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Isolation and characterisation of bacteria from the Eastern Mediterranean deep sea

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Abstract The Eastern Mediterranean deep sea is one of the most oligotrophic regions in the world's ocean. With the aim to classify bacteria from this special environment we isolated 107 strains affiliating to the *Gammaproteobacteria*, *Alphaproteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* from sediments of the Eastern Mediterranean Sea. As determined by 16S rRNA gene sequence analysis, *Actinobacteria* and *Firmicutes*, in particular members of the genus *Bacillus*, were dominant and represented a remarkable diversity with 27 out of a total of 33 operational taxonomic units obtained from the untreated sediment. The considerable percentage of operational taxonomic units (42%) which may be considered to be new species underlines the uniqueness of the studied environment. In order to selectively enrich bacteria which are adapted to the deep-sea conditions and tolerate broad pressure ranges, enrichments were set up with a sediment sample under in situ pressure and temperature (28 MPa, 13.5°C) using *N*-acetyl-D-glucosamine as substrate. Interestingly *Gammaproteobacteria* were significantly enriched and dominant among the strains isolated after pressure pre-incubation. Obviously, *Gammaproteobacteria* have a selective advantage under the enrichment conditions applied mimicking nutrient supply under pressure conditions and cope well with sudden changes of hydrostatic pressure. However, under the continued low nutrient situation in the Eastern Mediterranean deep-sea sediments apparently *Firmicutes* and *Actinobacteria* have a clear adaptative advantage.

Keywords Deep sea · Bacteria · Mediterranean sea · Pressure-incubation · Cultivation

Introduction

Studies on microbial communities in marine deep-sea sediments frequently focus on highly productive spots, like hydrothermally active regions. However, the major fraction of deep-sea sediments represents extremely oligotrophic environments subjected to high hydrostatic pressures. This holds especially true for the Eastern Mediterranean sea (Lampadariou et al. 2009). The extreme depletion of nutrients in surface waters results in low primary production. Only a minor fraction of the produced organic matter (1%) is reaching the deep-sea sediments (Lampitt and Antia

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1997). Organic carbon values of the Southern Cretan Margin sediments were found to be in the range of 0.07–1.55% of sediment dry weight (Polymenakou et al. 2008). The Eastern Mediterranean deep sea is furthermore characterised by comparably high temperatures of approximately 14°C opposite to most other deep-sea environments with temperatures of 2–4°C (Emig and Geistdoerfer 2004). The sediments of the Eastern Mediterranean deep sea thus combine different environmental features (extreme oligotrophy, high hydrostatic pressure of up to 52 MPa at the deepest point and comparably high deep-water temperature) which may be in favour of the development of unique types of bacteria. T-RFLP analysis revealed high values of bacterial diversity within Eastern Mediterranean sediments of the deep Ionian sea (Luna et al. 2004). Also environmental 16S rRNA gene libraries of Western Mediterranean deep-sea sediments revealed high bacterial diversity, while Archaea appear to be of minor abundance and diversity (Danovaro et al. 2009). Dominating bacterial sequences from deep-sea sediments of the Southern Cretan Margin were assigned to the *Acidobacteria* (18%), *Gammaproteobacteria* (13%), *Planctomycetes* (11%), *Actinobacteria* (11%), *Alphaproteobacteria* (10%) and *Deltaproteobacteria* (9%) (Polymenakou et al. 2009).

There is however, a considerable lack of cultivation-based studies of bacteria that is essential to enlighten their ecological role in natural environments (Das et al. 2006). Studies of cultured bacterial diversity from deep-sea environments are generally performed at atmospheric pressure and there are only few studies using in situ hydrostatic pressure incubation (e.g. Yayanos et al. 1979; Kato et al. 1995, 1996, 1998, 1999; Yanagibayashi et al. 1999). In contrast, low nutrient concentrations prevailing in marine deep-sea habitats have been taken into account in cultivation-based studies since the 1990s (Rueger and Tan 1992; Rappé et al. 1999). The use of low nutrient media turned out to significantly improve the cultivation efficiency (Carlucci et al. 1986; Rueger and Tan 1992; Koch 2001), for the growth of copiotrophic and fast growing bacteria is retarded in low-nutrient media giving a chance for growth of oligotrophic bacteria.

In the present study bacteria from Mediterranean deep sea sediments were isolated and characterised by culture-dependent studies using different media with low nutrient content and as well enrichments under in situ hydrostatic pressure with the chitin monomer *N*-acetyl-D-glucosamine (NAG) as supplement.

Materials and methods

Sample collection

All samples were obtained southwards of Crete during Meteor cruise 71 leg 2 in December 2006–January 2007. Sediment was collected from the Ierapetra basin (IB) at 4400 m (34°30.296N, 26°11.507E) and the Herodotos plain (HP) at 2800 m (33°42.989N, 26°20.329E) using a multiple corer from the Senckenberg Research Institute in Wilhelmshaven, Germany (DZMB, German Center for Marine Biodiversity Research) which has the possibility to hold 12 cores of 9.5 cm inner diameter. Of each sediment core the uppermost 5 cm were aseptically sub sampled. The deep-sea water column [>500 m referring to Kontoyiannis et al. (1999)] was sampled at the same locations by a rosette sampler equipped with 24 Niskin Bottles connected to a CTD probe. Sampling depth were 500, 1200, 2500 and 4000 m.

Isolation and cultivation

For standard isolation of aerobic bacteria from the sediment, dilutions of 10^{-1}–10^{-4} in sterile Mediterranean seawater were prepared and plated on five different agar media. MW-medium: 1.5% agar added to Mediterranean seawater; MWY-medium: 1.5% Bacto™ agar (BD, Germany), 0.01% Bacto™ yeast extract (BD, Germany) in Mediterranean seawater; TSB0.1-medium: 1.5% Bacto™ agar (BD, Germany), 0.01% BBL™ tryptic soy broth (BD, Germany), 3% NaCl in $Aqua_{demin}$; CFB-medium: 1.5% Bacto™ agar (BD, Germany), 0.1% BBL™ tryptone (BD, Germany), 0.05% Bacto™ yeast extract (BD, Germany), 0.05% $CaCl_2 \times 2H_2O$ (Merck, Germany), 0.05% $MgCl_2 \times 7H_2O$ (Merck, Germany) in Mediterranean seawater; Chitin-medium: 1.5% Bacto™ agar (BD, Germany), 0.2% chitin (Fluka Biochemica, Switzerland) in Mediterranean seawater. Water samples of different depths were treated equally using dilutions of 10^0 and 10^{-1}. Agar plates (two parallels each) were incubated at onboard

room temperature for up to three months and checked frequently for growth.

Pre-incubation of sediment at in situ hydrostatic pressure

In order to selectively enrich deep-sea bacteria that are adapted to broad ranges of hydrostatic pressure and spontaneous nutrient input, a sediment sample from 2800 m depth (Herodotos Plain) was pre-incubated at 28 MPa (13.5°C) in supplemented sea water and *N*-acetyl-D-glucosamine prior to plating on identical media and incubation at identical conditions as described above. For this purpose 10 ml of sediment were transferred into a plastic bag with 9 ml of sterile-filtered seawater (taken from the multicorer) and 1 ml solution of *N*-acetyl-D-glucosamine (100 μM). Using compression-proof steel tubes connected to a pumping system, which generates hydrostatic pressure by pumping water into the tubes in situ hydrostatic pressure (28 MPa) was impressed. A manometer attached to the pumping system allowed the adjustment of the respective hydrostatic pressure within the tubes. After reaching the desired pressure, the pump was disconnected and the tubes were incubated for 6 days at in situ temperature (13.5°C). During the incubation, maintenance of the hydrostatic pressure within the tubes was checked frequently by attachment of the manometer. After this incubation, 10^{-1} and 10^{-2} dilutions were plated directly on the above-mentioned agar media and incubated under the standard conditions used throughout these experiments.

Using a binocular microscope, all colonies appearing morphologically different were transferred to fresh agar medium until pure cultures were obtained. Strains were checked for purity by microscopy and identified by 16S rRNA gene sequencing. For long term storage, all strains were kept at −80°C using the Cryobank system (Mast Diagnostica GmbH, Germany).

Testing the growth properties of selected strains

Selected strains of all operational taxonomic units (strains are marked by an asterisk in Table 1) were grown (i) in nutrient-rich Marine Broth medium at in situ temperature (ii) in oligotrophic MW-medium (containing solely autoclaved oligotrophic Mediterranean seawater) using room temperature and (iii) in low-nutrient medium MWY (containing autoclaved oligotrophic Mediterranean Sea water with 0.01% yeast extract) at in situ temperature of 13.5°C.

DNA extraction, PCR and sequencing

DNA extraction, PCR and 16S rRNA gene sequencing was performed according to Gärtner et al. (2008). 16S rRNA gene sequences determined during this study were deposited in the EMBL Nucleotide Sequence Database and were assigned accession no. FM992709–FM992846 and FN179280.

Sequence analysis

Sequences were edited by ChromasPro v.1.33 and were compared to the NCBI database using BLAST [see http://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul et al. 1990)]. Subsequently, all sequences were aligned to the ARB database (see http://www.arb-home.de, (Ludwig 2004)) using the integrated aligner function. Type strains most closely related to the isolates according to BLAST were added to the ARB database when not already present. Alignments were refined manually and aligned sequences were added to the ARB tree using the quick-add-marked function (Parsimony). According to the results from Stackebrandt and Ebers 2006strains showing sequence similarities <98.7% were assigned to different operational taxonomic units (OTUs) using MOTHUR software (http://www.mothur.org/).

Phylogenetic trees

Phylogenetic trees were calculated applying maximum likelihood analysis using PHYML (Guindon and Gascuel 2003). Maximum Likelihood analysis was performed assuming the GTR model (Keane et al. 2006) with an optimised gamma distribution parameter alpha and 100 bootstrap replicates.

Results

The majority of the strains isolated during this study were affiliated to bacterial genera commonly found in the ocean. The 107 strains were grouped into 49 operational taxonomic units (OTUs showing ≥98.7% sequence similarity). A considerable percentage of 42% of the OTUs can be considered to represent new

Table 1 Origin, isolation conditions and next related validly described type strains of all strains

OTU	Strain (accession number)	Next related type strain (and accession number)	Identity	Isolation-source	Sampling-site	Isolation-media
Gammaproteobacteria						
1	D34 (FM992789); S09* (FM992775); W03x (FM992785)	*Pseudoalteromonas elyakovii,* KMM162^T (AF082562)	99.5–99.6	PE, S, W	HP	MWY, Chitin
2	D39* (FM992717); D48 (FM992783); D50x (FM992790); N102 (FM992770); N105 (FM992800)	*Alteromonas marina,* JCM11804^T (AF529060)	98.6–99.6	PE, W	HP, IB	TSB, MWY, MW
3	D42* (FM992781)	*Alteromonas marina,* JCM11804^T (AF529060)	97.7	PE	HP	Chitin
4	D33* (FM992780)	*Alteromonas litorea, JCM12188^T* (AY428573)	97.4	PE	HP	MWY
5	D45 (FM992719); D56* (FM992724)	*Alteromonas addita,* LMG22532^T (AY682202)	97.2–97.3	PE	HP	MWY
6	D47* (FM992720)	*Alteromonas addita,* LMG22532^T (AY682202)	96.5	PE	HP	MWY
7	D37 (FM992715); D38 (FM992716); D50 (FM992722); D43* (FM992782)	*Pseudomonas stutzeri,* ATCC17588^T (AF094748)	99.7–100.0	PE	HP	TSB, MWY, Chitin
8	W01 (FM992760); W29* (FM992767)	*Pseudomonas stutzeri,* ATCC17588^T (AF094748)	98.4	W	HP	MW
9	D06* (FM992773); D36 (FM992714)	*Marinobacter flavimaris,* DSM16070^T (AY517632)	99.3	PE	HP	MWY
10	D35x* (FM992844); S30b (FM992753)	*Marinobacter flavimaris,* DSM16070^T (AY517632)	98.8–98.9	PE, S	HP, IB	MWY, CFB
11	D95 (FM992736); S58* (FM992795)	*Microbulbifer agarilyticus,* DSM19200^T (AB158515)	99.3–99.4	*S*	HP, IB	MW, Chitin
12	D02 (FM992709); D49 (FM992721); S02* (FM992745); S57 (FM992758)	*Stenotrophomonas maltophilia,* DSM50170^T (AY484506)	99.0–99.6	PE, S	HP	CFB, TSB, MWY
Bacteroidetes						
13	W34 (FM992769), W32* (FM992768)	*Leeuwenhoekiella blandensis,* CECT7118^T (DQ294290)	97.8–98.9	W	IB	TSB, MWY
14	W27* (FM992766)	*Pontibacter korlensis,* NRRL B-51097^T (DQ888330)	96.5	W	IB	MWY
Alphaproteobacteria						
15	D82 (FM992732), S36 (FM992755), S42 (FM992756), N106 (FM992771), W04 (FM992761), W20 (FM992765), N109 (FM992798), W19* (FM992764)	*Erythrobacter flavus,* JCM11808^T (AF500004)	98.8–100.0	S, W	HP, IB	TSB, MWY, MW
16	D53* (FM992723)	*Sphingobium yanoikuyae,* ATCC51230^T (D16145)	99.4	PE	IB	CFB

Table 1 continued

OTU	Strain (accession number)	Next related type strain (and accession number)	Identity	Isolation-source	Sampling-site	Isolation-media
17	N108* (FM992772)	*Citreicella thiooxidans,* DSM10146^T (AY639887)	95.7	W	HP	MWY
18	W14* (FM992763)	*Paracoccus alcaliphilus,* DSM8512^T (AY014177)	94.9	W	HP	CFB
19	S30a (FM992752), W05x* (FM992793)	*Aurantimonas coralicida,* DSM14790^T (AJ86361)	99.7	W	HP	MWY
Actinobacteria						
20	S20 (FM992749), S32a (FM992754), S61 (FM992759), S71 (FM992746), S51* (FM992776)	*Micromonospora auratinigra,* DSM44815^T (AB159779)	98.3–99.2	S	HP, IB	CFB, MW, Chitin, MWY
21	S29* (FM992751)	*Micromonospora coxensis,* JCM13248^T (AB241455)	98.5	S	IB	CFB
22	S78* (FM992777)	*Pseudonocardia xinjiangensis,* DSM44661^T (EU722520)	98.3	S	HP	MW
23	S82* (FM992788)	*Pseudonocardia xinjiangensis, DSM44661^T* (EU722520)	97.5	S	HP	MW
24	S80*(FM992787)	*Blastococcus aggregatus,* DSM4725^T (L40614)	99.6	S	HP	MW
25	S35* (FM992796)	*Corynebacterium pseudodiphtericum, DSM44287^T (X81918)*	99.3	S	HP	CFB
26	D126 (FM992744), D81* (FM992731), D84 (FM992733),D85 (FM992734), D92 (FM992735), S26 (FM992750), S28 (FM992799)	*Streptomyces sampsonii,* ATCC25495^T (D63871)	98.2–100.0	S	IB	MWY, Chitin, CFB, TSB
27	S06* (FM992747)	*Streptomyces flavofuscus,* ATCC19908^T (DQ026648)	97.8	S	HP	TSB
28	D44 (FM992718), S37 (FM992797), S43* (FM992757)	*Micrococcus luteus,* ATCC4698^T (AF542073)	98.9–99.3	PE, S	HP, IB	MWY, MW
29	S08* (FM992748)	*Arthrobacter tecti,* LMG22282^T (AJ639829)	98.8	S	HP	Chitin
Firmicutes						
30	S40* (FM992845)	*Halobacillus karajiensis,* DSM14948^T (AJ486874)	98.3	S	HP	MW
31	S54* (FM992841)	*Bacillus aerophilus,* ATCC10840^T (AJ831844)	95.2	S	IB	CFB
32	D11 (FM992803), S14 (FM992821), S32 (FM179280), S04 (FM992843), S38 (FM992825), S03* (FM992831)	*Bacillus algicola,* KMM3737^T (AJ831844)	99.4–99.8	S	IB, HP	MWY, MW
33	D26* (FM992805), D92x (FM992834)	*Bacillus hawajinpoensis,* JCM11807^T (AF541966)	99.0–100.0	S	IB	CFB, MWY
34	D89* FM992834, S33 (FM992824)	*Bacillus decolorationis,* LMG19507^T (AJ315075)	97.0–97.8	S	IB, HP	TSB, Chitin

Table 1 continued

OTU	Strain (accession number)	Next related type strain (and accession number)	Identity	Isolation-source	Sampling-site	Isolation-media
35	D81a (FM992811), S19 (FM992822), D96 (FM992837), D79* (FM992809)	*Bacillus barbaricus,* DSM14730^T (AJ422145)	99.6	S	IB, HP	MW, CFB, Chitin
36	W15* (FM992786)	*Staphylococcus pettenkoferi,* CIP107711^T (AF322002)	99.9	W	HP	CFB
37	D35a (FM992801), D80 (FM992810), D88* (FM992813), S30 (FM992823), S52 (FM992829), W05 (FM992762)	*Bacillus subtilis,* DSM10^T (AJ276351)	99.7–100.0	PE, S, W	IB, HP	MWY, CFB
38	S49* (FM992828), S47 (FM992840)	*Bacillus licheniformis,* DSM13^T (X68416)	99.2–99.9	S	IB	CFB
39	S53* (FM992830)	*Bacillus indicus,* DSM16189^T (AJ583158)	100.0	S	IB	CFB
40	D94* (FM992835)	*Bacillus licheniformis,* DSM13^T (X68416)	93.9	S	IB	MWY
41	S10* (FM992819)	*Bacillus novalis,* DSM15603^T (AJ542512)	96.4	S	HP	Chitin
42	D32* (FM992832), S46 (FM992827)	*Bacillus boroniphilus,* DSM17376^T (AB198719)	99.0–99.9	S	HP, IB	TSB, CFB
43	S11* (FM992820)	*Bacillus foraminis,* LMG23174^T (AJ717382)	98.4	S	HP	Chitin
44	D87* (FM992812)	*Bacillus foraminis,* LMG23174^T (AJ717382)	98.3	S	IB	CFB
45	D29a* (FM992806)	*Bacilllus drentensis,* LMG21831^T (AJ542506)	99.7	S	HP	CFB
46	D52* (FM992833), D31 (FM992807), S05a (FM992818), S44x (FM992826)	*Bacillus muralis,* LMG20238^T (AJ628748)	98.5–99.6	PE, S	HP, IB	CFB, MWY
47	D04* (FM992802)	*Bacillus circulans,* DSM11^T (AY724690)	97.0	PE	HP	CFB
48	N104b* (FM992794)	*Bacillus massiliensis,* CIP108446^T (AY677116)	96.8	W	IB	MWY
49	S33x* (FM992838)	*Bacillus infantis,* JCM13438^T (AY904032)	99.7	S	HP	Chitin

S sediment, *W* water, *PE* pressurised sediment enrichment, *IB* Ierapetra basin, *HP* Herodotos plain

* Selected strains for growth experiments on MWY medium (one per OTU)

species (Table 1). The high proportion of presumably new bacteria is considered to underline the special features of the Mediterranean deep sea.

Phylogenetic affiliation of the strains

Strains isolated from the sediment

Using low-nutrient media, colony numbers of up to 2.1×10^2 CFU/cm^3 sediment were determined. Just slightly higher cell numbers (2.4×10^2 CFU/cm^3) sediment were obtained with sediment samples using the CFB medium which had the highest nutrient content. Altogether 65 strains were obtained from the Eastern Mediterranean sediment (37 strains from sediment of the Ierapetra basin and 28 strains from the Herodotos plain). The strains isolated from the IB and HP sediments revealed a similar distribution among phylogenetic groups (Figure S1). The majority of strains affiliated to the *Firmicutes* (34 of 65 strains, 52%) and *Actinobacteria* (21 of 65 strains, 32%) with only few representatives of the *Alpha*- and *Gammaproteobacteria* (Figs. 1, 2). *Firmicutes* and *Actinobacteria* were dominant among the isolated strains of the untreated sediment and represented highly diverse groups with 17 OTUs of *Firmicutes* and 10 OTUs of *Actinobacteria* (Table 1). Especially the genus *Bacillus* was isolated with a remarkable high number of OTUs (16 OTUs). Some of the *Firmicutes* and *Actinobacteria* were exclusively isolated using Mediterranean Sea Water medium (MW-medium) without additional nutrients (OTUs 22, 23, 24 and 30). Strains with close relationship to known species (≥98.7%) are summarised in Table 1. Fifteen strains represented thirteen putative new species. These strains affiliated to the genera *Micromonospora* (strains S32a and S29), *Pseudonocardia* (strains S82 and S78), *Streptomyces* (strains S06 and D92), *Halobacillus* (strain S40) and *Bacillus* (strains S54, D89, S33, D94, S10, S11, D87 and S44x).

Strains isolated from the deep-sea water

To demonstrate differences between strains inhabiting the sediment and the deep water, isolates from deep water samples of both stations (cell counts were $<1.7 \times 10^1$ CFU/ml deep-sea water, 19 strains obtained in total) were obtained and compared to those isolated from the sediment. According to their phylogenetic position, strains isolated from the deep water differed substantially from those isolated from the sediments. The 19 isolates grouped in 12 different OTUs: 3 (5 strains) *Gammaproteobacteria*, 2 (3 strains) *Bacteroidetes*, 4 (8 strains) *Alphaproteobacteria*, 3 (3 strains) *Firmicutes* (Fig. 1, 2; Table 1). Members of the *Bacteroidetes* were exclusively obtained from the deep water and all of them showed sequence similarities below 98.7% to known species. Other isolates from the deep sea water possibly can be assigned to new species assigned to the genera of *Pseudomonas* (strains W01 and W29), *Citreicella* (strain N108), *Paracoccus* (strain W14), *Leeuwenhoekiella* (strain W32) and *Pontibacter* (strain W27).

Strains isolated after hydrostatic pressure incubation

Pre-incubation was performed to selectively enrich bacteria supposed to be well adapted to the extreme conditions of the deep Mediterranean (high deep-sea temperatures, elevated hydrostatic pressure, oligotrophy). Noteworthy, CFU were increased at least tenfold compared to plating of sediment samples without pre-incubation. The 23 strains obtained after hydrostatic pressure incubation grouped into 15 different OTUs (10 *Gammaproteobacteria*, 3 *Firmicutes*, 1 *Alphaproteobacteria* and 1 *Actinobacteria*). Interestingly, the hydrostatic pressure pre-incubation yielded a considerable increase in the number of *Gammaproteobacteria* (78% of all isolates after hydrostatic pressure incubation of the sediment from Herodotos plain compared to 14% from this untreated sediment (Figs. 2, S1; Table 1). In contrast, the proportion of *Firmicutes* strains was drastically reduced (13% after hydrostatic pressure incubation compared to 50% from untreated sediment, Table 1, Figs. 2, S1). Noteworthy, 8 OTUs were exclusively obtained after this treatment and could not be isolated from the untreated sediment (Fig. S2). Most of these (7) are likely to represent new species and were assigned to the genera *Alteromonas* (strains D42, D39, D33, D45, D47 and D56) and *Bacillus* (strain D04).

Growth under selected in situ conditions

The strains isolated during this study originate from media with different nutrient concentrations. All tested strains were able to grow in oligotrophic

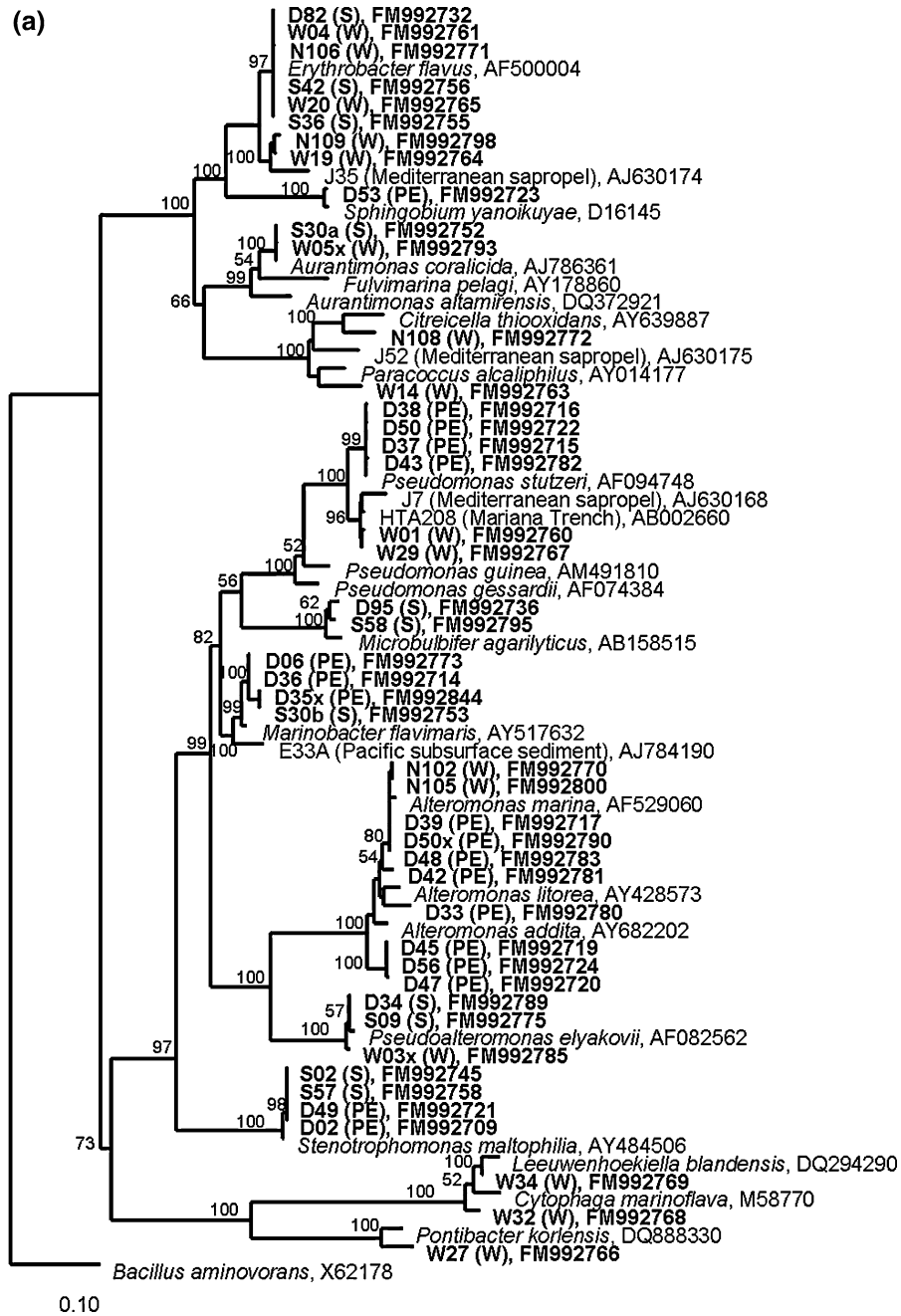

Fig. 1 **a** Maximum likelihood tree of the 16Sr RNA gene sequences of all Gram-negative strains obtained from sediment and water samples. Numbers on nodes represent bootstrap percentages > 50, calculated from 100 replicates. *S* Isolate from sediment' *PE* isolate from pressure experiment, *W* isolate from water column. **b** Maximum likelihood tree of the 16Sr RNA gene sequences of *Actinobacteria* strains obtained from sediment and water samples. Numbers on nodes represent bootstrap percentages > 50, calculated from 100 replicates. *S* Isolate from sediment, *PE* isolate from pressure experiment, *W* isolate from the water column. **c** Maximum likelihood tree of the 16Sr RNA gene sequences of *Firmicutes* strains obtained from sediment and water samples. Numbers on nodes represent bootstrap percentages > 50, calculated from 100 replicates. *S* Isolate from sediment, *PE* isolate from pressure experiment, *W* isolate from the water column

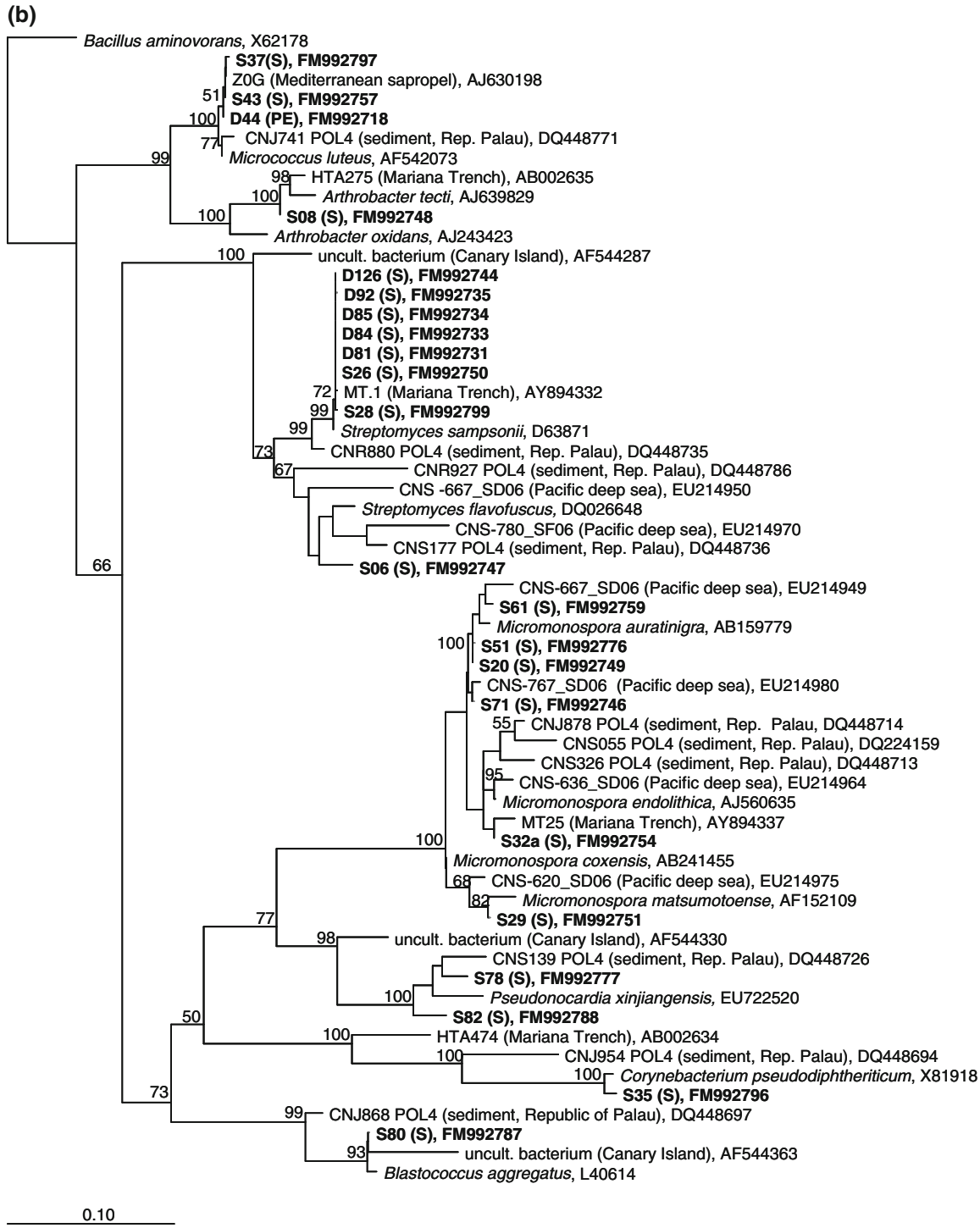

Fig. 1 continued

Mediterranean Sea water medium and also on Marine Broth medium with elevated nutrient concentrations. All strains could grow at room temperature as well as at the lower in situ temperature of 13.5°C. Apparently growth at the lower temperature was dependent on the medium and nutrient concentration. Cultivation

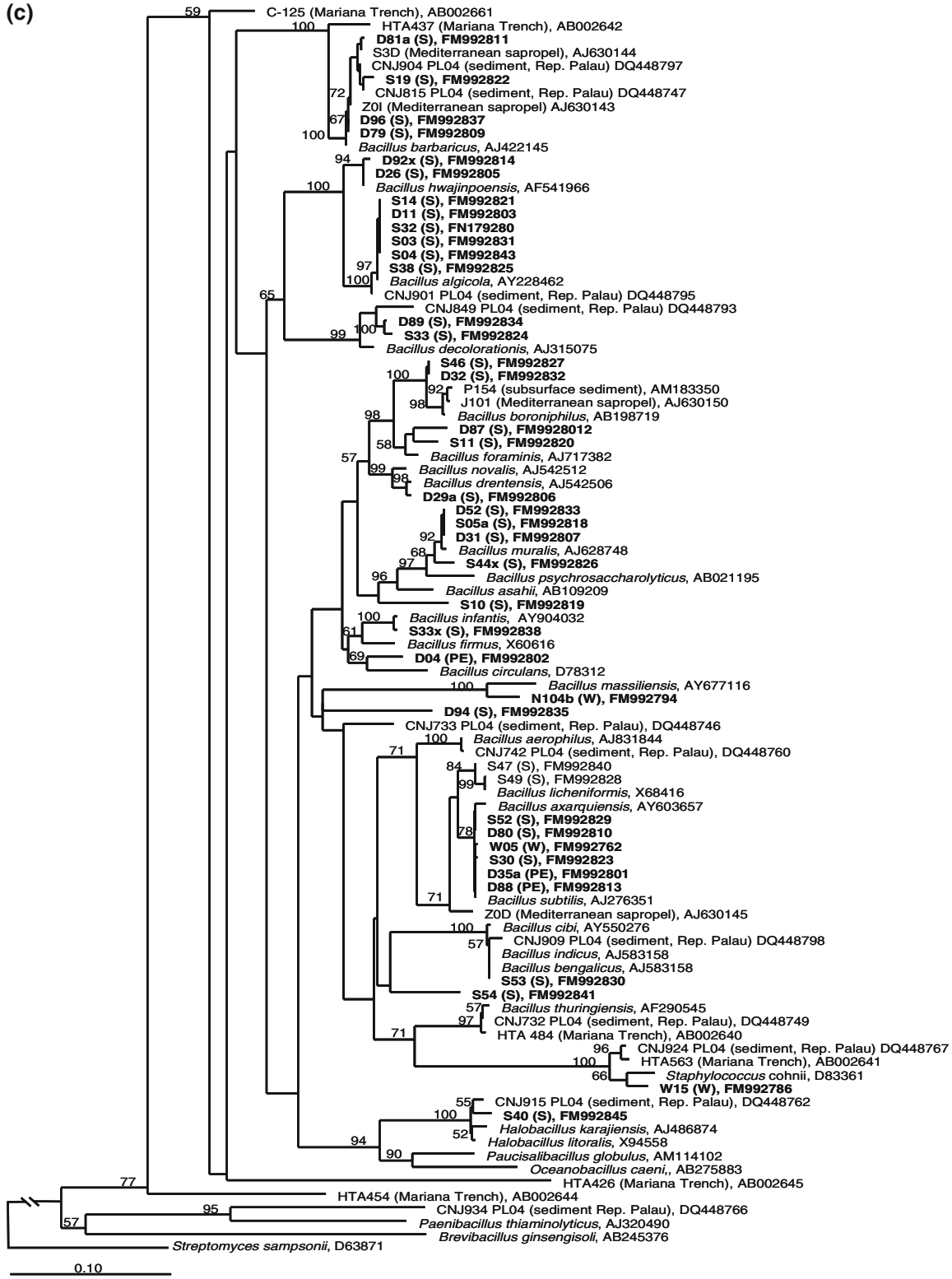

Fig. 1 continued

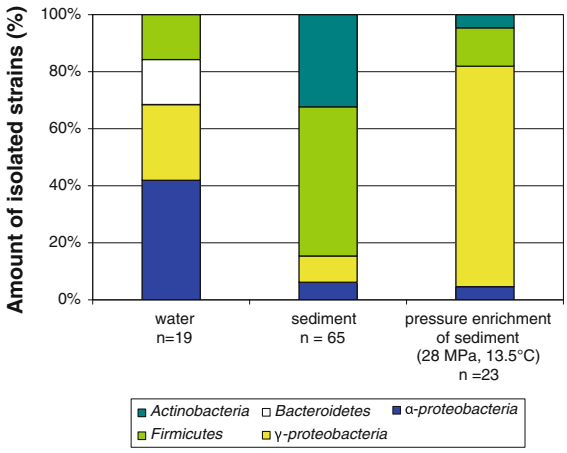

Fig. 2 Phylogenetic affiliation of all strains (%) obtained from water, sediment and the pressurised sediment enrichment

experiments using MWY, which contained 0.01% yeast extract, revealed that the great majority of the selected strains (93%) grew well under experimental low-nutrient conditions and in situ temperature of 13.5°C. Strains that did not grow under these conditions were *Bacillus* sp. D29a, *Corynebacterium* sp. S35 and *Staphylococcus* sp. W15. These strains did grow, however, at 13.5°C when the nutrient concentration was increased. Thus, most of the isolates were able to grow under in situ conditions (temperature and nutrient conditions) prevailing in the Eastern Mediterranean deep sea. This includes strains of *Alteromonas* spp. (D33, D42, D47 and D56), *Pseudomonas* sp. D43 and *Sphingobium* sp. D53, which were enriched and isolated exclusively after incubation at in situ hydrostatic pressure and in situ temperature with *N*-acetyl-D-glucosamine as energy and nutrient source. Most of the strains (14/23) obtained from the in situ hydrostatic pressure enrichment were isolated from low-nutrient seawater media (MW, MWY or Chitin-medium).

Discussion

Isolation from sediment samples

Gram-positive bacteria were clearly dominant among the strains isolated from the Eastern Mediterranean deep-sea sediment. This is in accordance to the frequent isolation of mainly *Firmicutes* and *Actinobacteria* from diverse marine habitats (Marteinsson et al. 1996; Colquhoun et al. 1998; Yanagibayashi et al. 1999; Siefert et al. 2000; Süß et al. 2004; Jensen et al. 2005; Koepke et al. 2005; Pathom-aree et al. 2006; Gontang et al. 2007; Stevens et al. 2007; Prieto-Davó et al. 2008). Members of the *Firmicutes* are moreover considered to be the most frequently isolated strains from subsurface sediments (D' Hondt et al. 2004).

In contrast to the high isolation frequency of Gram-positive bacteria, they were of minor relevance in 16S rRNA gene libraries of Mediterranean deep-sea sediments (Heijs et al. 2008). *Actinobacteria* accounted for 4–28% of total sequences in clone libraries of Cretan margin sediments, while *Firmicutes* accounted for a maximum of 3% (Polymenakou et al. 2009). Both taxonomic groups might well be under-represented in 16S rRNA gene based molecular approaches, most likely because they often resist commonly applied DNA extraction techniques or may be missed due to primer biases (McVeigh et al. 1996; Mincer et al. 2005; Carrigg et al. 2007). Nevertheless, it appears that *Actinobacteria* and *Firmicutes* represent only a small fraction of the microbial community. On the other hand, quantitative occurrence does not give any information about the ecological relevance of these groups. It was shown e.g. that spores of *Bacillus* strains are capable for manganese oxidation in hydrothermal sediments of Guaymas basin (Rosson and Nealson 1982; Dick et al. 2006). The high abundance of *Firmicutes* isolated during this study and in particular the remarkable high diversity of different operational taxonomic units of the genus *Bacillus* demonstrates the importance of culture-dependent techniques as an additional tool to assess the microbial diversity.

Members of the genus *Bacillus* are ubiquitary, but very little is known about the metabolic activity of *Bacillus* sp. in marine sediments. *Bacillus* species frequently recovered from different marine environments are *B. marinus, B. badius, B. subtilis, B. cereus, B. licheniformis, B. firmus, B. lentus* and *B. pumilis* (Ivanova et al. 1999). *B. marinus* e.g. was isolated from tropical Atlantic, Antarctic and Arctic deep-sea sediments and turned out to be psychrophilic or psychrotolerant (Rueger et al. 2000). Closest phylogenetic relatives to isolates assigned to *Bacillus* obtained during this study originated from various environments and varied in their physiological properties. Though inference of physiological properties

from those of closest phylogenetic relatives is possible to a limited extent, with some probability it may be used to delineate key properties of genetic relatives. With consideration of these limitations, it is interesting to see that *Bacillus licheniformis* as closest relative to isolate D94 is known for its specific strategy to cope with nutrient limitation (Hoi et al. 2006), which is important for adaptation to the extreme oligotrophy prevailing in the deep Eastern Mediterranean.

Interestingly, the Mariana Trench sediment was also dominated by Gram-positive bacteria and *Bacillus sp.* accounted for 25% thereof (Takami et al. 1997) (Figs. 1c,b). In addition, isolates from the present study assigned to *Micromonospora* and *Streptomyces* have close relatives to those recovered from the Mariana Trench (Pathom-aree et al. 2006) (Fig. 1b). This close affiliation of strains from different deep-sea sediments indicates their possibly wide distribution in deep sea sediments. More distantly related OTUs of our isolates might represent bacteria specifically adapted to the conditions of the Mediterranean Sea.

Isolation after enrichment under in situ hydrostatic pressure

Hydrostatic pressure incubation of the sediments clearly shifted the composition towards *Gammaproteobacteria* which is considered to reflect their ecological importance in the nutrient degradation of the deep sea. Apparently, they cope very well with changes of hydrostatic pressure as they have been decompressed during sampling, recompressed to in situ hydrostatic pressure and again decompressed for cultivation. It has already been demonstrated that decompression during retrieval of deep-sea samples affects microbial activity and microbial production rates (Seki and Robinson 1969; Jannasch et al. 1976; Bianchi et al. 1999). Especially cell membranes were shown to be damaged by changing hydrostatic pressure (Chastain and Yayanos 1991; Pagan and Mackey 2000; Park and Clark 2002). The gammaproteobacterial strains recovered by this approach might be assumed to be barotolerant. The majority of gammaproteobacterial OTUs (six out of ten) were exclusively isolated after this enrichment and are regarded as specifically selected by this treatment. This refers in particular to strains affiliating to the genus *Alteromonas*, which is commonly found in marine habitats including the deep sea (Yanagibayashi et al. 1999; Li et al. 1999a, b; Lopez-Lopez et al. 2005). Most of the barophilic bacterial strains identified to date (bacteria with optimal growth at hydrostatic pressure >0.1 MPa) affiliate to the *Gammaproteobacteria* (Yayanos et al. 1979; Kato et al. 1995; Delong et al. 1997; Kato et al. 1999; Lauro and Bartlett 2008). Lopez-Lopez et al. 2005 compared shallow water strains of *Alteromonas macleodii* to those obtained from the deep water by detailed sequence analyses of the 16S rRNA gene, the internal transcribed spacer and of house-keeping genes such as *gyrB* and *rpoB*. They demonstrated characteristic differences between deep-sea and shallow-water strains and ascribed them to different ecotypes (Lopez-Lopez et al. 2005; Lauro et al. 2007; Lauro and Bartlett 2008). This hints towards the existence of specifically adapted bacterial ecotypes in the deep sea (Lauro and Bartlett 2008).

In our experiments, the pressurised sediment was supplemented with *N*-acetyl-D-glucosamine (NAG), which is known to induce bacterial degradation of chitin, the (1-4)-linked β-homopolymer of NAG and a structural component of many organisms including fungi, protists, animals and plants. Chitin, as a valuable carbon and nitrogen source, is reported to be mainly degraded by chitinolytic bacteria, e.g. members of the genera *Pseudomonas, Aeromonas, Xanthomonas, Serratia, Cytophaga, Arthrobacter* as well as *Bacillus* and is supposed to be the most abundant biopolymer in the marine environment (Gooday 1990). It can be assumed that many bacteria adapted to the oligotrophic conditions of the Eastern Mediterranean deep sea are able to use a broad spectrum of available carbon sources, including chitin and its degradation products such as NAG. All strains from the pressurised enrichment were grown with NAG as sole carbon source and 11 additional strains of the untreated sediment were isolated from solid chitin media. These strains belong to the *Gammaproteobacteria*, the *Firmicutes* and the *Actinobacteria*. Apparently, the ability to degrade chitin and derivatives thereof is a beneficial and a widely distributed property of bacteria inhabiting the deep sea.

Growth at low nutrient concentrations

All strains tested during this study grew easily well with minor nutrient concentrations and as well at in

situ temperature of 13.5°C, conditions prevailing in the deep Mediterranean Sea. The bacterial isolates apparently are well adapted to low nutrient concentrations of oligotrophic deep-sea sediments as well as to spontaneous and occasional nutrient supply due to seasonal changes or sinking carcasses. An example is represented by isolates assigned to *Alteromonas* (D33, D42, D47 and D56). As demonstrated by their growth on low nutrient media, they coped well with minute nutrient concentrations but also successfully competed with others at in situ hydrostatic pressure in the presence of *N*-acetyl-D-glucosamine. Members of the *Gammaproteobacteria* are frequently appointed to be *r*-strategists that can stand long periods of starvation but they outcompete others when nutrients become available (Fuchs et al. 2000; Pinhassi and Berman 2003). This is in accordance with D' Hondt et al. (2004) who showed that *Gammaproteobacteria* are common in sediments of the ocean margin where concentrations of organic matter and net metabolic rates are high. As it was already shown for the Cretan Sea, bacterial abundance and biomass are sensitive to sudden changes in nutrient availability (Danovaro et al. 2000). Our data indicate that *Gammaproteobacteria* might considerably contribute to detectable changes in the composition of the bacterial communities in the Eastern Mediterranean deep-sea sediments.

In contrast *Actinobacteria* and *Firmicutes* dominated the strains obtained from the untreated sediment and some of these were exclusively isolated using low nutrient media. The suitability of low-nutrient media for the isolation of Gram-positive bacteria was also approved by other culture-dependent studies (D' Hondt et al. 2004; Jensen et al. 2005; Gontang et al. 2007). The lifestyle of these bacteria apparently is adapted to minute nutrient concentrations (*k*-strategists) and under such conditions gives them advantage over the fast growing *Gammaproteobacteria*. This is supported by a study of D' Hondt et al. (2004) who consistently isolated *Actinobacteria* and *Firmicutes* from sediments of ocean margins and open ocean sites exhibiting low concentrations of organic matter and net metabolic rates. As biopolymer degradation was shown to be of special importance in the Mediterranean deep Sea (Martin-Cuadrado et al. 2009), *Actinobacteria* might be considered as important players in the degradation of complex biopolymers of the studied environment.

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