs 12–14). In these instances, bacteria did not seem to be degraded (Figs 12 and 14), and these phagosome-like structures did not resemble classic lysosomes observed in bacteriocytes from other mytilids as in Figs 9 and 10. Bacteria were thus mostly extracellular but a few were intracellular, at least in BC 1007 and BC 289. In BC 289, however, a larger proportion of bacteria were present in the vacuoles compared with BC 1007, which contained a larger proportion of extracellular bacteria (Figs 12 and 14). In FISH images (using the Bthio-193 probe), the thickness of the bacterial layers was the same despite the fact that BC 1007 displayed a thicker layer of extracellular bacteria in TEM micrographs. This was due to the fact that both intracellular and extracellular bacteria are labelled with the probe, resulting in an overall similar FISH signal.

Individually, the bacteria were small and rod shaped (1 μm length, 0.3 μm thick) and their cell envelopes displayed an outer and a plasma membrane typical of gram-negative bacteria (Figs 15 and 16). No stacked intracellular membranes typical of methanotrophic symbionts were seen. Electron-dense and electron-transparent granules, non-membrane bound, are often found in the periplasmic space. Such electron-transparent granules could represent sulphur granules as observed commonly in gill-endosymbionts of marine invertebrates including some mussels (Nelson *et al.*, 1995; Cavanaugh *et al.*, 2005). In specimens BC 1007 and BC 289, bacterial morphotypes were similar, whether located in microvilli or in vacuoles (Fig. 12).

Discussion

Bacteria associated with the gills of mussels from sunken wood

Of the six morphotypes collected from sunken wood in the Bohol Sea (Philippines), bacterial 16S rRNA gene sequences were recovered from 4 morphotypes (representing five specimens). Each specimen harboured, at least, one to two



Figs 5–8. Fluorescent hybridizations on transverse sections of mytilid gill filaments revealed positive hybridizations expressed by yellow fluorescence for classical FISH experiments (Figs 5 and 7) and green fluorescence for CARD-FISH experiments (Figs 6 and 8). Both probes Eub 338 (Figs 5 and 7) and Bthio193 (Figs 6 and 8) hybridized positively in the gill filaments of the mytilids. Positive hybridizations with the universal probe indicate that eubacteria are associated with the gill filaments of the mytilids. The specific probes confirmed that sulphur-oxidizing symbionts were associated with the gill filaments. In both cases, fluorescence was restricted to the lateral zone (LZ) of the filaments while the ciliated zone (CZ) remained negative, meaning that the bacterial symbionts were specifically associated with the lateral zone.

16S rRNA gene phylotypes related to thiotrophic gammaproteobacterial symbionts of bathymodioline mussels. Specimens BC 1007 (two specimens) and BC 294 hosted very similar phylotypes, suggesting the possible presence of a single shared bacterium, a condition similar to that reported in *Bathymodiolus azoricus* and *B. puteoserpentis* from the northern Mid-Atlantic Ridge vent sites (Won *et al.*, 2003; Duperron *et al.*, 2006).

The interpretation of thiotrophic bacteria is supported by their close phylogenetic relationship with thiotrophs from vent and seep mussels, by the occurrence of APS reductase-encoding genes in at least BC 288 and both specimens of BC 1007 and by the presence of electron-lucent granules located in the periplasmic space of bacteria in all six specimens investigated using TEM. Such granules are usually considered to be sulphur granules in most of the thioautotrophic endosymbionts described in marine invertebrates (for a review, see Lechaire *et al.*, 2006). By contrast, the electron-dense, nonmembrane bound, granules observed could represent polyphosphate compounds as described in bacteria from *Riftia pachyptila* tube wall (Lechaire *et al.*, 2002).

Many species of bathymodioline mussels from cold seeps and hydrothermal vents are reported to harbour methanotroph-, *Methylophaga*- and even Bacteroidetes-related symbionts (Cavanaugh *et al.*, 1987; Duperron *et al.*, 2007; Duperron *et al.*, 2008). In the present study, evidence

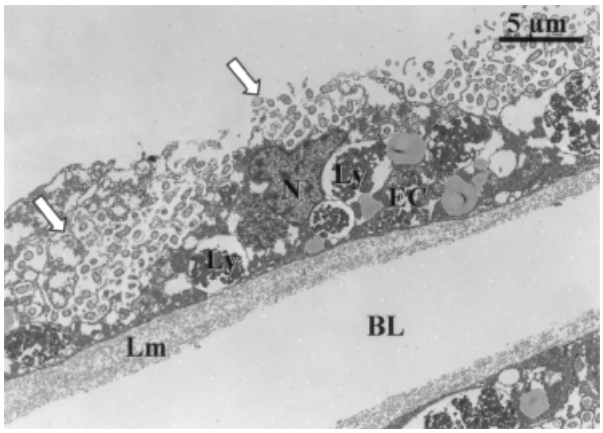


Fig. 9. TEM. Gill filaments and ECs from 'wood mytilids'. TEM view of gill filament from BC 279. ECs harbour numerous bacteria located between microvilli (arrows). The various bacterial shapes observed (rod-shaped or ovoid-shaped figures) could be due to the section orientation. BL, blood lacuna; Lm, basal lamina; Ly, lysosome; N, nucleus.

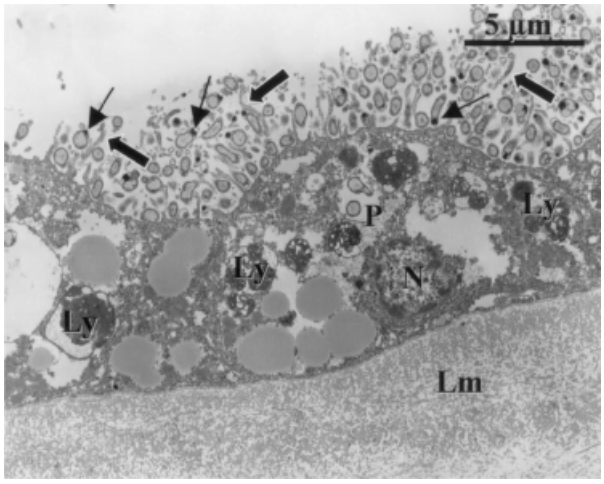


Fig. 10. TEM. Gill filaments and ECs from 'wood mytilids'. The cytoplasm of an EC from mytilid BC 288 is filled with secondary lysosomes (Ly), characterized by their heterogeneous aspect. A few bacteria can be seen inside an immature phagosom (P). Extracellular bacteria characterized by electron-dense granules (small arrows) are located on the apical surface of the host cells in contact with microvilli (arrows). Lm, basal lamina; N, nucleus of the host cell.

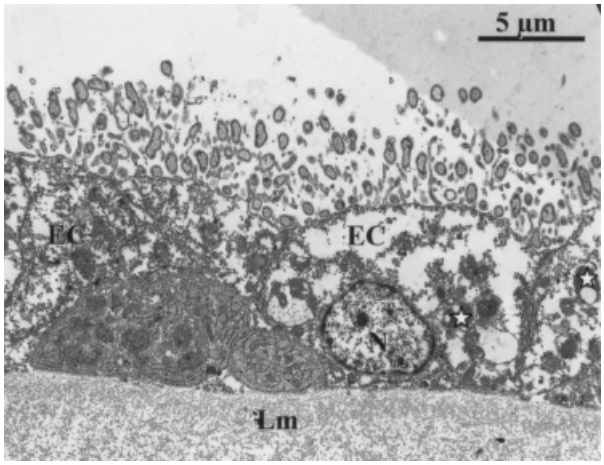


Fig. 11. TEM. Gill filaments and ECs from 'wood mytilids'. ECs from the individual BC 294. The coat of bacteria consists of a few layers of extracellular bacteria. Only a few lysosome-like structure (stars) can be observed in the cytoplasm of the host cells (EC). Lm, basal lamina; N, nucleus.

was not found for the presence of symbionts other than thiotrophs; all 16S rRNA gene phylotypes recovered had thiotrophic symbionts as their best BLAST hits; no 'methanotroph-like' morphotype (i.e. large bacteria with internal stacked membranes) was observed using TEM; the *pmoA* gene encoding the α subunit of particulate methane monooxygenase could not be amplified; and FISH results indicated that the bacteria hybridized with the thiotroph-specific probe Bthio-193.

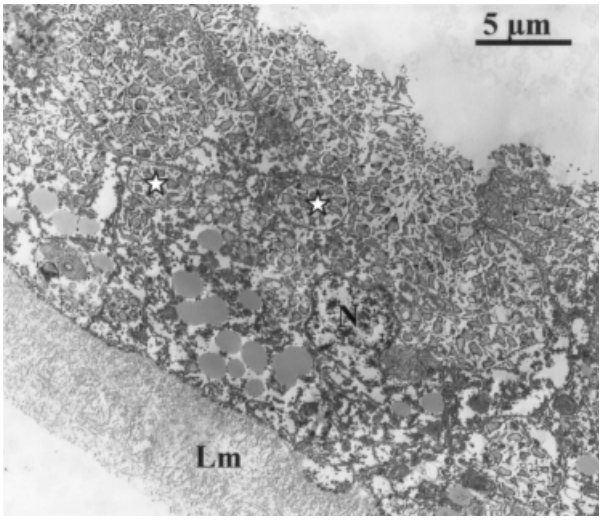


Fig. 12. TEM. Gill filaments and ECs from 'wood mytilids'. In mytilid BC 1007, the extracellular bacterial layer is thick while apparently intact bacteria can be seen inside phagosomes (star). Lm, basal lamina; N, nucleus of the host cell.

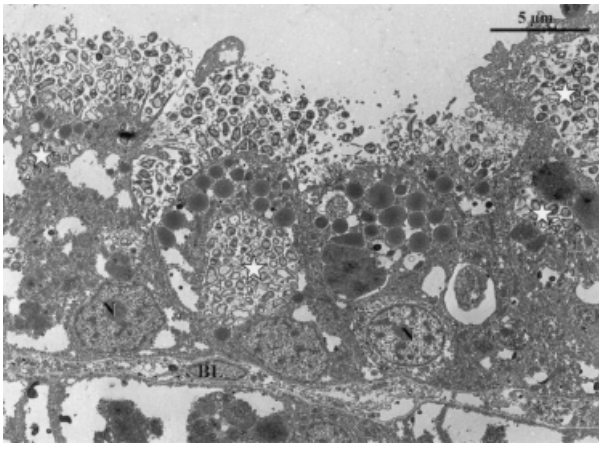


Fig. 13. In morphotype BC 289, bacteria can be observed outside the cell but also inside vacuoles (stars). BL, blood lacuna; N, nucleus.

The results from TEM and FISH indicate that bacteria only occur in the gill tissue of mussels. Contrary to what is observed in most bathymodioline mussels from cold seeps, hydrothermal vents and whale fall ecosystems, the bacteria of the wood-associated mussels investigated here occurred mostly extracellularly based on TEM observations in all specimens from this study. Thus, these bacteria live in close contact with the flow of water circulating through the lateral zone of the gill filaments. In mytilids, the extracellular association of symbiotic bacteria is unusual. One example of extracellular symbiosis was documented in a species of *Bathymodiolus* from hydrothermal vents at Juan de Fuca, but the authors were not very confident regarding symbiont

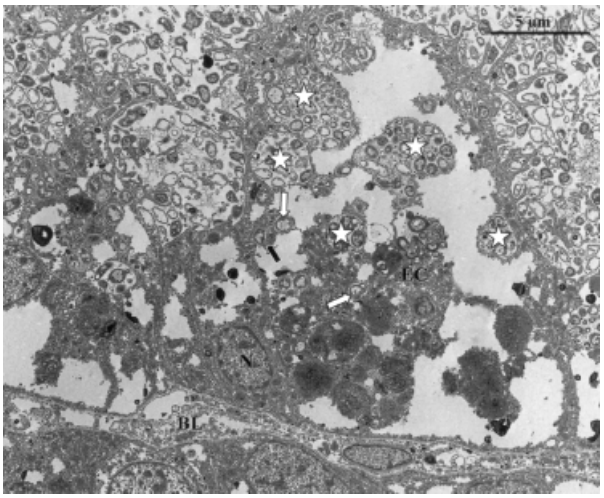


Fig. 14. Higher magnification of an EC from mytilid BC 289. Inside the EC, few large vacuoles (stars) contain numerous bacteria with a shape similar to that of extracellular ones. These bacteria probably became enclosed in the vacuoles by phagocytosis of extracellular bacteria. BL, blood lacuna.

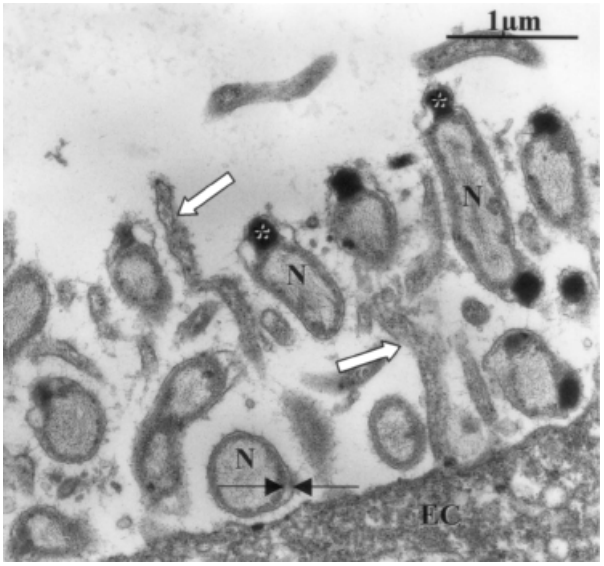


Fig. 15. Ultrastructure of bacteria. Bacteria, extracellularly located between the microvilli (arrows) of the host cells, possess an outer and a plasma membrane (small arrows) typical of Gram-negative bacteria. The DNA (N) occupies most of the volume of the bacterial cytoplasm. The periplasmic space does not seem to contain vesicles (such as sulphur granules) usually observed in thioautotrophic bacteria encountered in symbiotic bivalves but possesses electron-dense granules (asterisks). EC, epithelial cell.

localization because of poor preservation of the unique gill sample analysed (McKiness *et al.*, 2005). Recently, a few mussels collected from wood falls were described as living in association with extracellular bacteria located on their gill filaments (Gros & Gaill, 2007; Gros *et al.*, 2007). Except for

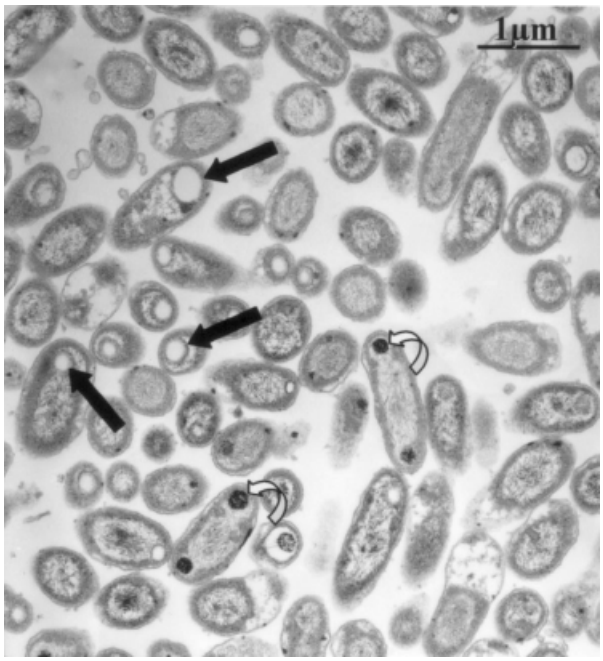


Fig. 16. Ultrastructure of bacteria. TEM. Some bacteria harbour osmiophilic dense granules (curved arrows) between the two membranes delimiting the periplasmic space of Gram-negative bacteria. On the other bacteria, numerous electron-transparent granules, probably representing sulphur granules, are observed (straight arrows).

these recent reports, all other bathymodiolines, including small *Idasolas* from whale falls, were shown to harbour intracellular symbionts in bacteriocytes located within gill filaments (Deming *et al.*, 1997; DeChaine & Cavanaugh, 2005; Stewart *et al.*, 2005). The presence of thioautotrophic gill ectosymbionts in Bivalves has only been reported for the family *Thyasiridae* (Fujiwara *et al.*, 2001). Because ECs from mussels from this study display high densities of extracellular bacteria and villosities that distinguish them from typical mussel ECs, it is suggested these cells could be qualified as 'epibacteriocytes'.

Ecological and evolutionary aspects of the interaction

In sunken wood ecosystems, the wood itself represents a huge source of carbon and energy for any heterotrophic organism able to consume plant material. This is exemplified by the abundance of cellulolytic bacteria, wood-boring bivalves such as *Xylophaga* sp. and putative symbiont-bearing wood-consuming cocculiniforms (Pailleret *et al.*, 2007; Palacios *et al.*, 2007). The anaerobic degradation of the wood may produce hydrogen sulphur, which could support chemosynthetic communities in a manner similar to bacterial decomposition of lipids from whale bones in the deep sea (Leschine, 1995; Deming *et al.*, 1997). Very recently, *in situ*

chemical data obtained from sunken woods in a very shallow tropical environment (i.e. mangrove swamp) have confirmed that sunken woods are sulphur-emitting systems, although data from deep sea sunken wood are not yet available (M.C.Z. Laurent, pers. commun.). Sulphur diffusing from the wood could be the energy source to the bacteria associated with the gills of the mussels. Whether sulphur uptake is coupled to autotrophy in these bacteria, as in symbioses of vent and seep mussels, remains to be confirmed.

Although bacteria occur in the gills, as observed in other bathymodiolines, the extracellular localization of bacteria is surprising when compared with other known mussels, and raises the question of whether the relationship is mutualistic. Indeed, how the host could benefit from the primary production of their epibionts is not clear. A possible mechanism for carbon transfer is suggested by TEM observations. Some bacteria seem to enter host ECs by phagocytosis and are subsequently degraded as shown in another mytilid associated with wood falls (Gros & Gaill, 2007; Gros *et al.*, 2007).

Bathymodioline mussels were recently suggested to have taken 'wooden steps' to deep-sea vents and seeps (Distel *et al.*, 2000). The occurrence of extracellular bacteria in the gills of all mussel morphotypes investigated herein is significant because ectosymbiosis probably represents the earliest type of association on the evolutionary route to endosymbiosis (Lallier, 2007). Gill ECs of the mussels, displaying villosities where extracellular bacteria occur, could illustrate an intermediate form between 'normal' ECs of symbiont-free species and bacteriocytes with intracellular bacteria of vent and seep bathymodiolines. The possible co-occurrence of the same bacteria as ecto- and endosymbionts in certain morphotypes may support the hypothesis of a continuous, progressive, process of symbiont internalization leading to true endosymbiosis found in more integrated models such as vent and seep mussel species, although a good host phylogeny would be necessary to conclude. Bacteria associated with wood mussels are distributed in several clades. They are thus not actual representatives of any putative 'ancient lineage' of extracellular symbionts as ancestors of modern intracellular symbionts. Extracellular bacteria are as closely related to thiotrophic endosymbionts of other mussels as endosymbionts from different mussel species are to one another, indicating that very similar bacteria occur extra- and intracellularly in different mussel species. Short branch lengths, low posterior probabilities and the presence of a multifurcation at the base of the mussel-associated thiotroph clade are, however, evidence that 16S rRNA gene alone is not sufficient to investigate the evolution of thiotrophic symbionts accurately.

This study provides the first indication that strong interactions exist between thiotrophic bacteria and metazoans in sunken wood ecosystems. Whether sunken woods sustain other thiotroph-associated metazoans besides mytilids remains to be investigated, both in shallow and in deep environments. To understand the factors that govern the distribution of mytilids and their effect on associated bacteria, an accurate description of the physico-chemical parameters characterizing deep sunken wood ecosystems, using *in situ* sensors, is needed. Autonomous sensors are available to monitor sulphur and related parameters (pH, T°) and were successfully deployed in various chemosynthetic habitats (hydrothermal vents and cold seeps) (Le Bris *et al.*, 2001; Le Bris *et al.*, 2006a, b). The authors plan to deploy such miniature *in situ* sensors with experimentally immersed wood in order to monitor sulphur at the wood interface during its decomposition in relation to the process of colonization by organisms such as mytilids in deep environments. Finally, a robust evolutionary framework, based on reliable and resolved host and symbiont phylogenies, has to be built to test whether mussels discussed in the present study, with their intriguing extracellular bacteria, are actual representatives of a more primitive type of host–symbiont interaction than found today at seeps, vents or whale falls. To achieve this, marker genes such as bacterial Internal Transcribed Spacers (ITS, a more resolutive marker than 16S rRNA gene) and host cytochrome oxidase I (COI) will be investigated.

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