MaCuMBA

Marine Microorganisms: Cultivation Methods for Improving their Biotechnological Applications

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Contents

Partner(s) involved in Deliverable production: UCC (12), UW (13), DTU (14), UMIL (15)

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Summary

In this deliverable we present experiments aimed at allowing genetic manipulation of marine bioactive bacteria. DTU has despite many attempts and systematic changes to the procedures, not been successful in mutagenizing these strains. UCC has successfully mutagenized strains of *Pseudovibrio* and *Pseudomonas*, UW has successfully mutagenized *Synecochoccus*, and UMIL has successfully manipulated strains of *Virgibacillus, Bacillus*, and *Halomonas*. Overall, conjugation has shown to be the most efficient way of genetically manipulating marine bacteria.

Strategies and results

UCC (12)

Two different *Pseudovibrio* strains, WM33 and W74, have been used to verify the genetic accessibility of strains belonging to this genus. After several unsuccessful attempts at transformation of W74, using different modifications (different culture volume, different amount of the transposome and different voltage), transformants were eventually achieved on selective plates with the Epicentre EX-Tn5 Tnp Transposome Kit. PCR analysis is ongoing to determine the site of insertion. Thus far, transformation attempts using WM33 have proven unsuccessful, despite using two independent transposon systems, the EX-Tn5 Tnp Transposome Kit of Epicentre, and plasmid pRL1063a. Currently, UCC is focusing on introducing native low molecular weight plasmids isolated into this isolate to determine whether incompatibility or competence is the underlying cause of lack of transformants.

Large scale random mutagenesis of a marine sponge *Pseudomonas* isolate using the same system has yielded over 10,000 mutants for analysis. Several of these have been characterized and the insertion site identified.

UW (13)
Blanca Perez Sepulveda, Joseph Christie-Oleza, and Dave Scanlan have worked on improving the efficiency of genetic tools functioning in marine *Synechococcus*. We have recently developed a new conjugation protocol that involves the role of "helper bacteria" (see WP5). This involves construction of a 'single cross over' *Synechococcus* gene knockout construct in the suicide delivery plasmid pGP704, which replicates in *E. coli* S17.1 λpir. Conjugation is performed between *Synechococcus* and *E. coli* but also in the presence of a helper bacterium *Roseobacter pomeroyi* DSS-3. The *Roseobacter* strain is used to "help" the *Synechococcus* mutants (those that have conjugated and recombined) to grow. Hence, *R. pomeroyi* needs to be resistant to Kanamycin (used to select for the *Synechococcus* mutant) and possesses SacB in order to eliminate the heterotroph once the mutant *Synechococcus* is growing nicely. This approach has been successfully used to create an axenic *Synechococcus* sp. WH7803_1017 gene mutant, a gene encoding a hypothetical protein that was highly up-regulated when *Synechococcus* interacted with a marine heterotroph. More recently we have used the same approach to knock out a type III polyketide synthase gene and shown that a novel metabolite produced in axenic, but not co-culture, is no longer produced in the Type III PKS mutant.

We have also developed an electroporation protocol for marine *Synechococcus* and have shown that cultures grown in ammonium containing artificial seawater medium depleted of phosphorus and a 5:1 ratio of *Synechococcus* to *E. coli* when transforming produce a higher frequency of mutant colonies.

DTU (14)
Several attempts have been made to genetically manipulate marine bioactive bacteria. We have tried to generate competent cells with different saline and non-saline wash buffers and different cryoprotectants but without success. With conjugation using *E. coli* as donor, we sometimes obtained a few colonies (primarily when using random mutagenesis), but the number of transformants was low.

We have attempted to manipulate the Alteromonad *Pseudoalteromonas luteoviolacea* S4054 as well as several Vibrionales species, e.g. *Photobacterium halotolerans* S2753. Below is listed some of the approaches and their outcome:

1. *Pseudoalteromonas luteoviolacea* S4054:
    a. $CaCl_2$-competent – unsuccessful.
    b. Electrocompetent with glycerol – unsuccessful.
    c. Electrocompetent with sorbitol – unsuccessful.
    d. Conjugation for random mutagenesis with miniTn10-plasmid, *E. coli* donor – successful but only few colonies were recovered.
    e. Conjugation for site-directed mutagenesis using a plasmid formerly used by other groups (Shikuma et al. 2014, Wang et al. 2015) to mutagenize other strains of *P. luteoviolacea* – not successful yet, but work continues.

2. *Photobacterium halotolerans* S2753:
    a. Electrocompetent with HEPES + glycerol – unsuccessful.
    b. Electrocompetent with HEPES + sucrose – partly successful, only very few colonies were recovered.
    c. Conjugation – partly successful, only very few colonies were recovered. We attempted to isolate mutants devoid of antivirulence compounds and mutants were screened against *S.aureus* reporter

fusions (lacZ fusions to the spa protein and hemolysin) (Nielsen et al. 2010, Mansson et al. 2011).

3. *Vibrio coralliilyticus* S:
    a. Electrocompetent with glycerol – unsuccessful.
    b. Conjugation with *E. coli* donor and a plasmid used by other groups to mutagenize *V. coralliilyticus* (de O Santos et al. 2011) – unsuccessful.

UMIL (15)

Objective of the activities was to assess the capability of UMIL-MACUMBA strains with biotechnological interest to be genetically manipulated by the insertion and expression of heterologous genes. In particular, artificial transformation in which cells are forced to incorporate exogenous DNA by physical or chemical treatments and conjugation strategies were employed. In case of artificial transformation, we decided to perform transformation experiments taking advantage of electroporation procedure.

Specifically, we focused our attention on the following strains:

- *Virgibacillus pantothenticus* 21D, isolated from the seawater-brine interface of the deep hypersaline anoxic basin Discovery (De Vitis et al., 2015);
- *Bacillus horneckiae* 15A, isolated from the seawater-brine interface of the deep hypersaline anoxic basin L'Atalante (De Vitis et al., 2015);
- *Halomonas aquamarina* 9B, isolated from the seawater-brine interface of the deep hypersaline anoxic basin Bannock (De Vitis et al., 2015);
- *Halomonas boliviensis* M2, isolated from mangrove crabs;
- *Pseudomonas plecoglossicida* PG21, isolated from mangrove crabs;
- *Halomonas axialensis* M10, isolated from mangrove crabs.

Biotechnological interest of the strains: Strains 21D, 15A, 9B were selected for their enantioselective ketoreductase/esterase activity on a key intermediate for prostaglandin synthesis; strain 21D was particularly of interest due to its activity in presence of remarkable concentrations of NaCl and $MgCl_2$ (De Vitis et al., 2015). Strains M2, PG21 and M10 were selected for their ω-transaminase (ω-TA) activity (see Fig. 1).

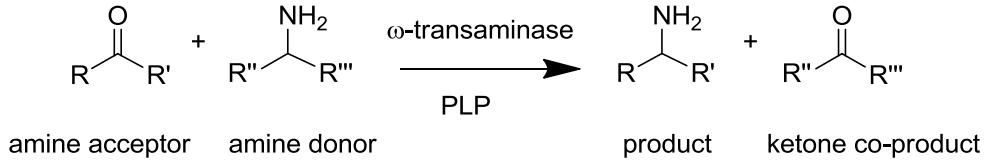

Figure 1. ω-TA-catalyzed reaction. PLP: pyridoxal-5'-phosphate.

Results: In the case of 21D and 15A, **artificial transformation** was performed by the use of plasmid pGFPratiometric (Tamagnini et al., 2008) which carries, besides the cassette encoding green fluorescent protein (Gfp), the chloramphenicol resistance, whereas in case of 9B and PG21, the plasmid pHM2-Gfp with the Gfp cassette and kanamycin resistance gene was employed (Favia et al., 2007). Unfortunately, no transformants were retrieved, except for PG21, obtaining the transformant strain PG21(pHM2-Gfp). In case of unsuccessful transformation trials, experiments were also repeated using different plasmid concentrations, competent cell densities and preparation procedures, but no successful results were obtained.

Conjugation procedure was attempted for strains 9B, M2, PG21 and M10 with the aim to insert into the bacterial chromosome a mini-Tn7 transposition system (Lambertsen et al., 2004). The procedure consists in the site-specific insertion in the recipient chromosome of a mini-Tn7 gene cassette, containing the Gfp cassette and the

antibiotic markers that were used to select the ex-conjugants (kanamycin, spectinomycin and chloramphenicol).
Briefly, a four-parental conjugation was employed using the following strains: i) the donor strain *Escherichia coli* AKN67, which contains a plasmid carrying a miniTn-7 transposon with the Gfp cassette and the antibiotic resistance genes; ii) the helper strain *E. coli* AKN68, carrying the plasmid that encodes for the transposase genes; iii) the helper strain *E. coli* #331, which contains the plasmid that can mobilise the other plasmids; iv) the recipient strain, i.e. the bacterium to be tagged. Ex-conjugants were selected on a medium added with 10% NaCl to prevent the donor growth. With this procedure, we were able to successfully label strains M10 and 9B.

Conclusion

Conjugation resulted in transformation of several bioactive marine strains, while attempts to genetically manipulate marine bacteria via artificial transformation were mostly unsuccessful (only for 1 out of several tested strains was successful).

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