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Role of bacteria in marine barite precipitation: A case study using Mediterranean seawater

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HIGHLIGHTS

- Bacteria isolated from Mediterranean seawater promote barite precipitation.
- Bacteria may play an essential role in barite formation in natural environments.
- Extracellular polymeric substances (EPS) production seems to affect mineral precipitation.
- Microbially induced barite precipitation may play an essential role in Ba cycling.

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ABSTRACT

Marine bacteria isolated from natural seawater were used to test their capacity to promote barite precipitation under laboratory conditions. Seawater samples were collected in the western and eastern Mediterranean at 250 m and 200 m depths, respectively, since marine barite formation is thought to occur in the upper water column. The results indicate that *Pseudoalteromonas* sp., *Idiomarina* sp. and *Alteromonas* sp. actually precipitate barite under experimental conditions. Barite precipitates show typical characteristics of microbial precipitation in terms of size, morphology and composition. Initially, a P-rich phase precipitates and subsequently evolves to barite crystals with low P contents. Under laboratory conditions barite formation correlates with extracellular polymeric substances (EPS) production. Barite precipitates are particularly abundant in cultures where EPS production is similarly abundant. Our results further support the idea that bacteria may provide appropriate microenvironments for mineral precipitation in the water column. Therefore, bacterial production in the past ocean should be considered when using Ba proxies for paleoproductivity reconstructions.

1. Introduction

Biogenic barium content in marine sediments has been traditionally considered as a reliable proxy for reconstructing past-ocean productivity (Paytan and Griffith, 2007 and references therein). At present, it usually occurs as discrete crystals of the mineral barite in ocean waters underlying high productivity regions. As barite subsequently accumulates in proportion to export production from the surface waters in marine sediments, Ba proxies (barite and algorithms based on Bio-Ba) have served to recognize productivity variations in the past. The settling of particulate Ba has been broadly demonstrated, as has its correlation with organic carbon fluxes and primary production (e.g., Dehairs et al., 1980, 1987, 1991, 1992, 2008; Bishop, 1988; Dymond et al., 1992; Planchon et al., 2013). Moreover, barite is very refractory under oxic conditions; thus, it is well preserved in oxic settings, and dissolution may occur only in anoxic sediments.

Since the early discoveries of Ba enrichment in marine sediments (Goldberg and Arrhenius, 1958; Chow and Goldberg, 1960), a vast body of literature on Ba proxies has appeared (e.g., Van Santvoort et al., 1996; Griffith and Paytan, 2012 and references therein). However, the mechanisms behind barite precipitation in seawater that is undersaturated in barite (e.g., Monnin et al., 1999) still remain unknown. Barite-supersaturated microenvironments may result from the decay of organic matter (e.g., Dehairs et al., 1980, 1990; Bishop, 1988; Ganeshram et al., 2003) or the dissolution of acantharian celestite ($SrSO_4$) skeletons, enriched in barium (e.g., Bernstein et al., 1987; Bernstein and Byrne, 2004). Yet the dissolution of acantharians would

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only contribute up to 20% of the observed barite precipitation, as demonstrated in the Southern Ocean by Jacquet et al. (2007). There is likewise a lack of clear correlation between acantharian abundance and barite concentration in sediments or in the water column (Bertram and Cowen, 1997). To date a direct biologic mediation of barite precipitation by living organisms has not been demonstrated. In this regard, the bacterial precipitation of barite is a promising field to explore.

Microbially induced precipitation of barite has been shown in diverse environments; González-Muñoz et al. (2003) were able to precipitate barite in culture experiments with a soil bacterium, and suggested that bacteria may have also played a significant role in barite precipitation in natural environments. Glamoclija et al. (2004), Sanchez-Moral et al. (2004) and Senko et al. (2004) reported barite precipitation in natural environments where microbes may have played a role either oxidizing sulfur compounds to generate sulfate or providing biofilms favoring biomineralization. Bonny and Jones (2007) suggested a re-evaluation of the capability of different microbial groups to mediate barite precipitation, which should be taken into account when using particulate marine barite as a paleoproductivity proxy. In a warm sulfur spring in Canada, Bonny and Jones (2008) reported barite crystals that nucleated on microbial cell surfaces and in microbial extracellular polymeric substances (EPS), suggesting that microbes are capable of adsorbing and bioaccumulating barium. Recently, González-Muñoz et al. (2012) demonstrated that marine bacteria promoted barite precipitation under laboratory conditions. These authors assayed marine bacteria for experimental work, using selected strains with proven biomineralization capability such as *Idiomarina* (González-Muñoz et al., 2008), as well as other strains of Gram-negative and Gram-positive bacteria. Other authors have shown that higher mesopelagic Ba in the Pacific coincides with greater bacterial activity, suggesting a potential relationship (Dehairs et al., 2008). Similarly, Jacquet et al. (2011) describe a correlation between mesopelagic Ba contents and bacterial activity in the Southern Ocean.

Additional work is required to fill gaps in our knowledge of barite production in seawater. As it seems to be correlated with enhanced bacterial activity and is produced throughout the mesopelagic layer, natural samples from the Mediterranean at depths of 200 m and 250 m were selected to isolate bacteria for the present study. Our objective was to explore the potential role of marine bacteria living in the upper water column in barite precipitation, as well as the mechanisms involved in such precipitation.

2. Material and methods

2.1. Sampling

Water column samples were obtained from two Mediterranean stations (Fig. 1): the Algero-Balearic basin station RL33CT (37° 44.05′ N 2° 12.43′ E) and the Ionian basin station RL81CT (34° 43.00′ N 22° 12.90′ E), from hereon referred to as stations WM (Western Mediterranean) and EM (Eastern Mediterranean) respectively. These samples were collected during the Ristretto e Lungo cruise M83/3 (Research vessel "Meteor") using a CTD (conductivity, temperature and depth) rosette with Niskin bottles of 12 l volume. One water sample was gathered at 250 m depth in the WM station, and another at 200 m depth in the EM station. Sampling depths were selected in view of indications of barite formation in the upper water column. The distribution of barite particles suggests that much of it would come from decaying organic matter aggregates, occurring mainly in the upper water column, where most organic matter is regenerated (e.g., Dehairs et al., 1980).

2.2. Culture media

For marine bacteria isolation and cultivation, "Marine Broth" (MB, DIFCO laboratories, USA) culture medium was used in both liquid and solid forms (jellified with 2% purified agar-agar, DIFCO laboratories, USA). The barite production assay was conducted using solid culture media enriched with Ba (CMBa) (González-Muñoz et al., 2012) initially containing no free sulfate so as to avoid inorganic barite precipitation. As barite is thought to precipitate in microenvironments where the Ba concentration may be higher than in seawater, the media were enriched in Ba accordingly. If sulfate were available in such conditions, inorganic precipitation would occur; hence a sulfate free medium is required to test mediating biogenic precipitation. We used two types of CMBa that differed in NaCl concentrations: 1) CMBa1 composed of yeast extract 0.4%, $BaCl_2 \cdot 2H_2O$ 2 mM, NaCl 1.9%, purified agar 2%, pH 7; and 2) CMBa2 composed of yeast extract 0.4%, $BaCl_2 \cdot 2H_2O$ 2 mM, NaCl 3.5%, purified agar 2%, pH 7. As in previous studies of barite precipitation by bacteria (González-Muñoz et al., 2012), two different NaCl

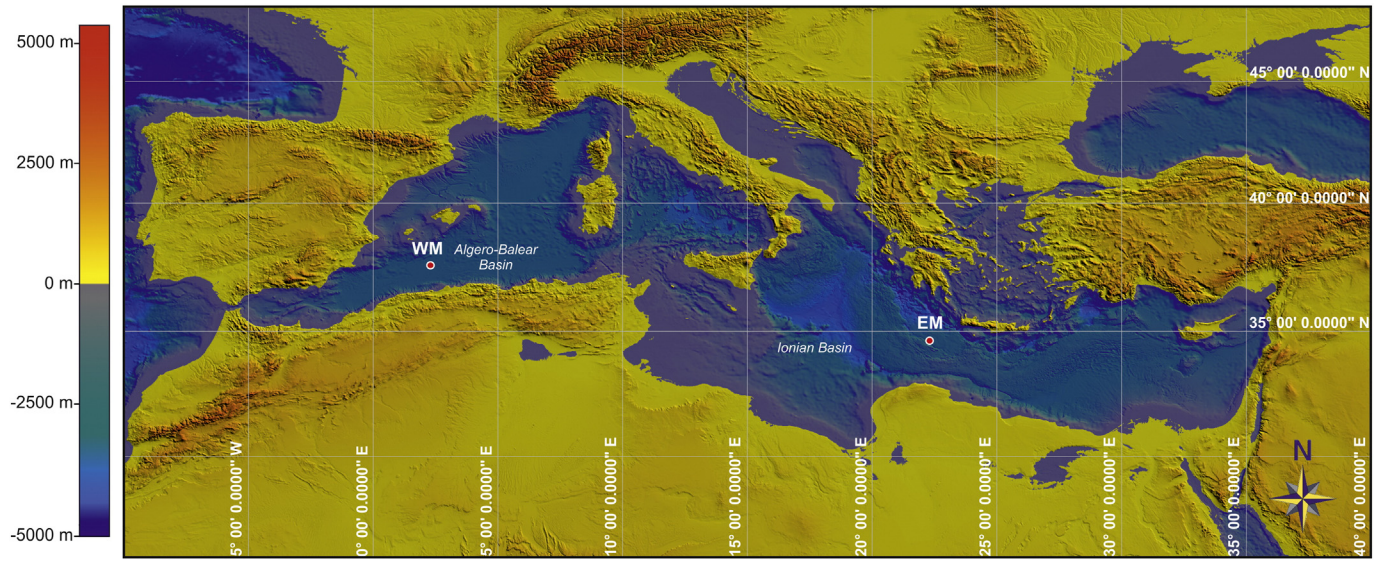

Fig. 1. Bathymetric map of the Mediterranean showing the position of the CTD stations from which the water samples analyzed in this study were recovered (WM in the Algero-Balearic basin, Western Mediterranean, and EM in the Ionian basin, Eastern Mediterranean, respectively).
Source map: Amante and Eakins (2009).

concentrations were used: that of the "Marine Broth" medium as well as the concentration closest to seawater salinity, to further test any potential salinity effect on bacterial growth and precipitation.

2.3. Isolation of bacteria

From each water sample, 100 ml was aseptically filtered using a filter with 0.45 μm pore size (Millipore) in a Kitasato system with a vacuum pump. The filter was recovered with tweezers and gently shaken in 10 ml of NaCl solution (3.5%). Afterwards, appropriate serial dilutions for both samples were performed and 100 μl aliquots of each dilution were inoculated and spread on MB solid medium and incubated at 28 °C; although this is an optimal growth temperature, incubation at a wide range of temperatures has been shown to produce similar results in terms of precipitation (González-Muñoz et al., 2012). Colonies were observed at two time intervals (6 and 20 days), and counted after the incubation to determine colony forming units (CFUs). The selection of colonies was based on visual characteristics such as color, shape and morphology. The purity of selected isolates was confirmed by repeated streaking on the same medium. Pure bacterial suspensions were stored in glycerol at −80 °C.

2.4. Identification of bacteria

Genomic DNA was extracted from bacterial cells using the RealPure Genomic DNA Extraction kit (Durviz, Valencia, Spain), following the manufacturer's instructions. The quantity of DNA was determined employing a Nanodrop spectrophotometer (NanoDrop ND1000, Thermo Fisher Scientific). Additional quality analyses entailed electrophoresis of the extracted DNA (5 μl) on 0.7% agarose gel at 100 V for 40 min, staining with a Gel Red solution (70 μl/l; stock: 10,000×) for 20 min, and visualizing by means of an UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer).

To obtain fingerprint patterns of all isolates and cluster them in different groups, repetitive extragenic palindromic-polymerase chain reactions (rep-PCR) were performed using primers REPIR-I and REP2-I (de Bruijn, 1992), as described in detail in Jroundi et al. (2010). PCR products of all isolates were electrophoresed on 1.5% agarose gel and the DNA fingerprints were compared by visual inspection of the banding patterns. The representative strains of each cluster were identified on the basis of their 16S rDNA amplification using the two opposing primers, fD1 and rD1 (Jroundi et al., 2010). PCR products were purified with the Qiagen PCR product purification system and directly sequenced using the same fD1/rD1 primers with an ABI PRISM 3130xI Genetic Analyzer. The sequences were compared with those from the GenBank using the BLASTN tool (Altschul et al., 1997) available at EMBL-EBI database (http://www.ebi.ac.uk) and the EzTaxon-e tool, an extension of the original EzTaxon database (Chun et al., 2007) available at EzBioCloud (http://eztaxon-e.ezbiocloud.net). Phylogenetic analyses were performed by means of the BioEdit computer program (package version 7.1.11; Hall, 2007), which uses the neighbor-joining algorithm, to be visualized with MEGA 4.0.2 (Tamura et al., 2007).

The 16S rDNA sequences obtained in this study were deposited in the GenBank database. The accession numbers are shown on the phylogenetic tree (Fig. 2).

2.5. Barite production assays

Representative strains of each rep-PCR group were inoculated in MB liquid culture medium (test tubes with 5 ml/tube) and incubated under shaking (200 rpm) for 24 h at 28 °C. Afterwards, cultures were centrifuged (15,871 ×*g* for 5 min), washed 3 times with a NaCl solution (1.9%, the same concentration as in MB) and finally re-suspended in a 5 ml final volume of the NaCl solution. Drops of 20 μl were inoculated on CMBa1 and CMBa2 solid media and incubated at 28 °C for more than one month. Plates were periodically observed by optic microscopy (4× and 10× objectives) to detect the presence of crystals.

2.6. Analysis of precipitates

- *X-ray diffraction* (XRD): Prior to XRD analyses, bacterial cells were killed by exposition to a germicide UV lamp for 2 h. Portions of the agar containing dead bacterial cells and precipitates were dried for 24–48 h at 37 °C. The mineralogical composition of the precipitates in these portions was determined using an X'Pert Pro diffractometer with an X'Celerator detector. As the sample size was small, a zero background sample holder was used. The diffractograms were interpreted using X-powder software (http://www.xpowder.com; Martín-Ramos, 2004).
- *High Resolution Scanning Electron Microscopy* (HRSEM): For morphological and compositional analyses of the precipitates by HRSEM, agar pieces with dead bacterial cells (treatment with UV for 2 h) were immersed in distilled water and melted in a microwave oven (600 W for 50 s). After centrifugation (30 s at 6261 ×*g*), the precipitates were recovered and washed with distilled water to eliminate the remaining culture medium, salts and cellular debris. Precipitates were transferred to a clean glass plate, dried for 24 h at 37 °C, then prepared and sputtered with carbon for HRSEM observation. Morphological and chemical analyses of the precipitates were performed with an AURIGA model microscope (Carl Zeiss SMT) coupled with energy-dispersive X-ray microanalysis (EDX) and Electron Backscatter Diffraction (EBSD), Oxford Instruments.

3. Results

3.1. Bacterial isolation and identification

The counts of bacterial colonies gave an average bacterial load of $(3.6 \pm 1.2) \times 10^5$ CFU/ml in the EM water sample and of $(7.19 \pm 1.0) \times 10^4$ CFU/ml in the WM water sample. Different heterotrophic bacteria were isolated from the two water samples. Rep-PCR grouped the 78 isolates (35 from EM water and 43 from WM water) into 32 REP patterns (Table 1). A phylogenetic tree derived from the nearly complete 16S rRNA gene sequences (Fig. 2) of a representative strain of each REP pattern showed the isolates to pertain to six genera, namely *Marinobacter*, *Alteromonas*, *Pseudoalteromonas*, *Idiomarina*, *Bacillus* and *Brevibacillus*.

The 35 isolates recovered from the EM water sample (Ionian basin) mainly belonged to phyla Proteobacteria (62.8%) and Firmicutes (37.2%) (Table 2). Within the Gammaproteobacteria, 15 isolates (42.8%) were affiliated with the genus *Marinobacter* from the family Alteromonadaceae, this being the most abundant genus. The strains were clustered in five rep-PCR groups (IV, V, VI, VII and VIII), and *Marinobacter algicola* DG893^T was found to be the closest relative species, with similarities ranging from 94% to 99%. Within the same family, the genus *Alteromonas* was represented by seven isolates (20%), which clustered in three rep-PCR groups (I, II and III) showing similarity of over 98% with *Alteromonas stellipolaris* LMG 21861^T (groups II and III) and more than 97% with *Alteromonas addita* R10SW13^T (group I). More than 34% of the isolates were related to the genus *Bacillus* and they clustered in six rep-PCR groups (X, XI, XII, XIII, XIV and XV), showing similarities with *Bacillus anthracis* ATCC 14578^T ranging from 99.85% to 100%. One additional isolate (rep-PCR group IX) was affiliated with the genus *Brevibacillus* from the family Peanibacillaceae (2.6%) and showed a similarity of more than 97% with *Brevibacillus formosus* NRRL NRS-863^T.

From the WM water sample (Algero-Balearic basin), 43 isolates were identified as members of the same two phyla found in the EM water sample. Within the Gammaproteobacteria, three families

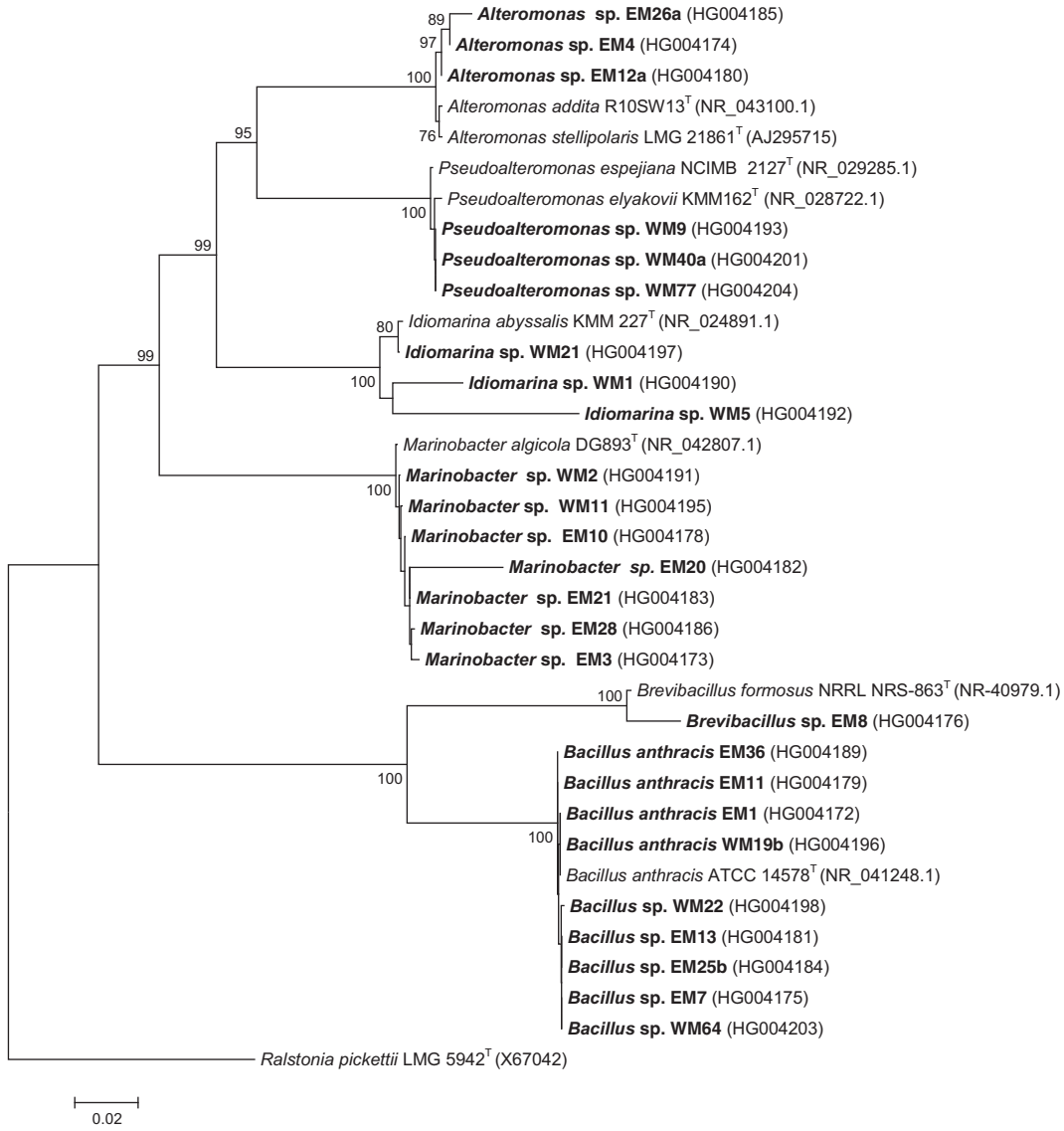

Fig. 2. Neighbor-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences of the studied strains from Mediterranean seawater samples and phylogenetically related species within the genera *Pseudoalteromonas*, *Alteromonas*, *Idiomarina*, *Marinobacter*, *Brevibacillus* and *Bacillus*. The rep-PCR representative strains are in bold letters. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 70 are not shown. Bar, 2 nucleotide substitutions per 100 nucleotides. The tree is rooted on *Ralstonia pickettii* LMG 5942^T.

dominated: Idiomarinaceae (25.6%), Pseudoalteromonadaceae (20.9%) and Alteromonadaceae (12.9%). One isolate (rep-PCR group IV) affiliated with the genus *Alteromonas* showed more than 98% similarity with the closest relative species, *A. stellipolaris* LMG 21861^T. Three rep-PCR groups (V, VI, and VII) were related to the genus *Marinobacter*, the closest relative species being *M. algicola* DG893^T. Nine isolates (20.9%) belonged to the genus *Pseudoalteromonas* and clustered in three rep-PCR groups (I, II, and III) showing almost 99.8% similarity with the type strain *Pseudoalteromonas espejiana* NCIMB 2127^T. Within the family Idiomarinaceae, the genus *Idiomarina* dominated with three different rep-PCR groups (VIII, IX, and X), and *Idiomarina abyssalis* NCIMB 2127^T as the closest relative species, with similarities ranging from 87.7% to 99.3%. Seventeen isolates were highly related to the members of the Firmicutes, *Bacillus* (32.6%) and *Brevibacillus* (7%). The isolates belonging to the genus *Bacillus* clustered in six rep-PCR groups (XII, XIII, XIV, XV, XVI, and XVII), the closest relative species being *B. anthracis* ATCC 14578^T with similarities ranging from 99.85% to 100%. One rep-PCR group (XI) was related to the genus *Brevibacillus*, the closest relative species being *B. formosus* NRRL NRS-863^T, having 97% similarity.

3.2. Barite production assays

No precipitates were observed under optic microscopy among the killed cell controls. In the bacterial colonies of *Pseudoalteromonas* sp., precipitates appeared after the 6th day of incubation (Table 3). They were easily recognized as a concentration of small dense points that were particularly abundant at 15 days and even detectable with the naked eye at one month. This strain showed conspicuous mucus on both solid and liquid culture media, and HRSEM observations also revealed grid-like structures most likely corresponding to EPS (Fig. 3). The isolate *Idiomarina* sp. was able to precipitate barite crystals but they were abundant only after one month of incubation. *Alteromonas* sp. also produced barite precipitates at the border of the colonies but after longer incubation and these precipitates were less abundant. On the other hand, *Brevibacillus* sp. and *Marinobacter* sp. showed low

Table 1
Phylogenetic classification of marine strains identified in this study.

Strains[a]	Rep-PCR pattern	Closest relative species on basis of 16S rRNA gene	Accession numbers of the closest type strains	Similarity %	Family	Class	Phylum
Algero-Balearic basin water sample (WM)							
WM9, WM8b, WM19a	I	*Pseudoalteromonas espejiana*	NR_029285.1	99.78	Pseudoalteromonadaceae	Gammaproteobacteria	Proteobacteria
WM40a, WM57, WM40b, WM35	II	*Pseudoalteromonas espejiana*	NR_029285.1	99.78	Pseudoalteromonadaceae	Gammaproteobacteria	Proteobacteria
WM77, WM16	III	*Pseudoalteromonas espejiana*	NR_029285.1	99.78	Pseudoalteromonadaceae	Gammaproteobacteria	Proteobacteria
'WM32b	IV	*Alteromonas stellipolaris*	NR_025433.1	98.69	Alteromonadaceae	Gammaproteobacteria	Proteobacteria
WM2	V	*Marinobacter algicola*	NR_042807.1	98.1	Alteromonadaceae	Gammaproteobacteria	Proteobacteria
WM11, WM7, WM10	VI	*Marinobacter algicola*	NR_042807.1	99.11	Alteromonadaceae	Gammaproteobacteria	Proteobacteria
[2]WM48	VII	*Marinobacter algicola*	NR_042807.1	98.57	Alteromonadaceae	Gammaproteobacteria	Proteobacteria
WM1, WM46, WM49, WM56	VIII	*Idiomarina abyssalis*	NR_024891.1	95.74	Idiomarinaceae	Gammaproteobacteria	Proteobacteria
WM5, WM6a, WM14a, WM8a	IX	*Idiomarina abyssalis*	NR_024891.1	87.69	Idiomarinaceae	Gammaproteobacteria	Proteobacteria
WM21, WM30, WM17a	X	*Idiomarina abyssalis*	NR_024891.1	99.38	Idiomarinaceae	Gammaproteobacteria	Proteobacteria
[3]WM3, WM31a, WM31b	XI	*Brevibacillus formosus*	NR_40979.1	97.04	Paenibacillaceae	Bacilli	Firmicutes
[4]WM17b, WM27, WM6b	XII	*Bacillus anthracis*	NR_041248.1	99.92	Bacillaceae	Bacilli	Firmicutes
[5]WM13	XIII	*Bacillus anthracis*	NR_041248.1	99.92	Bacillaceae	Bacilli	Firmicutes
[6]WM14b, WM29	XIV	*Bacillus anthracis*	NR_041248.1	100	Bacillaceae	Bacilli	Firmicutes
WM19b, WM20, WM4a, WM4b	XV	*Bacillus anthracis*	NR_041248.1	99.92	Bacillaceae	Bacilli	Firmicutes
WM22, WM25, WM24	XVI	*Bacillus anthracis*	NR_041248.1	99.85	Bacillaceae	Bacilli	Firmicutes
WM64	XVII	*Bacillus anthracis*	NR_041248.1	99.85	Bacillaceae	Bacilli	Firmicutes
Ionian basin water sample (EM)							
EM26a, EM26b, EM27	I	*Alteromonas addita*	NR_043100.1	97.73	Alteromonadaceae	Gammaproteobacteria	Proteobacteria
EM4, EM67	II	*Alteromonas stellipolaris*	NR_025433.1	98.69	Alteromonadaceae	Gammaproteobacteria	Proteobacteria
EM12a, EM66b	III	*Alteromonas stellipolaris*	NR_025433.1	98.69	Alteromonadaceae	Gammaproteobacteria	Proteobacteria
EM3, EM5	IV	*Marinobacter algicola*	NR_042807.1	98.77	Alteromonadaceae	Gammaproteobacteria	Proteobacteria
EM10, EM9b	V	*Marinobacter algicola*	NR_042807.1	98.57	Alteromonadaceae	Gammaproteobacteria	Proteobacteria
EM20, EM18, EM17a	VI	*Marinobacter algicola*	NR_042807.1	94.01	Alteromonadaceae	Gammaproteobacteria	Proteobacteria
EM21, EM22	VII	*Marinobacter algicola*	NR_042807.1	99.04	Alteromonadaceae	Gammaproteobacteria	Proteobacteria
EM28, EM29, EM65, EM66a, EM31, EM44	VIII	*Marinobacter algicola*	NR_042807.1	98.70	Alteromonadaceae	Gammaproteobacteria	Proteobacteria
EM8	IX	*Brevibacillus formosus*	NR_40979.1	97.04	Paenibacillaceae	Bacilli	Firmicutes
EM1	X	*Bacillus anthracis*	NR_041248.1	100	Bacillaceae	Bacilli	Firmicutes
EM7	XI	*Bacillus anthracis*	NR_041248.1	99.85	Bacillaceae	Bacilli	Firmicutes
EM11	XII	*Bacillus anthracis*	NR_041248.1	99.92	Bacillaceae	Bacilli	Firmicutes
EM13, EM14	XIII	*Bacillus anthracis*	NR_041248.1	99.85	Bacillaceae	Bacilli	Firmicutes
EM25b, EM30, EM33, EM2	XIV	*Bacillus anthracis*	NR_041248.1	99.85	Bacillaceae	Bacilli	Firmicutes
EM36, EM48, EM32	XV	*Bacillus anthracis*	NR_041248.1	99.92	Bacillaceae	Bacilli	Firmicutes

[a] For a given strain, the letters WM and EM stand for Western Mediterranean and Eastern Mediterranean respectively, the names of the locations from which water samples were collected. Strains in bold were chosen as representative strains of each rep-PCR group. 1 to 6: The representative strains of these groups are EM4, EM10, EM8, EM36, EM11 and EM1, respectively

growth on any of the media used in this study (CMBa1 or CMBa2). All isolates related to *B. anthracis* species were eliminated for the sake of biological safety.

3.3. Analyses of precipitates

XRD analysis confirmed that barite is the mineral phase precipitated in the bacterial cultures. Diffractograms also revealed halite as a consequence of desiccating the culture media (Fig. 4). HRSEM showed that barite aggregates were mostly spherical or ellipsoidal in shape, with a size ranging from 0.5 to 2 μm. In general the morphologies resembled previously reported barite that was microbially mediated in culture experiments mostly spherulitic, and occasionally dumbbell-shaped aggregates. As the size of the aggregates reflects the rate of growth, barite aggregates in the bacterial colonies of *Pseudoalteromonas*

Table 2
Number of isolates (N) related to the corresponding genera and their percentages in each water sample.

Genus	Algero-Balearic basin water sample (WM)		Ionian basin water sample (EM)	
	N	%	N	%
Alteromonas	1	2.3	7	20
Pseudoalteromonas	9	20.9	0	0
Idiomarina	11	25.6	0	0
Marinobacter	5	11.6	15	42.8
Brevibacillus	3	7	1	2.9
Bacillus	14	32.6	12	34.3
Total of 6 genera	43	100	35	100

Table 3
Bacterial growth on both CMBa culture media (CMBal and CMBa2) and barite production by the tested strains.

Strains	Growth		Barite production	
	CMBa1	CMBa2	CMBal	CMBa2
Alteromonas sp. EM4, EM12a and EM26a	++	++	P	P
Pseudoalteromonas sp. WM9, WM40a and WM77	++	++	P	P
Idiomarina sp. WM1, WM5 and WM21	++	++	P	P
Marinobacter sp. WM2, WM11, EM3, EM10, EM20, EM21 and EM28	+	+	–	–
Brevibacillus sp. EM8	+	+	–	–

+: Low growth; ++: Moderate growth; P: Crystal production; –: Unknown.

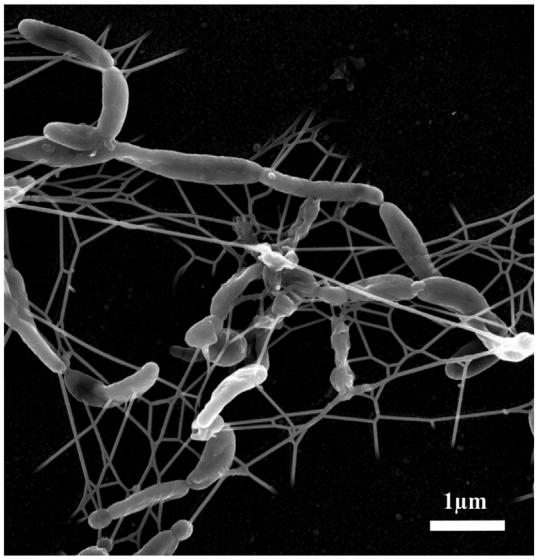

Fig. 3. Scanning electron micrographs (backscattered) showing cells of *Pseudoalteromonas* sp. strain WM40a that present abundant grid-like structures, most likely corresponding to EPS. Samples were prepared using the CO_2 critical point method.

sp. were larger than those of *Idiomarina* sp. and *Alteromonas* sp. colonies. EDX analyses showed the composition of barium, sulfur and oxygen, although phosphorous was also abundant in some precipitates (Fig. 5). The compositions also generally agree with previous experimental results in terms of P enrichment in the initial phases of crystallization (González-Muñoz et al., 2012). Thus, larger spheroidal aggregates had more abundant Ba and S as compared to P. In contrast, smaller and less compact aggregates had relatively high P content with respect to S and Ba.

4. Discussion

The precipitation of barite under laboratory conditions in association with bacteria isolated from natural seawater represents a step forward towards demonstrating the significance of bacteria in the precipitation of barite in natural environments. Laboratory experiments (e.g., Bonny and Jones, 2008; González-Muñoz et al., 2003, 2012) and field observations (e.g., Bonny and Jones, 2007 and references therein) have revealed that the precipitation of barite can be microbially mediated. Furthermore, our experimental work showed that the sizes, morphologies, and presence of high P concentration at the initial stages of crystallization are consistent with microbial precipitation (Fig. 5). In fact, the formation of P-rich precursors is a common step in bacterial biomineralization. Nucleation of an amorphous phosphate phase has been previously reported for the microbial precipitation of barite (González-Muñoz et al., 2003, 2012), aragonite, apatite and iron oxides, both under laboratory conditions and in natural environments (Rivadeneyra et al., 2010 and references therein). As suggested by González-Muñoz et al. (2012), phosphoryl groups in structural polymers as EPS or cell membranes may act as sorbing constituents during the precipitation process, resulting in amorphous or poorly crystalline P-rich precursors. Our results show that in precipitates from *Idiomarina* sp. and *Alteromonas* sp. which appeared later than those from *Pseudoalteromonas* sp. and where smaller aggregates were observed, P is particularly abundant in comparison to S. This further suggests that crystals produced at initial stages have higher P contents.

Moreover, marine barite is usually found in oceanic sinking aggregates in association with decaying organic matter (e.g., Bishop, 1988; Dymond et al., 1992; Dehairs et al., 2000), and in such microenvironments a high bacterial activity is expected from organic matter degradation. A correlation between higher mesopelagic Ba and bacterial activity has been demonstrated in several regions from the Pacific and the Southern Oceans. It has been proposed that mesopelagic particulate biogenic Ba reflects bacterial degradation of organic matter, and is related to oxygen consumption and bacterial carbon respiration (Dehairs et al., 2008; Jacquet et al., 2011; Planchon et al., 2013). Thus, the precipitation of barite in association with bacteria isolated from natural seawater, at depths where particulate Ba is more abundant, further points to a connection between high bacterial activity and marine barite formation.

In terms of the phylogenetic groups retrieved in this study, our results are not very different from those obtained in offshore oligotrophic waters from the Mediterranean sea, and from the Pacific and Atlantic oceans. The rep-PCR fingerprint technique, used in this study, has enough sensitivity to accurately detect genotype microbes at the strain or subspecies levels. This is a valuable tool used to group, identify, track and examine diversity among environmentally important microorganisms (Versalovic et al., 1991, 1998; Sadowsky and Hur, 1998; Rademaker et al., 2004; Healy et al., 2005; Ishii et al., 2009). Bacteria with the same rep-PCR pattern belong to the same bacterial species or even the same strain (de Bruijn, 1992; Ishii and Sadowsky, 2009), while the same species with different fingerprint patterns indicate that the isolates belong to different strains from the same species. The bacteria isolated from the two areas of our study showed similar phylogenetic distributions and taxonomic resolutions: the communities were dominated by Gammaproteobacteria (60.4% in the western sample and 62.8% in the eastern sample), although members of Firmicutes (39.6% in the western sample and 37.2% in the eastern sample) were also abundant. These results confirm that these bacteria are common in seawater from different oceans (Sawabe et al., 2000; Van Trappen et al., 2004; Ivanova et al., 2005; López-López et al., 2005; Green et al.,

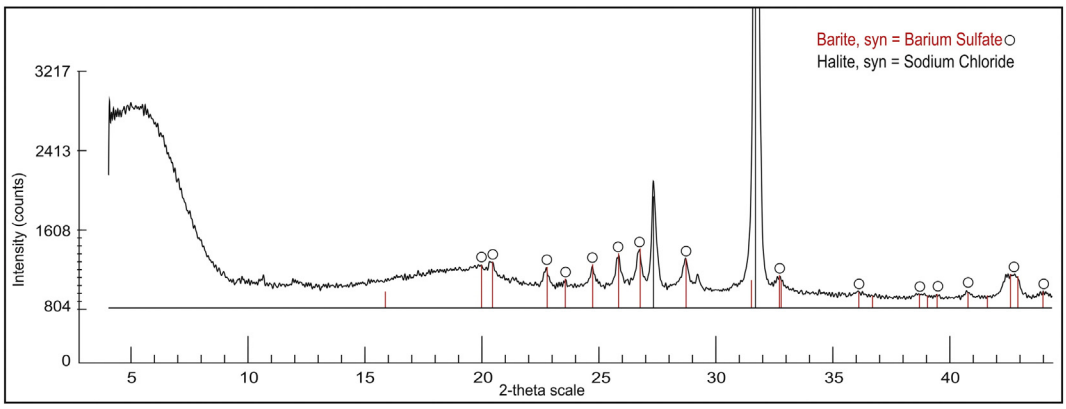

Fig. 4. X-ray diffraction diagram of the precipitates formed in culture experiments by *Pseudoalteromonas* sp. (sample WM40a); barite peaks are indicated with red bars. Abundant halite precipitates as a result of desiccating the medium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

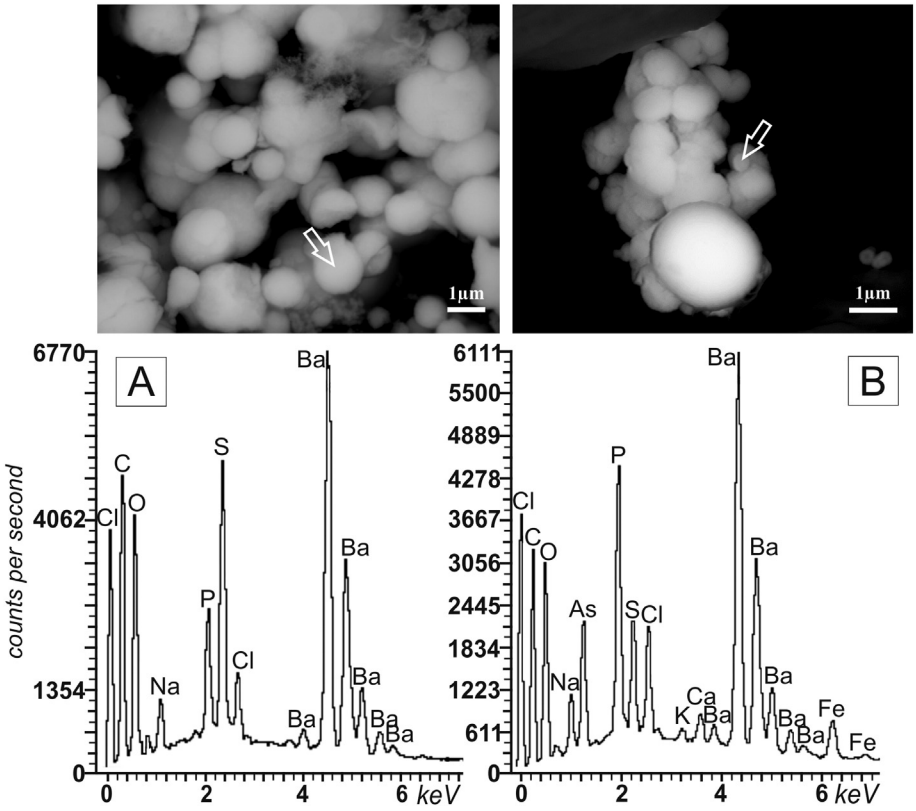

Fig. 5. Scanning electron micrographs (backscattered images) showing typical morphologies and sizes of the barite precipitates (*Pseudoalteromonas* sp. WM40a), and acquired energy-dispersive X-ray spectroscopy spectra: A) from one large spherical aggregate that shows relative abundance of sulfur in relation to phosphorous; B) from one small aggregate, probably corresponding to initial stages of crystallization, that shows a significantly higher phosphorous concentration (see arrows).

2006; Keim et al., 2006; Ki et al., 2009; Porob et al., 2013; Lekunberri et al., 2014). The genus *Bacillus*, within the Firmicutes, is one of the most frequently isolated from marine waters (Eilers et al., 2000). The sequences found here matched very well with *B. anthracis*, though this species belongs to the *B. anthracis* group (*B. anthracis*, *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus toyonensis*), a taxonomic group that includes species/subspecies that are not distinguishable by the 16S rRNA sequence. The same observation was made by Ki et al. (2009), who isolated several marine *Bacillus* species from diverse marine environments but failed to separate, on the basis of 16S rRNA sequences, the species of *B. cereus* group in individuals. Accordingly, discerning the members of *B. anthracis* group found here would call for further studies. The genus *Brevibacillus* is related to the genus *Bacillus*, which is distributed widely throughout nature. The habitat of both genera overlaps, occurring in diverse environments such as rocks, dust, aquatic environments, and guts of various insects and animals (Nicholson, 2002).

Gammaproteobacteria represent a large phylogenetic group of cosmopolitan species (Bowman et al., 1997; Pinhassi et al., 1997; Suzuki et al., 1997; Eilers et al., 2000). Our results are consistent with those of Lekunberri et al. (2014), who used culture-dependent methods to show that this group of bacteria dominated in the waters in the NW Mediterranean sea (Blanes Bay Microbial Observatory, approximately 70 km north of Barcelona). Mullins et al. (1995) found that the Gammaproteobacteria subclass contains the largest number of sequences from cultured marine isolates. In our study, this is reflected by the high degree of similarity between our sequences and those of members of the genera *Alteromonas*, *Idiomarina*, *Marinobacter* and *Pseudoalteromonas*. The occurrence of these genera could be an effect of the peculiar depth conditions of the chlorophyll maximum (DCM), with a much higher abundance of phytoplankton and perhaps a higher availability of organic nutrients; or it could signal a change in physical conditions, e.g. water temperature and light intensity. The isolates representing *Marinobacter* sp. appear to branch at some distance from the other Gammaproteobacteria (Fig. 2) and are related to halotolerant bacteria. The genus *Idiomarina* has a widespread distribution in marine and hypersaline habitats (Martínez-Cánovas et al., 2004), and different species were isolated from the Pacific Ocean deep-sea (Ivanova et al., 2000), hydrothermal vents at a depth of 1296 m (Donachie et al., 2003), surface water of the central Baltic Sea (Brettar et al., 2003), hypersaline water in Spain (Martínez-Cánovas et al., 2004), and wetland and seashore sand in Korea (Choi and Cho, 2005; Kwon et al., 2006). Recently, several species of this genus have served to demonstrate the capability of marine bacteria to precipitate barite (González-Muñoz et al., 2012). The predominant occurrence of *Alteromonas* and *Pseudoalteromonas* spp. in marine waters all around the world had been suggested previously (Acinas et al., 1999; Eilers et al., 2000; Schäfer et al., 2000; Lekunberri et al., 2014). In particular, *Alteromonas* species were abundant in Mediterranean offshore samples, in the surface and down to 400 m (Acinas et al., 1999). This was supported by our rep-PCR DNA fingerprints and further identification on the basis of the 16S rDNA, as we recovered *Alteromonas* sp. from both eastern and western Mediterranean waters at respective depths of 250 m and 200 m. Other authors describe the significant presence of this marine organism in samples from the Atlantic (Klochko et al., 2012), and in many other tropical and temperate seas (García-Martínez et al., 2002). The genus *Pseudoalteromonas* is common to the marine environment, including seawater, algae, and marine invertebrates (Baumann et al., 1972, 1984; Baumann and Baumann, 1981; Gauthier and Breittmayer, 1992; Sawabe et al., 2000). It was described for the first time by Gauthier et al. (1995) as a heterotrophic, Gram-negative, aerobic, rod-shaped, and polarly flagellated marine bacteria. All pseudoalteromonads require Na^+ ions for growth (Mikhailov et al., 2006), and could therefore be regarded as obligate marine

microorganisms. The sequences found herein matched very well with *P. espejiana*, characterized by non-pigmented, translucent and highly mucoid colonies, all of which confirm the phenotypic characteristics of this microorganism described previously by a number of authors (Chan et al., 1978; Bowman, 1998). Some members of the genus *Pseudoalteromonas* are highly effective in biofilm formation (Saravanan et al., 2006; Huang et al., 2007; Iijima et al., 2009) and accordingly might be hosted by marine fauna and flora for defense, by means of biofouling (Lindquist and Fenical, 1991; Holmström et al., 1996, 2002; Dobretsov et al., 2006, 2007). Interestingly, several bacterial strains of the genus *Pseudoalteromonas*, including *P. espejiana*, commonly produce EPS. It would appear that this characteristic provides a range of survival strategies for the cells. This EPS acts as protective barriers against antibiotics and against predation by protozoa (Costerton et al., 1987), reduces the diffusion of some substances to and from the cells, and functions as enhancers for nutrient and ion uptake (Geesey, 1982).

EPS production would therefore play a key role in barite precipitation, as is the case for other mineral phases (Bontognali et al., 2014; Tourney and Ngwenya, 2014). Ba would bind to phosphate groups on EPS and bacterial cells, locally increasing its concentration (González-Muñoz et al., 2012). As a second step, the replacement of phosphate by sulfate occurs. Under experimental conditions, bacteria also oxidize sulfur compounds and generate the sulfate needed for barite precipitation. However, in seawater, where sulfate is available, the major role played by bacteria would be providing binding sites for cations in the EPS and cell membranes. It is also important to note that living organisms such as plankton contain a relatively large pool of labile Ba, which is readily released during decomposition, as demonstrated by Ganeshram et al. (2003), who also suggested that Ba enrichment rather than sulfate induced barite precipitation. Thus, in natural seawater where sulfate is available, bacteria could contribute to Ba enrichment and saturation in microenvironments where barite may precipitate.

As the assayed bacterial strains are common and abundant in seawater, it is likely that bacteria promote Ba saturation in natural decomposing organic matter microenvironments. Barite formation may involve the Ba binding to phosphate groups on EPS and bacterial cells, the increasing of Ba concentrations at nucleation sites followed by the replacement of phosphate by seawater sulfate and barite crystal growth. It is known that organic matter degradation, particularly in sinking organic aggregates, is linked to microbial activity. Thus, bacterial production would significantly impact barite precipitation, and this would be a major process to take into account when using Ba proxies for paleoceanographic reconstructions.

5. Conclusions

Several bacterial strains isolated from natural Mediterranean sub-surface seawater, at 200 m and 250 m depths, promoted barite precipitation under laboratory conditions. Our results show that barite precipitates are particularly abundant in cultures where EPS production is also high. In fact, EPS production and bacterial growing ability under experimental conditions would appear to affect the rates of barite production. Therefore, the role of EPS in barite precipitation in the water column is an important issue for future research. Microbially induced precipitation of barite under laboratory conditions triggered by bacteria that are common in marine seawater suggests that bacterially induced precipitation may also occur in the oceans. Therefore, bacterial production in the past and the mechanisms involved in barite precipitation should be considered in paleoproductivity reconstructions.

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