MaCuMBA

Marine Microorganisms: Cultivation Methods for Improving their Biotechnological Applications

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Database of cultivable microorganisms capable of natural transformation

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RE Restricted to a group specified by the consortium (including the Commission Services)	
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Summary

Objective:

The aim of the deliverable was to provide a database of cultivable microorganisms capable of natural transformation. Natural transformation is a process in which competent bacterial cells are able to acquire free exogenous DNA and incorporate it stably into the genome or convert it in an autonomous extrachromosomal replicon. Conversely, in artificial transformation cells are forced to incorporate exogenous DNA by physical or chemical treatments.

Rationale and experimental procedures:

In order to evaluate the natural competence of several marine isolates, a collection of different aerobic bacteria, isolated from i) deep hypersaline anoxic basins, DHABs (in particular from the deep seawater-brine interface of Urania, Discovery, Bannock and L'Atalante and from the brine of Kryos), and ii) mangrove ecosystem (crab organs and marine sediments) was screened (Tab. 1). Briefly, the bacteria were exposed to different plasmids and putative transformants were selected exploiting the antibiotic resistance conferred to the bacterium (originally sensitive) by the plasmids. Bacteria representative of different taxonomic groups were considered, particularly high G+C sporeforming bacteria of the family Bacillaceae, including the genera *Bacillus*, *Halobacillus* and *Virgibacillus*, and members of the classes Alpha- and Gamma-Proteobacteria (Tab. 1).

Tab. 1. List of tested strains and efficiency of transformation.

Strain	Species	Phylogenetic taxon	Origin	Plasmid used in transformation experiments	Transformation efficiency
10U	*Bacillus acidogenesis*	*Bacillaceae*	Urania*	pSM1890, pEG922	0
21U	*Bacillus jeotgali*	*Bacillaceae*	Urania	pSM1890; pEG922	0
25U	*Halobacillus karajiensis*	*Bacillaceae*	Urania	pSM1890; pUB110	0
8D	*Bacillus baekryungensis*	*Bacillaceae*	Discovery	pSM1890; pUB110;	0
9D	*Bacillus firmus*	*Bacillaceae*	Discovery	pSM1890; pUB110; pEG922	0
11D	*Bacillus firmus*	*Bacillaceae*	Discovery	pSM1890; pUB110	0
13D	*Halobacillus trueperi*	*Bacillaceae*	Discovery	pSM1890; pUB110; pEG922	0
31D	*Bacillus pantothenticus*	*Bacillaceae*	Discovery	pSM1890; pEG922	0
20B	*Virgibacillus pantothenticus*	*Bacillaceae*	Bannock	pSM1890; pEG922	0
6B	*Ruegeria algicola*	Gammap.	Bannock	pSM1890	0
11B	*Aererehalobacter ostenderi*	Gammap.	Bannock	pSM1890	0
16B	*Halomonas alimentaria*	Gammap.	Bannock	pSM1890	0
3A	*Alteromonas marina*	Gammap.	L'Atalante	pSM1890	0
18A	*Idiomarina abyssalis*	Gammap.	L'Atalante	pSM1890	0
5U	*Pseudoalteromonas issachenkonii*	Gammap.	Urania	pSM1890	0
7U	*Pseudoalteromonas porphyrae*	Gammap.	Urania	pSM1890	0
9U	*Exiguobacterium auriantiacum*	Gammap.	Urania	pSM1890	0
24U	*Salinisphaera shabanense*	Gammap.	Urania	pSM1890	0
5D	*Alteromonas macleodii*	Gammap.	Discovery	pSM1890	0
6D	*Halomonas cupida*	Gammap.	Discovery	pSM1890	0
5M-7	*Erythrobacter vulgaris*	Alphap.	Mangrove Sediment	pHM2(GFP)	3,51E-10
SWA5%45	*Lutibacterium anuloederans*	Alphap.	Mangrove Sediment	pHM2(GFP)	0
25M-6	*Pimelobacter simplex*	Alphap.	Mangrove Sediment	pHM2(GFP)	1,09E-09
UU14	*Ruegeria pelagia*	Alphap.	*Uca urvillei* Gill	pHM2(GFP)	0
AG16	*Idiomarina loihiensis*	Gammap.	Kryos Brine	pHM2(GFP)	0
AG11	*Marinobacter adhaerens*	Gammap.	Kryos Brine	pHM2(GFP)	3,91E-10
HOR24	*Acinetobacter calcoaceticus*	Gammap.	Crab Gill	pHM2(GFP)	0

HOR19	*Acinetobacter junii*	Gammap.	Crab Gill	pHM2(GFP)	0
HOR58	*Acinetobacter oleivorans*	Gammap.	Crab Gill	pHM2(GFP)	0
HOR46	*Shewanella decolorationis*	Gammap.	Crab Gill	pHM2(GFP)	4,39E-07
SWA5%18	*Microbulbifer elongatus*	Gammap.	Mangrove Sediment	pHM2(GFP)	0
SWA0%6	*Microbulbifer hydrolyticus*	Gammap.	Mangrove Sediment	pHM2(GFP)	0
SWA0%3	*Microbulbifer mangrovi*	Gammap.	Mangrove Sediment	pHM2(GFP)	0
SWA5%34	*Marinobacter sediminum*	Gammap.	Mangrove Sediment	pHM2(GFP)	0
SWA5%26	*Microbulbifer salipaludis*	Gammap.	Mangrove Sediment	pHM2(GFP)	1,12E-08
47M-5	*Halomonas alkaliphila*	Gammap.	Mangrove Sediment	pHM2(GFP)	0
34M-6	*Marinobacter adhaerens*	Gammap.	Mangrove Sediment	pHM2(GFP)	8,73E-07
9M-6	*Microbulbifer agarilyticus*	Gammap.	Mangrove Sediment	pHM2(GFP)	0
49M-5	*Microbulbifer donghaiensis*	Gammap.	Mangrove Sediment	pHM2(GFP)	0
SWA5%4	*Microbulbifer mangrovi*	Gammap.	Mangrove Sediment	pHM2(GFP)	0
52M-5	*Microbulbifer mangrovi*	Gammap.	Mangrove Sediment	pHM2(GFP)	0
SWA0%1	*Microbulbifer salipaludis*	Gammap.	Mangrove Sediment	pHM2(GFP)	3,89E-08
46M-5	*Microbulbifer salipaludis*	Gammap.	Mangrove Sediment	pHM2(GFP)	1,26E-08
43M-5	*Microbulbifer taiwanensis*	Gammap.	Mangrove Sediment	pHM2(GFP)	0
30M-6	*Microbulbifer variabilis*	Gammap.	Mangrove Sediment	pHM2(GFP)	1,50E-07
PG38	*Pseudomonas putida*	Gammap.	*Perisesarma guttatum*	pHM2(GFP)	0
PG4	*Shewanella algae*	Gammap.	*Perisesarma guttatum*	pHM2(GFP)	0
PG36	*Pseudomonas putida*	Gammap.	*Perisesarma guttatum*	pHM2(GFP)	1,32E-09
PG3	*Photobacterium ganghwense*	Gammap.	*Perisesarma guttatum*	pHM2(GFP)	0
PG21	*Pseudomonas plecoglossicida*	Gammap.	*Perisesarma guttatum* Gill	pHM2(GFP)	1,07E-09
UU64	*Pseudomonas sp.*	Gammap.	*Uca urvillei* gills	pHM2(GFP)	0
UU1	*Pseudomonas pachastrellae*	Gammap.	*Uca urvillei* gills	pHM2(GFP)	1,83E-10
UU28	*Shewanella haliotis*	Gammap.	*Uca urvillei* gills	pHM2(GFP)	0
UU11	*Pseudomonas putida*	Gammap.	*Uca urvillei* gills	pHM2(GFP)	1,26E-10
UU42	*Citrobacter freundii*	Gammap.	*Uca urvillei* gills	pHM2(GFP)	0
UU63	*Pseudomonas putida*	Gammap.	*Uca urvillei* gills	pHM2(GFP)	0
UU21	*Vibrio hepatarius*	Gammap.	*Uca urvillei* gills	pHM2(GFP); pSEVA431(GFP)	0
90-11-287	*Vibrio anguillarum*	Gammap.	*Reference strain*	pSEVA431(GFP)	0
UU24	*Vibrio fortis*	Gammap.	*Uca urvillei gills*	pSEVA431(GFP)	0
UU92	*Vibrio hepatarius*	Gammap.	*Uca urvillei* gills	pHM2(GFP); pSEVA431(GFP)	0

* DHABs: Urania, Bannock, L'Atalante, Discovery
"0": no transformant was recovered

Transformation assays were performed in liquid (mixing cells and plasmidic DNA in suspensions) or in solid conditions (leaning the mix of cells and plasmidic DNA on nitrocellulose membrane filters), incubating the mixtures for different times. Different plasmids were employed: i) pSM1890, able to replicate in *Escherichia coli* and in *Bacillus*, carrying the cassette that encodes from a green fluorescent protein (GFP) and the genetic resistance determinants to gentamicin and spectinomycin (Richter and Smalla, 2007); ii) pUB110, a replicative plasmid in *Bacillus*, able to confer the resistance to kanamycin (Boe et al., 1989) ; iii) pEG922, capable of conferring tetracycline resistance in Bacillaceae sensitive strains (Baum, 1994); iv) pHM2(GFP) carrying a kanamycin antibiotic selection marker and the GFP cassette (Favia et al., 2007) and iv) pSEVA431(GFP), carrying the genes for the streptomycin-spectinomycin resistance (we inserted the GFP cassette from pHM2-GFP into the SpeI restriction site of pSEVA431 plasmid, Silva-Rocha et al., 2013). After fixed times of contact, cells were plated on media with the addition of appropriate antibiotic to counterselect transformants. Molecular analysis were performed to assess transformation success that was also verified by fluorescent microscopy when applicable.

Last part of activities was dedicated to verify the natural competence in *Vibrio* isolates. Strains of *Vibrio cholerae* and *Vibrio fischeri* have been reported to acquire a competent status after growth on chitin (Meibom et al., 2005; Sun et al., 2013.; Pollack-Berti et al., 2010). Chitin is one of the major

carbon source for vibrios in the estuarine environments, in which they live associated to fragments of crustacean exoskeletons. Upon growth in chitin-supplemented media, these bacteria import DNA from the environment in their cytosol. If in the imported molecules are present homolog DNA sequences, they can integrate them into the bacterial chromosome through homologous recombination. However, it has not been tested yet if the bacteria could acquire plasmids through the same mechanism. For this reason, we tested a chitin-mediated transformation protocol (Marvig and Blokesch, 2010) on *Vibrio* strains, mixing the bacterial cultures with plasmid DNA.

Results:

A summary of the tested strains and the transformation efficiency obtained in natural transformation experiments is reported in table 1.

Natural transformation assays performed with suitable Bacillaceae strains using plasmid pSM1890 or pUB110 (i.e. with strains sensitive to the antibiotic markers carried on plasmids) gave no positive results. These experiments were performed in liquid conditions, using different times of contact (1.5h, 2.5h, 5h, and 7.5h).

Natural competence in solid conditions was tested on tetracycline-sensitive Bacillaceae strains using plasmid pEG922 and 11 isolates belonging to Gamma-Proteobacteria class (6B, 11B, 16B, 3A, 18A, 5U, 7U, 9U, 24U, 5D and 18B) by using plasmid pSM1890. Also in these cases different contact times (5h, 8h and overnight) were used, but no transformants were retrieved in any of the assays.

Kanamycin-sensitive strains isolated from mangrove crabs, mangrove sediment and strains AG11 and AG16 isolated from Kryos brine were used in natural transformation assays with the plasmid pHM2(GFP) or pSEVA431(GFP) taking advantages of nitrocellulose filters (Tab. 1). In this case transformants were obtained: they acquired the plasmid conferring resistance to kanamycin and expressing beta-galactosidase enzyme (allowing blue colour screening). Natural transformation frequency was calculated as the number of kanamycin resistant blue-colony transformants over the total number of cells. The success of transformation was also monitored evaluating GFP expression by epifluorescence microscopy. The transformant strains showed nevertheless to be unstable, losing the plasmid after few transfers. This could be probably due to a transient maintenance and a following loss of plasmids or to degradation phenomenon toward the cell-internalized DNA.

Finally, regarding *Vibrio* strains, we performed experiments on naturally-sensitive strains toward kanamycin and streptomycin. We were not able to obtain any transformants. This could be explained by a fragmentation of the DNA that could happen, while it is imported in the cells. If so, the re-circularization of the plasmid in the cytosol could be the critical step that prevents the persistence of the plasmid in the bacterial cell.

The same isolates used in transformation protocol with plasmid pHM2(GFP) were tested in artificial transformation assays by electroporation using pHM2(GFP) also in this case. The procedure allowed to genetically label two strains, *Pseudomonas pseudoalcaligenes* UU63 and *Shewanella decolorationis* HOR46. In a different trial, artificial transformation was also verified in marine cyanobacteria *Synechococcus* sp. WH7803 and *Synechococcus* sp. WH8102.

Conclusion:

We screened for natural transformation 60 strains isolated from extreme marine environments belonging to different phylogenetic groups, using different plasmids and operative procedures,

including non-conventional methods like the chitin-mediated competence acquired by members of the *Vibrio* genus.

Thirteen strains (21.6% of the collection) were able to acquire exogenous plasmid DNA by natural transformation, thus can be considered naturally competent. All the transformants nevertheless demonstrated to be unstable, since unexpectedly the inserted plasmid was lost or degraded in few strain transfers. We conclude that a significant fraction of our isolate collection is naturally transformable, indicating that horizontal gene transfer in extreme marine environment is probably an important mechanism of evolution by genome shuffling. Given the instability of the transformants obtained in the laboratory, nevertheless natural transformation cannot be considered a routine method for the genetic manipulation of the strains.

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Partner(s) involved in Deliverable production: UMIL (partner 15) has worked on this Deliverable. UMIL and UW (partner 13) provided also data on artificial transformation.