MaCuMBA

Marine Microorganisms: Cultivation Methods for Improving their Biotechnological Applications

Project number: 311957
Start of the project (duration): August 1st, 2012 (48 months)

Collaborative Project
Seventh Framework Programme
Cooperation, KBBE

Deliverable D2.15

Cultivation system for deep-sea sediment microorganisms

Organisation name of lead contractor: UBO-LUBEM

Due date of deliverable: M12
Actual submission date: M24

Revision: V.1

Project co-funded by the European Commission within the Seventh Framework Programme (2007-2013)	
Dissemination Level	
PU Public	**X**
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v.1		

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Summary

Objective(s): Development of cultivation systems to isolate microorganisms from deep-sea sediments (Task 2.7 – Subtask 5)

Rationale: The deep biosphere is considered to be a huge reservoir of carbon on Earth and a third realm for microbial life. Sub-seafloor sediments provide a habitat for large numbers of microbial cells that consitute a large fraction of Earth's living biomass. The subsurface microbiota is diverse and complex, hosting metabolically active communities down to depths of more than one thousand meters below the seafloor. It harbors representatives from the three domains of life, i.e., numerous endemic and/or as yet uncultured *Archaea* and *Bacteria*, in addition to bacterial endospores, protists and fungi belonging to the *Eukarya.* Such deep-sea representatives form an untapped resource of biotechnological potential. Aim of this deliverable is to improve the isolation rate and growth efficiency of deep-sea sediment microorganisms by using cultivation system mimicking *in situ* conditions. Partners involved in this deliverable have selected 3 strategies: (i) Using hydrostatic pressure as a key physical parameter to isolate and characterize deep-sea fungal strains. (ii) Using a microcultivation method mimicking the natural sedimentary context and favoring the attached lifestyles of prokaryotes in deep-sea sediments. (iii) Designing complex isolation media where some growth factors can offer the selection of specific group of actinomycetes with potential capability to produce bioactive secondary metabolites.

Results:
UBO-LUBEM and UBO-LM2E (Partner 3) have developed well-defined methodologies based on elevated hydrostatic pressure to isolate and visualize 183 endemic fungal strains, and a microcultivation method to isolate and visualize endemic prokaryotes. PharmaMar (Partner 19) has designed complex isolation media dedicated to the isolation of 1464 deep-sea sediment actinomycetes with biotechnological potential.

Partner(s) involved in Deliverable production:

Partner name / number	Hydrostatic pressure	Microcultivation	Complex media
UBO-LUBEM (P.3)	X		
UBO-LM2E (P.3)		X	
PharmaMar (P.19)			X

D2.15 – Part I: Systems mimicking high-pressure conditions of the deep subsurface

High-pressure is a key feature of deep-sea environments. The incubation of deep subsurface microorganisms under high-hydrostatic pressure conditions is necessary to isolate and characterize endemic strains adapted to *in situ* conditions. Enrichment cultures were performed with subsurface sedimentary rocks from the Canterbury basin, off the coast of New Zealand. Enrichment cultures were kept under aerobic and anaerobic conditions in sterile syringes. All media used at aerobic conditions were saturated with dissolved oxygen. Media performed in anaerobic conditions were supplemented with resazurin (0.5 g/l) as anaerobiosis indicator. Each syringe contained 5 ml of culture medium and 200 µl of sediment sample. Syringes were then transferred in high-hydrostatic pressure incubation system (Fig. 1), and finally incubated at different temperatures: (i) samples from 5.6 to 37.1 mbsf were incubated at 25°C / 4 MPa, (ii) samples from 137.13 to 765 mbsf were incubated at 30°C / 11 MPa, and (iii) samples from 1477.76 to 1864.06 mbsf were incubated at 45°C / 37 MPa.

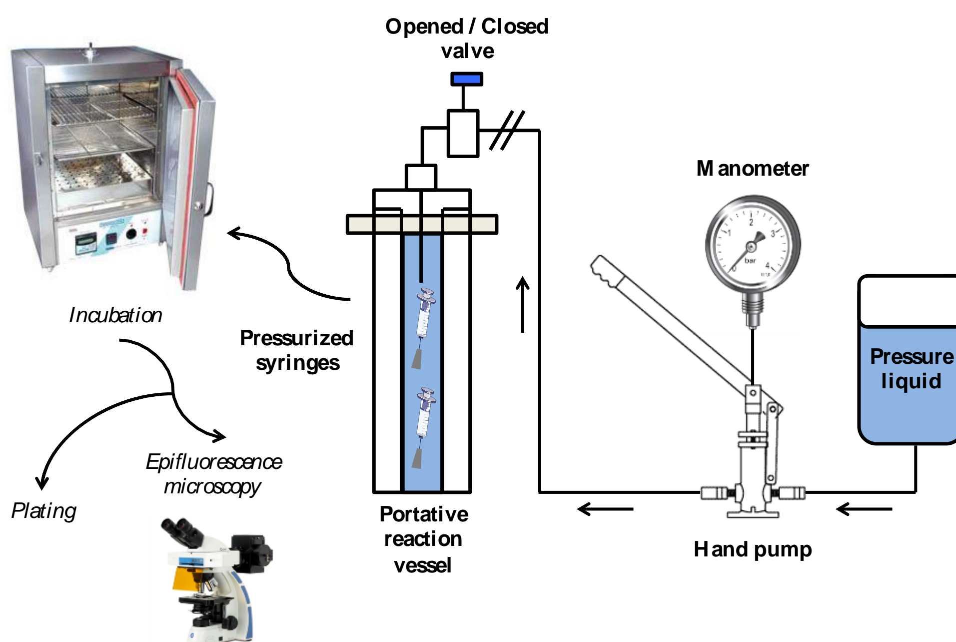

Figure 1: Schematic drawing of the 'portable' high-pressure incubation system (Top-Industrie, France). Syringes loaded with culture media and sediment slurry are sealed with a silicon caps and placed in the pressure cylinder. The reaction vessel is pressurized using a hydraulic pump coupled with a manometer. The system is closed with a valve allowing the portable reaction vessel to be incubated at specific temperature in a separate incubator.

After 14 days of enrichment under hydrostatic pressure, epifluorescence microphotographs of initial enrichment cultures were processed to reveal fungal cells (Fig. 2). In parallel, 100 µl of culture were spread on agar plate and incubated at the same temperature until fungal growth was visible. Effectiveness of this system mimicking *in situ* conditions for the isolation of deep-sea fungi has been evaluated using atmospheric pressure as control (Table 1). The number of fungal strains that have been isolated with this system (146 strains) was more than 3-times higher than the number obtained from enrichments under atmospheric pressure (40 strains). In this study, hydrostatic pressure can be seen as a "starter" enhancing fungal growth of deep-sea piezo-sensitive, piezo-tolerant and piezo-philic representatives under pressure before cultivation at atmospheric pressure for identification and characterization.

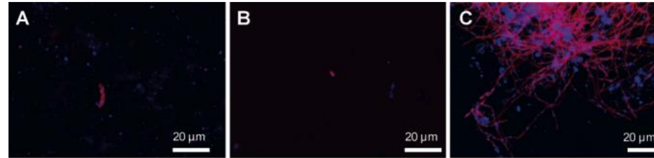

Figure 2: Epifluorescence microphotographs of initial enrichment cultures and subsequent liquid subcultures hybridized with the Cy3-labelled probe Euk516 specific for the 18S rRNA and stained with the general DNA stain DAPI. (A-C) Identification of fungus-like eukaryotic cells with fluorescently-labeled 18S rRNA oligonucleotide probes in initial enrichment cultures with sediments from 21 m CSF-A on PDB 3%, at 4 MPa (A), with sediments from 37 m CSF-A on PDB 3%, at 11 MPa (B), and with sediments from 765 m CSF-A on PDB 0%, at 11 MPa (C).

Table 1. Culture collection of fungi from deep-sea sediments using systems mimicking *in situ* conditions.

Depth (mbsf)	Total number of strain	Number of strain isolated under hydrostatic pressure	Number of strain isolated under atmospheric pressure
5.60	26	16	10
11.60	17	12	5
21.10	13	12	1
24.60	25	25	0
34.10	38	31	7
37.10	26	15	11
137.13	13	13	0
403.10	12	11	1
765.00	12	11	1
1477.76	0	0	0
1884.06	1	0	1
	183	**146**	**37**

References

Ciobanu, M.-C., Burgaud, G., Dufresne, A., Breuker, A., Redou, V., Ben Maamar, S., Gaboyer, F., Vandenabeele-Trambouze, O., Lipp, J.S., Schippers, A., Vandenkoornhuyse, P., Barbier, G., Jebbar, M., Godfroy, A. & Alain, K. Microorganisms persist at record depths in the subseafloor of the Canterbury Basin. *The ISME Journal*. In press.

D2.15 – Part II: Microcultivation method mimicking the natural sedimentary context.

Enrichment cultures were performed with subsurface sedimentary rocks from the Canterbury basin, off the coast of New Zealand. Microcolonies of 7-10 fermentative prokaryotic cells were grown anaerobically at 60-70°C from calcareous chalk/limestone collected at 1827 and 1922 mbsf (meters below the sea floor) (Fig. 3), using a microcultivation method that combines a polycarbonate membrane as a growth support and sediment slurry as substrate (Fig. 4). This cultural strategy mimics the natural sedimentary context and favors the attached lifestyle. Membranes with colonies were successfully transferred in liquid media and subcultured 6 to 9 times to $1/40^{th}$ or $1/50^{th}$. Within these subcultures, mean cell densities were low, around 4×10^5 cells ml^{-1} and growth rates were slow. Cultures were composed of viable tiny rods, coccobacilli and cocci of 300 to 800 nm in diameter, that often form aggregates and that contained ATP (Ciobanu *et al.*, in press). The major lineages identified in DNA and RNA libraries from these subcultures belonged to *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Actinobacteria* and *Armatimonadetes*. The large majority of the environmental sequences affiliating with our sequences were recovered from environments with similar physical-chemical characteristics (hot and reduced habitats) than the sub-seafloor of the Canterbury basin (Lin *et al.*, 2006; Mason *et al.*, 2010).

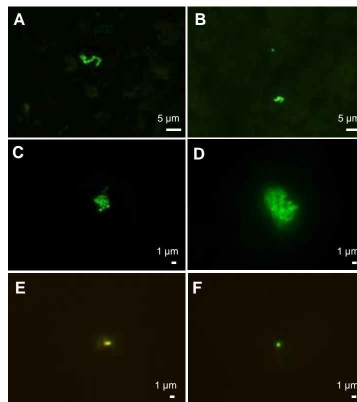

Fig. 3. Epifluorescence microphotographies of initial enrichment cultures and subsequent liquid subcultures stained with SYBR®Green I (A-D) or with the dual staining LIVE/DEAD®Bacterial Viability Kit (E-F). (A-B) Microcolonies observed on polycarbonate membranes (initial enrichment cultures) after 15 days of incubation with sediments from 1922 mbsf on H_2/CO_2 + YE. (C-D) Cellular aggregates observed in the 7th liquid subcultures performed after the initial enrichment with sediments from 1827 mbsf on YE + peptone + casamino acids. (E-F) Cell structural integrity was observed in the 7th liquid subcultures performed after the initial enrichment with sediments from 1827 mbsf on acetate + YE (H) and with sediments from 1922 mbsf on YE + peptone + casamino acids (I). Legend: YE, yeast extract.

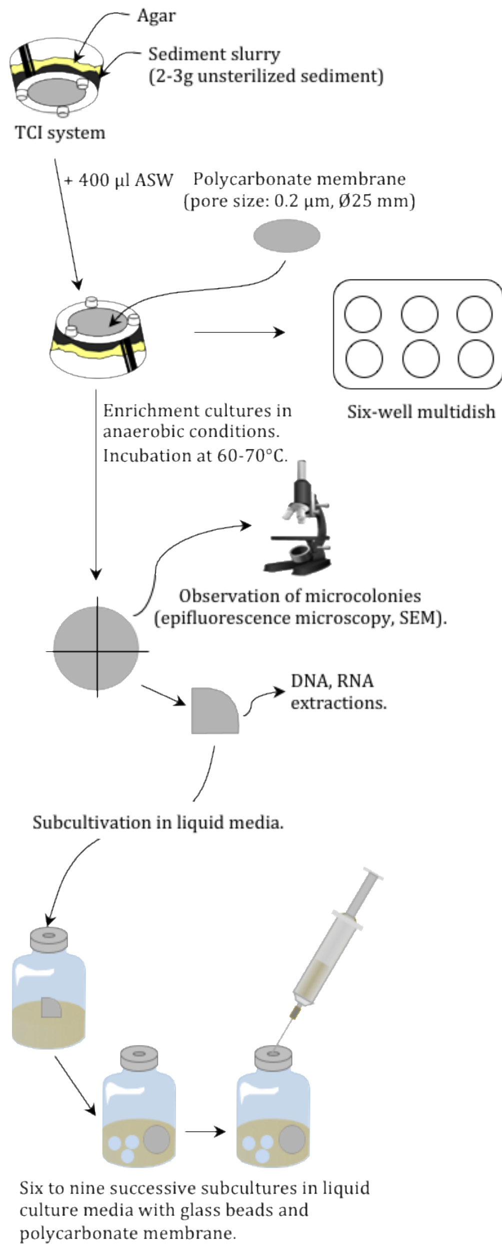

Fig. 5. Culture flow-diagram (TCI, tissue culture insert; ASW, artificial seawater). Microcultivation in a sediment slurry membrane system.

References

Ciobanu, M.-C., Burgaud, G., Dufresne, A., Breuker, A., Redou, V., Ben Maamar, S., Gaboyer, F., Vandenabeele-Trambouze, O., Lipp, J.S., Schippers, A., Vandenkoornhuyse, P., Barbier, G., Jebbar, M., Godfroy, A. & Alain, K. Microorganisms persist at record depths in the subseafloor of the Canterbury Basin. *The ISME Journal*. In press.

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Mason, O. U., Nakagawa, T., Rosner, M., Van Nostrand, J. D., Zhou, J., Maruyama, A., Fisk, M. R., and Giovannoni, S. J. (2010). First investigation of the microbiology of the deepest layer of ocean crust, *PLoS One*, 5, e15399.

D2.15 – Part III: Cultivation systems for deep-sea sediment actinobacteria

For the isolation of deep-sea actinobacteria, all the isolation plates were supplemented with nalidixic acid (0.2 g/l) and cycloheximide (0.2 g/l) to avoid fast-growing bacteria and fungi.
In both cases, we have used a standard isolation solid medium (BEN) composed of L-Asparagine, glycerol and marine salts and several induction media:

- IG2.- Induction_1: BEN complemented with humic acid and a mixture of different vitamins B
- IG2.- Induction_2: BEN complemented with SiO2, trace elements, vitamins B and amino acids
- MUR.- Induction_1: The same as IG2
- MUR.- Induction_3: Induction_2 plus gluconate and N-acetyl-glucosamine

39 samples from Cadiz Gulf and Gibraltar Strait, Spain (IG2) and 24 samples from Coco Island, Costa Rica (MUR) were homogenised and 100 µl were seeded in each plate. Incubation during 2 to 4 weeks at 27°C at atmospheric pressure to avoid scale-up issues for drug discovery. In total, we have used 585 isolation plates in IG2 and 360 in MUR.

Table 2. Results period 0 to 18 months of molecular characterization of isolates

Expedition	Isolation media	Pure colonies isolated	DNA fingerprinting differents (Collection)
IG2	Standard BEN	636	**431**
	Induction_1	492	**330**
	Induction_2	678	**452**
MUR	Standard BEN	322	119
	Induction_1	219	94
	Induction_3	331	145

In order to analyzed the biodiversity obtained, the unique strains are amplified by partial 16S rRNA and the sequences are compared with EzTaxon data base (www.ezbiocloud.org). During the period 0 to 18 months, **1.464 taxonomies (1.118 from IG2 and 346 from MUR)** have been determined. 99% belonged to the Phylum Actinobacteria.

In the table 2 are summarized the biodiversity obtained from IG2 and the table 3 the biodiversity of MUR.

Table 3. List of taxonomies obtained from IG2 isolates

Phylum	Genera	# Species	# Strains
Actinobacteria	*Actinomadura*	3	4
	Agromyces	2	2
	Arthrobacter	3	3
	Citricoccus	1	1
	Dietzia	1	4
	Georgenia	1	1
	Glycomyces	3	3
	Isoptericola	2	2
	Kitasatospora	1	1
	Kocuria	2	2
	Micrococcus	1	1
	Micromonospora	18	68
	Myceligenerans	1	4
	Nocardia	6	10
	Nocardioides	1	3
	Nocardiopsis	7	23
	Nonomuraea	5	9
	Promicromonospora	4	8
	Pseudonocardia	1	1
	Rhodococcus	2	2
	Saccharomonospora	2	5
	Saccharopolyspora	2	6
	Streptomyces	214	946
	Streptosporangium	1	1
Firmicutes	*Bacillus*	4	4
Proteobacteria	*Defluviimonas*	1	1
	Erythrobacter	1	1
	Mesorhizobium	1	1
	Nitratireductor	1	1

Table 4. List with deep-sea actinobacteria isolated from MUR samples

Phylum	Genera	# Species	# Strains
Actinobacteria	*Actinomadura*	4	6
	Micromonospora	29	139
	Microtetraspora	2	13
	Nocardia	7	27
	Nonomuraea	4	5
	Planctatinospora	2	2
	Streptomyces	45	133
	Streptosporangium	3	6
	Verrucosispora	4	15
	TOTAL ACTINOBACTERIA ISOLATED IN MUR	100	346