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RESEARCH ARTICLE

Diversity and physiology of polyhydroxyalkanoate-producing and -degrading strains in microbial mats

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Abstract

Photosynthetic microbial mats are sources of microbial diversity and physiological strategies that reflect the physical and metabolic interactions between their resident species. This study focused on the diversity and activity of polyhydroxyalkanoate-producing and -degrading bacteria and their close partnership with cyanobacteria in an estuarine and a hypersaline microbial mat. The aerobic heterotrophic population was characterized on the basis of lipid biomarkers (respiratory quinones, sphingoid bases), polyhydroxyalkanoate determination, biochemical analysis of the isolates, and interaction assays. Most of the polyhydroxyalkanoate-producing isolates obtained from an estuarine mat belonged to the *Halomonas* and *Labrenzia* genera, while species of *Sphingomonas* and *Bacillus* were more prevalent in the hypersaline mat. Besides, the characterization of heterotrophic bacteria coisolated with filamentous cyanobacteria after selection suggested a specific association between them and diversification of the heterotrophic partner belonging to the *Halomonas* genus. Preliminary experiments suggested that syntrophic associations between strains of the *Pseudoalteromonas* and *Halomonas* genera explain the dynamics of polyhydroxyalkanoate accumulation in some microbial mats. These metabolic interactions and the diversity of the bacteria that participate in them are most likely supported by the strong mutual dependence of the partners.

Introduction

Photosynthetic mats are microbial ecosystems of intense carbon, sulfur, and oxygen cycling (Canfield & Des Marais, 1994). The upper layers of microbial mats are mainly formed by cyanobacteria (filamentous and unicellular) and by heterotrophic bacteria belonging to *Alpha-*, *Gammaproteobacteria*, and members of the *Bacteroidetes* phylum (Caumette *et al.*, 1994; Fourçans *et al.*, 2004, 2006; Villanueva *et al.*, 2004, among others. See Table 1 for details). Few studies have focused on the diversity and role of aerobic heterotrophic bacteria in the carbon cycle of microbial mats (Van Trappen *et al.*, 2002; Jonkers & Abed, 2003; Abed *et al.*, 2006), although it has been suggested that aerobic heterotrophs are specialized in the degradation of photosynthates excreted by primary producers in the photic zone (Bateson & Ward, 1998; Epping *et al.*, 1999; Jonkers & Abed, 2003). In fact, aerobic heterotrophic bacteria utilize photosynthates, derived from cyanobacterial photosynthesis, as well as complex polymeric carbon compounds resulting from the death of cyanobacterial cells (Lange, 1976; Stal, 1995). The microhabitat surrounding cyanobacteria in the photic zone assures the availability of oxygen, nutrients, and nitrogen sources, which are prerequisites for successful aerobic degradation, while the close proximity between primary producers and consumers facilitates their interaction and reinforces their interdependence (Abed *et al.*, 2006; Kirkwood *et al.*, 2006).

Carbon cycling is closely related to the dynamics of polyhydroxyalkanoates in microbial mat communities (Rothermich *et al.*, 2000; Villanueva *et al.*, 2007). Polyhydroxyalkanoates are intracellular lipid storage compounds synthesized in the presence of excess carbon sources, but limiting supplies of other compounds. Polyhydroxyalkanoates are accumulated by many types of bacteria as a response to environmental stress and nutrient-imbalanced growth, in addition to acting as a carbon and energy sink (Dawes & Senior, 1973).

Table 1. Bacterial diversity at the topmost layer of two microbial mat systems (2 mm depth)

Quinone composition*		DNA and culture-based methods†	
Ebro Delta estuarine mat	Camargue hypersaline mat	Ebro Delta estuarine mat	Camargue hypersaline mat
Quinone-10 (33%) *Alphaproteobacteria* *Rhodobacter* sp., *Stappia* sp., *Sphingomonas* sp., *Roseobacter* sp. *Roseospira* sp., *Rhodomicrobium* sp.	Menaquinone-9 (58%) *Bacteroidetes*, *Firmicutes*, *Rhodospirillum*, *Actinobacteria*, *Mycobacteria*	Phylum *Cyanobacteria* Unicellular *Synechocystis*, *Cyanothece*† *Gloeocapsa* Filamentous *Phormidium*, *Leptolyngbya*† *Microcoleus*†, *Spirulina*	Phylum *Cyanobacteria* Unicellular *Synechocystis*, *Chroococcus* *Gloeocapsa*, *Microcystis* Filamentous *Pleurocapsa*, *Oscillatoria* *Microcoleus*, *Halomicronema* *Leptolyngbya*, *Pseudoanabaena*
Quinone-8 (20%) *Gammaproteobacteria* *Marichromatium*, *Pseudoalteromonas* sp., *Beggiatoa* sp., *Thiomicrospira* sp.	Menaquinone-10 (12%) *Bacteroides*, green nonsulfur bacteria (*Chloroflexi*)	Phylum *Bacteroidetes* Uncultured *Bacteroidetes*†	Phylum *Bacteroidetes* *Bacteroidetes*, *Psychroflexus*
Quinone-9 (11%) *Gammaproteobacteria* *Marinobacter* sp., *Halomonas* sp. *Vibrio* sp., *Pseudomonas* sp. *Rhodospirillum* sp.	Menaquinone-7 (7%) *Shewanella* sp. (γ), *Bacteroidetes*, *Chlorobi*, *Firmicutes*, *Delta-*, *Epsilonproteobacteria*, *Euryarchaeota*	Phylum *Proteobacteria* *Roseobacter*† (α), *Rhodobacter*† (α), *Thiorhodococcus* (γ), *Thiorhodovibrio* (γ), *Lamprocystis* (γ) *Rhabdochromatium* (γ), *Ectothiorhodospira* (γ), *Marichromatium* (γ), *Thiocystis* (γ) Uncultured *Gammaproteobacteria*†	Phylum *Proteobacteria* *Roseospira* (α), *Neisseria* (β) *Halochromatium* (γ), *Marinobacter* (γ) *Desulfobacter* (δ), *Desulfonema* (δ) *Desulfosarcina* (δ)
Menaquinone-6 (10%) *Bacteroidetes*, sulfate-reducing bacteria (δ), *Euryarchaeota* Menaquinone-8 (6%) *Halobacteriaceae*, *Euryarchaeota*, *Deltaproteobacteria* Menaquinone-10 (5%) *Bacteroides*, *Chloroflexi*	Quinone-8 (6%) *Gammaproteobacteria* Menaquinone-8 (5%) Menaquinone-6 (4%) Quinone-9 (3%)	Others *Chlorobium*, *Prosthecochloris* (phylum *Chlorobi*)	Others *Chloroflexus* (phylum *Chloroflexi*) *Mycoplasma* (phylum *Firmicutes*) *Halanaerobium* (phylum *Firmicutes*) *Spirochaeta* (phylum *Spirochaetes*)

*Total quinone: 13 and 25 nmol g^{-1} dry weight in Ebro Delta and Camargue mats, respectively (SD was ± 5%; $n = 4$ replicates).

†Camargue data obtained by 16S rRNA gene cloning, terminal restriction length polymorphism and denaturing gradient gel electrophoresis (DGGE) in Fourçans *et al*. (2004, 2006); and Villanueva *et al*. (2004). Ebro Delta data obtained by culture, DGGE methods (Urmeneta *et al*., 2003; Martínez-Alonso *et al*., 2005; Llirós *et al*., 2008), and 16S rRNA gene cloning in this study [operational taxonomic unit (OTU), closest relative, sequence accession number, % clones: OTU-1 *Microcoleus* sp. (GU213185), 70%; OTU-2 *Cyanothece* sp. (GU213189), 6%; OTU-3 *Bacteroidetes* (GU213190), 4.5%; OTU-4 *Leptolyngbya* (GU213188), 6.5%; OTU-5 uncultured gammaproteobacterium *Haliea* sp. (GU213187), 7.5%; OTU-6 *Alphaproteobacteria* sp. (GU213186), 5.5%].

Pure cultures of polyhydroxyalkanoate-producing bacteria have been studied to gain a better understanding of the involvement of these compounds in the environmentally significant physiological processes of prokaryotes (van Germerden *et al*., 1989; Urmeneta *et al*., 1995). The exact composition of the polyhydroxyalkanoates found in nature is also of considerable interest. While early studies reported that polyhydroxybutyrate was the representative lipid storage polymer, new techniques using LC and GC as well as MS have revealed more complex naturally occurring polyhydroxyalkanoates (Comeau *et al*., 1988). Specifically, studies carried out in estuarine and other sediments revealed the presence of different β-hydroxy fatty acids of six, eight, and 10 hydroxy units (Findlay & White, 1983). In other environmental samples, 5-hydroxy repeating units (3-hydroxyvalerate, 3-HV) were more prevalent than those containing 3-hydroxybutyrate (3-HB) (Rothermich *et al*., 2000). Recent studies have evaluated the potential of microbial mats as high-level polyhydroxyalkanoate production systems under natural conditions and as a source of bacterial polyhydroxyalkanoate producers (Berlanga *et al*., 2006; López-Cortés *et al*., 2008), because polyhydroxyalkanoates are of technological and commercial interest in the manufacture of biodegradable plastics (Lenz, 1995). In this context, the diel cycle of polyhydroxyalkanoate dynamics has been investigated in hypersaline and in estuarine microbial

mats (Navarrete *et al.*, 2000; Rothermich *et al.*, 2000; Villanueva *et al.*, 2007). For example, an analysis of lipid biomarkers in Ebro Delta mat samples suggested that heterotrophic microorganisms accumulate polyhydroxyalkanoate from the excess carbon that is generated and excreted by photosynthetic microorganisms (Villanueva *et al.*, 2007). However, those observations were not in accord with the typical nocturnal production of polyhydroxyalkanoate, as reported in hypersaline microbial mats with a high content of anoxygenic phototrophs (Rothermich *et al.*, 2000).

In the current study, the diversity of aerobic heterotrophic bacteria in microbial mats was surveyed with respect to their role in the production and degradation of polyhydroxyalkanoate, as part of a broader analysis of the metabolic and phylogenetic associations between heterotrophs and phototrophs. The isolation and characterization of strains was combined with lipid analyses. The latter allowed rapid screening to detect polyhydroxyalkanoate producers and degraders among aerobic heterotrophs. In addition, diel polyhydroxyalkanoate dynamics in two microbial mat systems and the medium- and long-chain polyhydroxyalkanoates present in mat samples were evaluated. Most of the polyhydroxyalkanoate-producing isolates belonged to the *Alpha-* and *Gammaproteobacteria* [*Lanbrezia* (*Rhodobacterales*), *Halomonas*], and they were characterized by their versatility of growth under different conditions and substrates. Microbial characterization and interaction assays suggested that syntrophic associations might account for the dynamics of polyhydroxyalkanoate accumulation in microbial mats. This study not only improves our knowledge of the dynamic of carbon cycling in complex microbial communities but also demonstrates again their potential as a source of versatile polyhydroxyalkanoate-producing and -degrading microorganisms with biotechnological applications.

Materials and methods

Sampling

Samples were obtained from estuarine mats located in the Ebro Delta (northeastern Spain, 40°47′N, 0°56′E) (Guerrero *et al.*, 1993) and from hypersaline mats in the Camargue (Rhone Delta, Southern France, 44°40′N, 4°51′E) (Caumette *et al.*, 1994) (for a more detailed description of the physicochemical characteristics of the sampling sites, see Supporting Information, Table S1). Microbial mat samples for the isolation and characterization of species were transported on ice and used immediately. Mat samples following the diel cycle were collected in both sampling sites in July 2005 within a week's difference between sampling trips. Samples for polyhydroxyalkanoate analysis and DNA extraction were collected as cores removed from the upper part of the mat with a cork borer (core diameter, 16 mm; thickness, 1 mm) and frozen in liquid nitrogen. Core samples for DNA extraction were cut on a microtome into 500-μm-thick layers and then pooled to extract a 2-mm-deep mat sample.

Culture conditions and isolation

Several strains of filamentous cyanobacteria isolated from microbial mats samples were maintained as described previously (Urmeneta *et al.*, 2003). These cyanobacterial strains were used for the isolation of aerobic heterotrophic strains and in interaction assays with them. Heterotrophic bacteria were isolated from micromanipulated cyanobacteria as follows: a subsample of the cyanobacterial layer from a freshly sampled Ebro Delta microbial mat was removed and homogenized. Mineral medium for the growth of cyanobacteria (MN, Urmeneta *et al.*, 2003) was prepared as agar plates, which were subsequently inoculated with filamentous cyanobacteria (*Lyngbya* sp.). Cyanobacterial filaments were micromanipulated using a Skerman micromanipulator (Skerman, 1968). Bacteria growing in proximity to the filaments were cultured on seawater yeast peptone agar (SWYP: 5 g tryptone, 3 g yeast extract, 750-mL-filtered sea water, 250 mL H_2O, L^{-1}, pH 6.8) and on MN agar to evaluate the growth of the associated heterotrophic bacteria on rich and MN medium, respectively.

Isolation of polyhydroxyalkanoate-producing and -degrading heterotrophic bacteria from microbial mat samples

The top layers (visible cyanobacterial layer, approximately 2 mm) of Ebro Delta and Camargue microbial mat samples were homogenized in an isotonic solution. Dilutions were cultured onto SWYP medium and MN medium for marine bacteria (modified 3M+ medium; Östling *et al.*, 1991) supplemented with 0.2% glucose, 0.2% fructose, or 1% sodium gluconate. Cultures were grown under oxic conditions at 20 and 30 °C. *Sphingomonas* sp. were isolated from homogenized mat samples cultured on MN agar plates containing glucose or fructose and supplemented with 200 μg streptomycin mL^{-1} (Vanbroekhoven *et al.*, 2004). Polyhydroxyalkanoate-producing strains were detected by culturing the bacteria in medium supplemented with 0.5 mg Nile red L^{-1} in dimethylsulfoxide (Berlanga *et al.*, 2006). Polyhydroxyalkanoate accumulation was further confirmed by Nile blue staining (Ostle & Holt, 1982) and by HPLC determination in isolated strains and in sediment samples. Cultures for polyhydroxyalkanoate analysis by HPLC were prepared as follows: microbial strains were incubated for 48 h in 3M+ medium, after which they were centrifuged, resuspended in the same medium with nitrogen limitation, and incubated for an additional 48 h. Bacterial pellets were lyophilized and digested with 2 mL of 2 N

NaOH at 100 °C for 45 min. After the tubes had cooled, 1 mL of 5 N HCl and 1 mL of 1 M phosphate buffer (pH 7) were added. The samples were centrifuged at 2000 ***g*** for 20 min, and the supernatant was filtered. These extracts were used directly for HPLC (Hewlett-Packard series II-1040-M) in a system equipped with an HPLC Fast Analysis column (100 × 7.8 mm; Bio-Rad, Richmond, CA) Hewlett-Packard series 1050 as described previously (Urmeneta *et al.*, 1995). A more sensitive analysis of polyhydroxyalkanoate in microbial mat samples was performed by GC–MS of the glycolipid fractions, as described previously (Elhottová *et al.*, 2000; Villanueva *et al.*, 2007). The mass spectra of *N*-*t*-butyldimethylsilyl-*N*-methyltrifluoroacetamide-3OH (MTBSTFA-3OH) fatty acid derivatives of 3-HB and 3-HV released monomers were obtained by preparing extracts of lyophilized cells of *Cupriavidus necator* CECT 4635 and of *Chromobacterium violaceum* CECT 494 that had been grown in MN medium supplemented with 1% sodium gluconate and 1% sodium valerate as carbon sources, respectively (Kolibachuk *et al.*, 1999). Released monomers of polyhydroxyoctanoic and polyhydroxyhexanoic were detected by comparison with DL-β-hydroxycaprylic acid (Sigma-Aldrich, St. Louis, MO) or based on the deduced *m/z* fragments $(M-57)^+$ at 303. The polyhydroxyalkanoate-degrading capacity of the isolates was tested by culturing them onto MN medium for nonmarine control strains (*C. necator* CECT 4635 and PH4$^-$ mutant DSM 541; Hareland *et al.*, 1975) or onto 3M+ agar plates with an overlay prepared as follows: a mixture containing 0.5 g poly-3-hydroxybutyrate (Sigma-Aldrich), 5 mL water, and 5 mL 0.1% Triton X-100 was sonicated for 2 h, followed by the addition of 100 mL of MN medium agar. The mixture was then added as an overlay onto agar-base plates. Negative results of polyhydroxyalkanoate degradation were concluded when, after 2 weeks of incubation, degradation halos had failed to appear.

Characterization of the isolates

Strains were characterized based on the results of the following biochemical and physiological tests: oxidase, catalase, anaerobic growth, Gram staining, morphology, pigmentation, temperature, pH and salinity ranges of growth, sensitivity to antibiotics, biochemical tests included in API 20NE, as well as enzymatic activities by the API ZYM assay (bioMérieux, France) and on plates (DNAse, gelatinase, protease, chitinase, amylase, cellulose, lipase, alginase, and hemolysis). Incubations were performed in triplicate and negative results were concluded after 72 h. Lyophilized bacterial cell pellets and/or microbial mat samples were used to analyze fatty acid methyl esters (FAMEs), respiratory quinones, and sphingoid bases for their lipid content, as described previously (Leung *et al.*, 1999; Geyer *et al.*, 2004; Villanueva *et al.*, 2007). Ubiquinones, menaquinones, and demethylmenaquinones with *n* isoprene units in their side chains were abbreviated as Q-*n*, MK-*n*, and DMK-*n*, respectively.

Interaction assays

Growth inhibition of other bacteria and the test bacterium was detected using the overlay method on agar plates, as described by Rao *et al.* (2005). An interaction activity assay between filamentous cyanobacteria and an isolated heterotroph was designed based on the inhibition of cyanobacterial photosystem II by 3-(3′,4′-dichlorphenyl)-1, 1-dimethylurea (DCMU) (Sigma-Aldrich). The filamentous cyanobacterium LPP (*Lyngbya–Phormidium–Plectonema*), group EBD-11 (Urmeneta *et al.*, 2003), was selected for its inability to grow on MN agar plates supplemented with 2 μM DCMU as well as its resemblance to the filamentous cyanobacterium (*Lyngbya* sp.) micromanipulated for the original isolation. Filamentous EBD-11 cells were transferred to liquid MN+DCMU and maintained in an incubator under light/dark conditions for 48 h. Afterwards, the cells were pelleted by centrifugation (1500 ***g***, 10 min) and washed three times. The washed pellet was suspended in MN+DCMU and spread onto MN or MN+DCMU agar plates inoculated previously with 100 μL of a saturated culture of *Pseudoalteromona*s sp. EBD. The inoculated agar plates were incubated under light/dark conditions for 10 days. Growth was followed by phase-contrast microscopy examination of the cultures every 24 h.

DNA extraction, 16S rRNA gene sequencing, and phylogeny reconstruction

Ebro Delta mat samples were cut on a cryomicrotome to yield 500-μm-thick slices that were pooled to cover 2 mm depth, from which DNA was extracted using the Power Soil™ DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA). Single colonies of the isolated strains were suspended in 50 μL TE buffer (pH 7.0), heated at 95 °C, and cooled, after which the cells were pelleted by centrifugation (13 000 ***g***, 10 min). A 1–2-μL aliquot of the supernatant without further purification served as the DNA template for PCR. Primers E8F (8–27) (5′-AGA GTT TGA TCC TGG CTC AG-3′) and Ty06R (1489–1505) (5′-TAC CTT GTT ACG ACT T-3′) were used to amplify partial bacterial 16S rRNA gene sequences (1497 bp) (Baker *et al.*, 2003). The PCR products were purified (Wizard® PCR Preps, Promega, Madison, WI) and then cloned using the pGEM® T vector system (Promega). Restriction analysis was carried out to select different sequence inserts for analysis by sequencing. The occurrence of chimeras was checked using the CHIMERA_CHECK program.

Results

Detection and dynamics of polyhydroxyalkanoates in microbial mats

Quantification of the polyhydroxyalkanoate content in two microbial mat systems during a diel cycle in the summer season revealed higher accumulation in Camargue hypersaline mats than in Ebro Delta estuarine samples per gram of dry weight (Table 2). In addition, the ratio polyhydroxybutyrate/polyhydroxyvalerate was lower. The dynamics of polyhydroxyalkanoate accumulation in the two systems during the diel cycle clearly differed, with polyhydroxyalkanoate accumulation reaching a maximum at 6:00 hours in Camargue mats, but in the afternoon in mats from the Ebro Delta. The detection of medium- and long-chain polyhydroxyalkanoates in sediment samples was optimized according to a previously published method (Elhottová *et al.*, 2000). MTBSTFA-3OH derivatives with *m/z* fragmentation patterns indicative of 3-HB and 3-HV were detected in both mat samples, whereas derivatives of polyhydroxyhexanoate (3-hydroxyhexanoic) were detected only in Ebro Delta mat samples (Fig. 1). Long-chain derivatives of polyhydroxyalkanoates, such as 3-hydroxyoctanoic, were not observed.

Microbial diversity in microbial mat photic zones

Microbial diversity in the topmost layer of the mats (cyanobacterial layer) was investigated by means of quinone analysis and 16S rRNA gene sequencing. The first two millimeters of the mat samples were defined arbitrarily as the area with more presence of cyanobacteria during the day hours in accordance with previous microbial diversity studies of Camargue mats (Fourçans *et al.*, 2004, 2006; Villanueva *et al.*, 2004) (Table 1). In Ebro Delta mat samples, ubiquinones predominated, especially Q-10 (*Alphaproteobacteria*) and Q-8, Q-9 (*Gammaproteobacteria*) (Collins & Jones, 1981), whereas mat samples from the Camargue were dominated by menaquinones such as MK-7, -8, and -10 (present in *Bacteroidetes*, *Delta-* and *Epsilonproteobacteria*, *Euryarchaeota*, and green nonsulfur bacteria), but mainly MK-9, which was associated with *Bacteroidetes*, *Firmicutes*, and *Rhodospirillaceae* (Collins & Jones, 1981). Total DNA was extracted from the first millimeter of Ebro Delta microbial mat cores, and the 16S rRNA gene amplified and sequenced using universal eubacterial primers. The results of the most abundant sequenced clones and their closest relatives are shown in Table 1. The most abundant sequences had a high homology with filamentous and unicellular cyanobacteria (*Microcoleus*, *Leptolyngbya*, *Cyanothece*). Sequences highly similar to aerobic heterotrophs belonging to *Alpha-* (*Roseobacter* sp., *Rhodobacter* sp.) and *Gammaproteobacteria* and to the *Bacteroides* group were also detected.

Table 2. Polyhydroxyalkanoate accumulation dynamics in microbial mats

	Ebro Delta mats		Camargue mats	
Time	Polyhydroxybutyrate	Polyhydroxyvalerate	Polyhydroxybutyrate	Polyhydroxyvalerate
18:00	225 ± 17	210 ± 19	120 ± 7	315 ± 30
24:00	145 ± 8	100 ± 8	110 ± 8	210 ± 18
6:00	75 ± 2	75 ± 5	220 ± 13	600 ± 48
12:00	20 ± 0.7	79 ± 4	100 ± 7	145 ± 8

Data given in $\mu g\,cm^{-2}$ of the mat core. Sampling time GMT+01:00. Reproducibility of polyhydroxyalkanoates analysis was within ± 10% (data obtained with $n = 4$ replicates of microbial mat cores).

Diversity of heterotrophs accompanying cyanobacteria

The diversity and role of aerobic heterotrophs involved in the polyhydroxyalkanoate dynamics in microbial mat systems were evaluated in two different approaches.

Isolation of polyhydroxyalkanoate-producing and -degrading heterotrophs

Heterotrophic strains from the cyanobacterial layer of the two mat systems were isolated by culturing fresh samples in rich SWYP agar and minimum marine media supplemented with carbon sources. Pure cultures were subcultured in the same growing media supplemented with Nile red. Strains found to be positive for polyhydroxyalkanoate accumulation were retested by Nile blue staining and by HPLC detection of polyhydroxyalkanoate. Strains able to degrade polyhydroxyalkanoate were identified by subculturing in

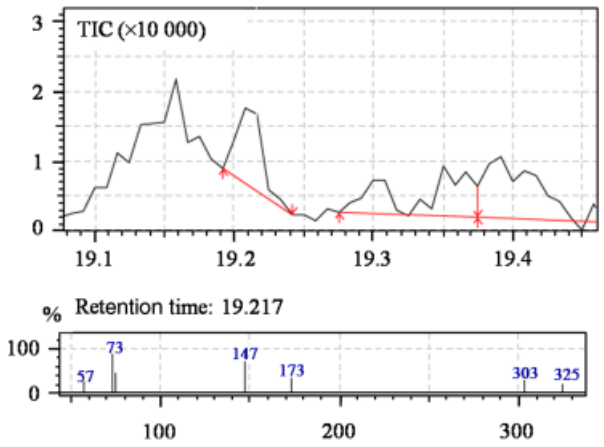

Fig. 1. Detection of polyhydroxyhexanoates in the glycolipid fraction of Ebro Delta microbial mats. Total lipids after MTBSTFA-3OH derivatization by GC–MS. Chromatogram and mass spectra of polyhydroxyhexanoate with the *m/z* fragments $(M\text{-}57)^+$ at 303.

Table 3. Polyhydroxyalkanoate-producer and -degrader aerobic heterotrophs isolated from Ebro Delta (EBD) and Camargue mat (CM) samples

EBD	CM
Polyhydroxyalkanoate producers	
Halomonas sp. PEB01 (GU213157)	*Sphingomonas* sp. PCM01 (GU213163)
Labrenzia sp. PEB02 (GU213158)	*Halomonas* sp. PCM02 (GU213167)
Virgibacillus sp. PEB03 (GU213159)	*Bacillus* sp. PCM04 (GU213168)
Bacillus sp. PEB04 (GU213160)	*Bacillus* sp. PCM05 (GU213169)
Labrenzia sp. PEB05 (GU213161)	*Bacillus* sp. PCM06 (GU213170)
Pseudomonas sp. PEB06 (GU213162)	
Polyhydroxyalkanoate degraders	
Halomonas sp. PEB01, 07-09	*Microbacterium* sp. DCD01 (GU213172)
Labrenzia sp. PEB02	*Bacillus* sp. DCD02 (GU213173)
Arthrobacter sp. DEB01 (GU213171)	*Agromyces* sp. DCD03 (GU213174)

Accession number of the 16S rRNA gene sequences indicated in parentheses. *Halomonas* sp. PEB07, PEB08, and PEB09 accession numbers are GU213164, GU213165, and GU213166, respectively.

polyhydroxybutyrate-overlay agar plates. Polyhydroxyalkanoate-producing and -degrading strains were taxonomically characterized by 16S rRNA gene sequencing, as indicated in Table 3.

Representatives of the α and γ classes of the phyla *Proteobacteria* and *Firmicutes* were chosen for further morphobiochemical characterization. The gammaproteobacterium *Halomonas* sp. PEB01 (producer Ebro Delta) and the alphaproteobacterium *Labrenzia* sp. PEB02 were the most abundant isolates from Ebro Delta mat samples (four strains, *Halomonas* sp. PEB01, 06, 07, and 08; sequence accession numbers are provided in Table 3), and all were able to produce and degrade polyhydroxybutyrate extracellularly. Polyhydroxyalkanoate-producing strains of the *Halomonas* genus were also abundant in Camargue samples as were members of *Bacillus*. *Halomonas* sp. PEB01 was characterized as a nonmotile rod with Q-9 quinone and with 18:1ω7*c* and 16:0 as the main FAMEs. Quinone-10 was the major ubiquinone and 18:1ω7*c* the major fatty acid in the nonsporing, motile rod *Labrenzia* sp. PEB02. *Halomonas* sp. PEB01 grew over a wide range of temperatures and salinities (4–44 °C and 0–10% NaCl), while *Labrenzia* sp. PEB02 grew at more restrictive temperatures (20–37 °C). Although both isolates showed α-glucosidase and esterase activities, *Labrenzia* sp. PEB02 displayed wider enzymatic capabilities (lipase, α-galactosidase, acid and alkaline phosphatase, etc., Table S2). In addition, *Halomonas* sp. PEB01 had a broader range of metabolized D-glucopyranoside substrates.

Table 4. Quantification of the detected sphingoid bases in Ebro Delta (EBD) and Camargue (CM) mat samples

	EBD	CM
C14:0	0	69.2
C15:0	0	30.8
C16:0	0	136.3
C18:0	23.1	120.4
C19:0	0	20.8
C21:1	46.9	51.2

Data in nmol g^{-1} of dry weight. Average of $n = 3$ samples. SD $< 5\%$.

A polyhydroxyalkanoate-producing strain identified as a member of the *Sphingomonas* genus (*Alphaproteobacteria*) was isolated from Camargue mat samples. *Sphingomonas* sp. PCM01 was a deep-yellow-pigmented rod, non-spore-forming, and nonmotile. The bacterium grew within a salinity range of 0–2.5% NaCl, at temperatures of 20–37 °C, and at pH 5–8. The major ubiquinone was Q-10 and the main FAMEs were 18:1ω7*c*, 17:1, and *cy*17:0. The isolated strain showed the highest sequence similarity (99%) with *Sphingomonas melonis* (AB055863; Buonaurio *et al.*, 2002), but differed from the latter in several phenotypic characteristics, such as the inability to grow in the presence of 3% NaCl or to assimilate phenylacetate and the absence of oxidation activity on glycerol. The high similarity between the 16S rRNA gene sequences and the phenotypic characteristics of *Sphingomonas* sp. PCM01 and *S. melonis* (AB055863) suggested that the former is a new strain of the same genus and species. As *Sphingomonas* sp. can be important polyhydroxyalkanoate producers in microbial mats, selective isolation was carried out in both mat systems based on pigmentation and resistance to high concentrations of streptomycin (Vanbroekhoven *et al.*, 2004). *Sphingomonas* candidate strains were detected in Camargue mats, but not in Ebro Delta samples. The yellow-pigmented isolated strains were confirmed by 16S rRNA gene sequencing as members of the *Sphingomonas* genus. Based on these results, a rapid screening method was developed for the detection of sphingoid bases in mat samples, as biomarkers of the presence of sphingolipid-containing bacteria with the potential to produce polyhydroxyalkanoate. The major sphingoid bases detected in *Sphingomonas* sp. PCM01 were C14:0, C18:0 (dihydrosphingosine), and C21:1. In Ebro Delta samples, only C18:0 and C21:1 were detected, while those from the Camargue contained C14:0, C15:0, C16:0, C18:0, C19:0, and C21:1 (Table 4). The total sphingoid content in Ebro Delta samples was 7×10^4 pmol g^{-1} dry weight, but almost an order of magnitude higher in Camargue mats, 4.3×10^5 pmol g^{-1} dry weight.

Two polyhydroxyalkanoate producers from Ebro Delta samples, identified as representatives of the phylum *Firmicutes* (PEB03 and PEB04), were also detected. Their closest

relatives by 16S rRNA gene sequencing were marine *Bacillus* belonging to *Virgibacillus* sp. and *Bacillus marisflavis*, respectively. The temperature range of growth was 20–45 °C for PEB03 and 20–35 °C for PEB04. The salinity growth range was 1–2.5% NaCl for the strain PEB03, whereas strain PEB04 tolerated up to 5% NaCl. Both strictly aerobic isolates grew at a pH between 7 and 8, and while both showed protease activity, only PEB04 displayed amylase activity (Table S2). Substrate assimilation was mainly restricted to glucose, fructose, and *N*-acetylglucosamine; however, strain PEB04 was also able to assimilate starch and glycogen (Table S3).

Heterotrophic strains isolated from the cyanobacterial layer of the mats on either rich or minimum medium were cultured in minimum medium supplemented with polyhydroxybutyrate in an overlay layer. Isolates able to produce degradation halos were isolated in pure culture and identified by means of 16S rRNA gene sequencing. The majority of the polyhydroxybutyrate degraders belonged to the *Halomonas* and *Labrenzia* genera, while the remainder displayed high homology to *Microbacterium*, *Bacillus*, and *Agromyces* in Camargue samples and to *Bacillus* and *Arthrobacter* in those from the Ebro Delta (Table 3).

Isolation of polyhydroxyalkanoate-producing heterotrophic strains accompanying filamentous cyanobacteria cultures

Polyhydroxyalkanoate-producing heterotrophs were isolated from different filamentous cyanobacterial cultures by following the same strategy as described above. Cyanobacterial cultures maintained in the laboratory were not axenic after multiple transfers to reduce the heterotrophic load. The filamentous cyanobacterial cultures consisted of members of the *Oscillatoriales* order (Table 5). In most cases, the heterotrophs isolated from those cultures comprised members of the *Gammaproteobacteria* with homology to the *Halomonas* genus, but also members of the *Alphaproteobacteria*. Among all the cyanobacterial cultures examined, only one heterotrophic strain was isolated on both rich and minimum media supplemented with different carbon sources. Again, all isolates were able to produce polyhydroxyalkanoates.

To better understand the role of cyanobacteria as a microhabitat for heterotroph proliferation, cyanobacterial filaments from fresh mat samples were micromanipulated and the heterotrophs surrounding or attached to the sheaths were characterized following subculture on SWYP agar plates aimed at isolating the growing morphologies; however, only a single heterotrophic strain could be isolated. Cells of the isolated strain were gram-negative rods, facultative anaerobes, 0.5–0.8 μm wide and 1.7–4 μm long. The strain was motile by means of one polar flagellum. It did not form endospores nor did it accumulate polyhydroxybutyrate. Growth was achieved within a salinity range of 1.5–10% NaCl, at temperatures of 4–35 °C, and at pH 5–10. Oil displacement activity was positive, suggesting biosurfactant activity. Q-8 was the major quinone, and 14:0, 16:0, *a*17:0, and *br*16:1 were the main FAMEs. The enzymatic activity of the isolated strain included esterase, lipase, acid phosphatase, DNAse, protease, amylase, and hemolysin (Tables S1–S2). The range of assimilable substrates was restricted to D-glucose, D-fructose, D-maltose, D-saccharose, *N*-acetylglucosamine, starch, and glycogen, which were also substrates utilized by the rest of the heterotrophs isolated in this study.

Analysis of the 16S rRNA gene sequence (accession number DQ218321) revealed that the isolate was a member of the *gamma*-subclass of *Proteobacteria*, showing the highest sequence similarity (99%) with *Pseudoalteromonas elyakovii* (Sawabe *et al.*, 2000). However, the isolate's capacity for growth at 4 °C, the absence of alginase activity, the use of certain carbohydrates, and its facultative anaerobic growth differentiated it from *P. elyakovii*. The isolated strain was also tested for its capacity to produce antibacterial and autoinhibitory compounds – a feature characteristic of some *Pseudoalteromonas* sp. The target bacterial strains were noncharacterized aerobic heterotrophic bacteria isolated

Table 5. Cyanobacterial strains and their heterotrophic partners after rounds of selection under laboratory conditions

Cyanobacteria	Closest relative	Heterotroph	Closest relative
EBD02 (GU213175)	*Geitlerinema* sp. (GQ402016)	H02E (GU213180)	*Nesiotobacter exalbescens* (AF513441)
EBD07 (GU213176)	*Leptolyngbya* sp. (EU249119)	H07E (GU213181)	*Halomonas aquamarina* (EU440965)
EBD09 (GU213177)	*Geitlerinema* sp. (EF372580)	H09E (GU213182)	*Citreimonas salinaria* (AY962295)
EBD11 (GU213178)	*Leptolyngbya* sp. (EU249119)	H11E (GU213183)	*Nesiotobacter exalbescens* (AF513441)
EBD14 (GU213179)	*Leptolyngbya* sp. (EU249119)	H14E (GU213184)	*Halomonas* sp. MAN K22 (AM945678)

Accession number of the 16S rRNA gene sequences indicated in parentheses.

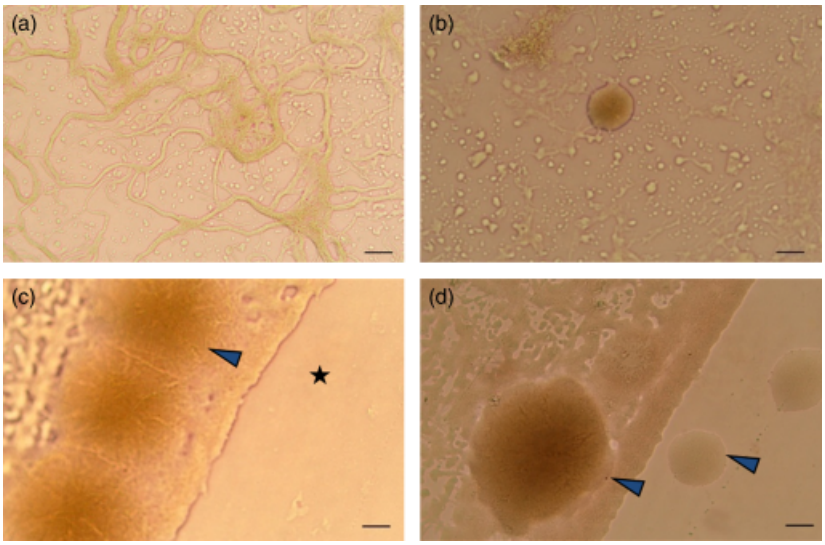

Fig. 2. Interaction assay between cyanobacteria and *Pseudoalteromonas* sp. EBD (scale bar = 50 μm). (a) Cyanobacteria in MN medium recovering from DCMU inhibition. (b) Cyanobacteria in MN+DCMU exhibit slow heterotrophic growth. (c) Cyanobacteria and *Pseudoalteromonas* sp. EBD, arrow: adequate heterotrophic growth in the inoculum area vs. low growth in the noninoculated zone (★). (d) Cyanobacteria and *Pseudoalteromonas* sp. EBD in medium supplemented with DCMU, a comparison of inoculated and noninoculated areas.

from Ebro Delta and Camargue microbial mats as well as the polyhydroxyalkanoate producers and degraders described above. Neither antibacterial nor autoinhibitory activity was detected using the supernatant obtained from the isolated strain.

Because the enzymatic and antimicrobial activities of *Pseudoalteromonas* sp. are relevant to niche occupancy, an experiment aimed at elucidating the nature of the interaction between the isolated strain and filamentous cyanobacteria was designed. In this interaction assay, MN plates were inoculated with *Pseudoalteromonas* sp. EBD and/or the filamentous cyanobacterium EBD-11 in the presence or absence of DCMU, an inhibitor of cyanobacterial photosystem II. After 10 days of incubation, MN and MN+DCMU plates inoculated with *Pseudoalteromonas* sp. EBD displayed small colonies that grew at the expense of the organic compounds present in the inoculum. Control plates inoculated only with cyanobacterium EBD-11 showed that the phototroph could return to its original state following DCMU inhibition (sublethal concentrations). In addition, those plates displayed small colonies of heterotrophic bacteria surrounding the cyanobacterial filaments (Fig. 2a). Control plates of MN+DCMU that had been inoculated only with the cyanobacterium revealed the same autochthonous heterotrophic population as that seen before (attached to the cyanobacterium even after the washes), which proliferated when the flux of photosynthates was blocked by DCMU. However, growth of the heterotrophic population was low and only detectable microscopically (Fig. 2b). In the set of MN and MN+DCMU plates inoculated with both the bacterial suspension of *Pseudoalteromonas* sp. EBD and the cyanobacterium EBD-11, agar zones that had not been in contact with the cyanobacterial inoculum contained small colonies of *Pseudoalteromonas* sp. EBD resembling those observed in the control plates inoculated only with the heterotroph (Fig. 2c and d). By contrast, in areas in which the cyanobacterial suspension was in contact with agar previously inoculated with *Pseudoalteromonas* sp. EBD, larger colonies of a heterotrophic bacterial strain developed. The growth of these colonies onto the inoculum area can be attributed either to higher *Pseudoalteromonas* sp. EBD growth or to other heterotrophs that surrounded the cyanobacterial inoculum. A comparison between the extent of heterotrophic colony growth and the control plates of MN+cyanobacterium suggested higher growth of *Pseudoalteromonas* sp. EBD at the expense of other assimilable organic compounds apart from the photosynthates (blocked by the DCMU). This possibility was tested by isolating some of the larger colonies growing on the inoculum area on SWYP agar. The identity of the recovered heterotrophic microorganisms was determined by Gram staining and 16S rRNA gene sequencing. The results suggested the affiliation of the strains with the *Pseudoalteromonas* genus. Background bacterial growth observed in the cyanobacterial inoculum was isolated in pure culture and the isolates were identified by 16S rRNA gene sequencing as members of the *Halomonas* genus.

Discussion

In this study, several heterotrophic bacterial strains were isolated and characterized based on their ability to accumulate and/or degrade polyhydroxyalkanoates and their metabolic association with cyanobacteria in the topmost layers of microbial mats. An integrated approach consisting of physicochemical characterization and lipid analysis allowed the diversity and phylogeny of these microbial groups to be determined. Members of the *Gammaproteobacteria*, i.e.,

Halomonas sp. and *Pseudoalteromonas* sp., appear to interact syntrophically with their phototrophic partners, with direct consequences on polyhydroxyalkanoate diel dynamics in stratified systems. A strong partnership between heterotrophic groups in the cyanobacterial layer may well lead to phylogenetic radiation and the maintenance of diversity in microbial mat ecosystems.

The amounts of polyhydroxyalkanoates quantified during the diel cycle of Camargue hypersaline mats were in agreement with a nocturnal polyhydroxyalkanoate-producing metabolism driven by anoxygenic phototrophs (Rothermich *et al.*, 2000). This was in contrast to Ebro Delta mats, where the maximum polyhydroxyalkanoate production occurs after active photosynthetic processes (Villanueva *et al.*, 2007). These data clearly show that differences in the bacterial populations and physicochemical characteristics of the two assemblages are reflected by differences in polyhydroxyalkanoate dynamics. The detection in mat samples of medium-chain length polyhydroxyalkanoates suggested the presence of active members of the *Pseudomonas* genus. It also reinforced that microbial mats may be a novel source of polyhydroxyalkanoate producers and degraders with a high tolerance to changing conditions and nutrient limitations (Berlanga *et al.*, 2006; López-Cortés *et al.*, 2008). The methods for polyhydroxyalkanoate analysis applied in this study were shown to be effective in the biopolymer's detection and in the rapid screening of polyhydroxyalkanoate producers in microbial mat samples. However, further studies are needed to assess the presence of other hydroxylated derivatives of valeric, hexanoic, heptanoic, and octanoic acids. Also, internal standards other than 3-hydroxyoctanoate should be used because low amounts of this compound may be present in environmental samples (Guezennec *et al.*, 1998).

Previous studies have assessed the microbial diversity in different microbial mat systems using several approaches. Here, 16S rRNA gene cloning and quinone analysis were combined in a rapid method to detect microorganisms for further isolation and characterization – in this case, with respect to the phylogeny of heterotrophic bacteria in the cyanobacterial layer of mat systems. The results suggested that Ebro Delta estuarine mats contain an abundance of heterotrophs belonging to the *Alpha-* and *Gammaproteobacteria*. By contrast, in the cyanobacterial layer of hypersaline Camargue mats, the predominance of menaquinones, together with previous results obtained with molecular methods, clearly suggested a dominance of heterotrophs, specifically those of the *Cytophaga–Flavobacterium–Bacteroides* (CFB) group and *Alphaproteobacteria* (Fourçans *et al.*, 2004; Villanueva *et al.*, 2004). Although representatives of the CFB group are important degraders of dissolved organic matter in marine environments (Cottrell & Kirchman, 2000), they do not accumulate polyhydroxyalkanoates. Recent studies suggested that bacteria belonging to the phylum *Bacteroidetes* play important roles in the degradation of organic matter during algal blooms (Pinhassi *et al.*, 2004). Their presence in the hypersaline mats of the Camargue was therefore assessed using molecular methods and by determining the contribution of MK-6 and -7 to the quinone pool at the upper layers of these mats. Members of the *Bacteroidetes* are also known to be specialists in the degradation of polymeric organic matter and to closely associate with exudate-producing cyanobacteria (Pinhassi *et al.*, 2004; Bauer *et al.*, 2006).

The survey of aerobic heterotrophs involved in mat polyhydroxyalkanoate dynamics demonstrated the important contribution of members of *Gammaproteobacteria* and of *Bacillus*-like groups. Polyhydroxyalkanoate degraders belonged to the *Halomonas*, *Labrenzia*, and *Arthrobacter* genera in Ebro Delta samples, while those in Camargue mats showed homology with *Microbacterium*, *Agromyces*, and *Bacillus*.

The combination of sphingolipid detection and selective isolation was successfully applied to assess the presence and capabilities of members of the *Sphingomonas* genus (*Alphaproteobacteria*), which may also contribute to polymer accumulation in hypersaline mat ecosystems. Application of a similar method by Vanbroekhoven *et al.* (2004) allowed the isolation of a *Sphingomonas* strain in Camargue mat samples, but no streptomycin-resistant, yellow-pigmented colonies were recovered from Ebro Delta mats. The isolated strain showed a high similarity to *S. melonis* (Buonaurio *et al.*, 2002) with respect to its phenotypic characteristics and 16S rRNA gene sequence, suggesting its classification in the same species. Because many of the members of *Sphingomonas* grow on the surface of a wide range of plants (Kim *et al.*, 1998), it may well be that the *Sphingomonas*-like isolate acts as an epiphyte on a phototrophic partner – a scenario that could explain the relationship between cyanobacteria and *Sphingomonas* members in the oxic zone of stratified microbial ecosystems. Indeed, other interactions between members of the *Sphingomonas* genus and cyanobacteria have been described previously, for example, the production of an anticyanobacterial compound by a *Sphingomonas* strain (Hibayashi & Imamura, 2003). The lower content of sphingoid bases in Ebro Delta mat samples suggests that members of *Sphingomonas* are not highly represented in estuarine mats. This finding is consistent with the differences in polyhydroxyalkanoate dynamics between these mats and the hypersaline mats from the Camargue. However, the predominance of sphingoid bases may, alternatively, be due to the presence of other bacterial genera, such as *Bacteroides* and *Sphingobacterium*, detected previously using molecular methods in Camargue mats (Fourçans *et al.*, 2004).

In this study, polyhydroxyalkanoate-producing heterotrophic strains that accompanied filamentous cyanobacteria

were isolated by two approaches: (1) multiple transfers of cyanobacterial cultures, inducing the artificial selection of heterotrophs growing onto cyanobacterial exudates and adapted to a unique cyanobacterial strain, and (2) reproduction of natural conditions, with micromanipulation of cyanobacterial filaments from fresh samples, followed by characterization of the heterotrophic bacteria surrounded or attached to their sheaths. In the first approach, members of the *Halomonas* genus and of *Alphaproteobacteria* were positively selected for growth at the expense of cyanobacterial photosynthates, thus supporting their dominance over possible competitors. It should be noted that most of the filamentous cyanobacteria obtained under these conditions still showed *Halomonas*-type growth, which suggests a close interaction between members of this genus and cyanobacteria, including polyhydroxyalkanoate production. Thus, *Halomonas* may be an optimum colonizer that grows at the expense of photosynthates released by the phototroph. However, the predominance of other heterotrophic groups in isolations from fresh samples or without selection based on polyhydroxyalkanoate production suggests that it is not a good commensalistic partner under natural conditions.

Differences in the 16S rRNA gene sequence and phenotypic characteristics of the isolated *Halomonas* strains from different cyanobacterial cultures indicated that, under laboratory conditions, the partnership is selected after multiple transfers, thus providing evidence of the speciation of heterotrophic genera such as *Halomonas*, *Labrenzia*, and other members of *Alphaproteobacteria* in the photic zone of microbial mats. Indeed, Jonkers & Abed (2003) reported the presence of members of the genera *Rhodobacter*, *Roseobacter*, *Marinobacter*, *Halomonas*, and populations affiliated with the CFB group in a hypersaline microbial mat. They suggested that the isolated bacteria were specialized in the degradation of photosynthates excreted by the primary producers.

Micromanipulation of filamentous cyanobacteria from fresh mat samples allowed isolation of a heterotrophic strain belonging to *Pseudoalteromonas* genus. One of the main features of this genus is that some of its members generate antibacterial proteins that are beneficial during competition in microbial biofilms (Rao *et al.*, 2005). The isolated *Pseudoalteromonas* sp. EBD is a versatile bacterium that grows under widely ranging conditions of pH, salinity, and temperature in addition to possessing enzymatic capabilities that allow growth under different environmental conditions (e.g. alkaline and acid phosphatase activities favoring the use of organic phosphorus). These adaptations provide the isolate with access to a more stable and lasting source of nutrients (Holmström & Kjelleberg, 1999; Ivanova *et al.*, 2003). The interaction assay between cyanobacteria and *Pseudoalteromonas* sp. EBD provided preliminary evidence that the heterotrophic isolate participates in the recycling of organic compounds generated by the phototrophic partner. The higher growth of *Pseudoalteromonas* sp. observed in DCMU-treated cyanobacterial cultures is consistent with the growth of *Pseudoalteromonas* at the expense of other compounds apart from the photosynthates (blocked by DCMU), such as structural components of the cyanobacterial sheath. The latter provides a microenvironment where essential nutrients are concentrated and used by the cyanobacteria, and where photosynthates produced by the phototrophs are available to the heterotrophs. The gelatinous sheath acts as a protective matrix that retains humidity and nutrients in addition to providing an attachment surface for heterotrophs. Accordingly, the latter do not attack the sheath when alternative sources of assimilable organic matter are available (Lange, 1976). If this is not the case, then *Pseudoalteromonas* may preferentially degrade the portion of the cyanobacterial sheath mainly formed by carbohydrates, especially glucose (Bertocchi *et al.*, 1990; De Philippis *et al.*, 2001) or other exopolymeric substances present in the mat biofilm matrix such as amino sugars, proteins, and uronic acids (Klock *et al.*, 2007; Braissant *et al.*, 2009), and then, by producing proteinases and lipases, the cell walls and membranes. Degradation would generate monosaccharides and low-molecular-weight organic compounds for both members of the association. This hypothesis is strongly supported by the range of enzymatic activities displayed by *Pseudoalteromonas*, as well as by the results of the physical contact and interaction assays performed in this study.

In previous studies, a cooperative relationship between members of a heterotrophic community, i.e., *Pseudoalteromonas* sp. and *Halomonas* sp., in the degradation of the brown algae *Fucus evanescens* was observed (Ivanova *et al.*, 2002a, b). The *Pseudoalteromonas* partner was essential to the initial stages of algal degradation because of its enzymatic capacities, while *Halomonas* (with less enzymatic activities) benefited from the low-molecular-weight compounds produced by the degradation activity of its partner. In those experiments, *Pseudoalteromonas* was considered to be a saprophytic organism that degrades the algal thallus. A similar association may explain the interaction between these two heterotrophic species in the oxic zone of microbial mats (Ivanova *et al.*, 2002a).

Previous studies have shown that some members of *Pseudoalteromonas* produce biologically active compounds that reflect their colonization strategies, symbiotic associations, etc. (Sawabe *et al.*, 1998; Rao *et al.*, 2005); however, the lack of bacteriolytic activity in the *Pseudoalteromonas* strain of this study remains to be explained. It is important to highlight that no inhibitory activity was found when *Halomonas* sp. EBD was used as the target, which may explain the involvement of a strain resistant to the bacteriolytic activity of its partner in the association. This study

demonstrated the presence of *Halomonas* and *Pseudoalteromonas* surrounding cyanobacterial filaments in the oxic zone of microbial mats. The coexistence of these two heterotrophic groups reflects their abilities to exploit distinct niches. Based on the observations described herein, we propose a succession process in which *Pseudoalteromonas* and members of the CFB group compete for polymers in the photic zone (Berkenheger & Fischer, 2004; Rao *et al.*, 2006), while the synthesis of inhibitory compounds prevents the displacement of these bacteria by others. Also possible is a three-component system in which *Halomonas* and other polyhydroxyalkanoate producers store the excess low-molecular-weight compounds released by the degraders in the form of reserve polymers. This system likely varies from one microbial mat system to another depending on the microbial diversity, which in turn is mainly influenced by environmental conditions. This is likely the case in hypersaline microbial mats (e.g. Camargue) and marine mats like the ones at Great Sippewissett Salt Marsh (MA), which display a polyhydroxyalkanoate cycle based on anoxygenic phototrophs and high-level accumulation of the polymer during the night (Rothermich *et al.*, 2000).

The role of heterotrophic bacterial associations in the cyanobacterial layer in microbial mats should be studied in detail to clarify the possible syntrophic relationships between its members in the recycling of nutrients and also to evaluate the responses of the two partners to changing conditions. Such studies would enhance our understanding of the structure–function relationship in marine microbial communities and the role of aerobic heterotrophs in carbon cycling. Likewise, the characterization of these isolates and their metabolic strategies offers new alternatives for the biotechnological application of polyhydroxyalkanoate-producing microorganisms.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Location, physicochemical conditions and microbial diversity in Ebro Delta and Camargue mats.
Table S2. Biochemical tests based on API 20NE, API ZYM and degradation on plates.
Table S3. Substrate assimilation.

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