Probing transfer of an IncP replicon to natural marine bacteria*

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Abstract.- An IncP plasmid probe (pUCV2), coding for Cm-r, Km-r and bearing Ap (am) and Tc (am) resistance determinants was constructed by transposition of Tn9 (Cm-r) onto plasmid pLM2 for an efficient selection of potential recipients among natural marine bacteria. Using a Dap- E. coli donor, transmission of pUCV2 to marine bacteria was tested. pUCV2 is transferred to about 4-8% of natural, marine bacterial cells capable of forming colonies on a low nutrient, marine agar medium. The following bacterial genera, commonly found in the marine environment, could be detected when twenty of the transconjugant colonies obtained were identified: Vibrio, Pseudomonas and Aeromonas.

Keywords: Horizontal transfer, IncP probe, marine bacteria.

Introduction

The carriage of plasmids by antibiotic-resistant marine bacteria was described in a pioneering study by Sizemore & Colwell (1977). However, only recently more attention has been devoted to the nature of such plasmids and their potential for horizontal transfer. In this vein, resistance plasmids have been described for bacteria in the marine air-water interface (Hermansson et al. 1987), in marine sediments (Sobeky et al. 1997, 1998) and other marine habitats (Dahlberg et al. 1997). This seems relevant to the emergence of antibiotic-resistant marine bacteria in aquafarming as its has been revealed by studies of Sandaa et al. (1992), who found transferable drug resistance among bacteria from fishfarm sediments and showed dispersal of a promiscuous plasmid from Aeromonas salmonicida to bacteria in marine sediments (Sandaa & Enger 1994).

Besides plasmids in indigenous marine bacteria, those carried by allochthonous bacteria that enter the marine environment are also considered a potential source of genetic variation for marine bacteria, in the event that heterologous transmission takes place and the allochthonous plasmids are stabilized in the marine bacterial recipients. This situation was perceived in an early study by Patt et al. (1972), who showed transfer of Escherichia coli plasmids to marine bacteria. In this regard, promiscuous plasmids in Gram negative bacteria, such as the IncP replicon RP4 (Smith & Thomas 1989) are prime candidates for such a role because they are capable of transfer to different genera of Proteobacteria (Hodgson 1989), a dominant group among marine bacteria (González & Moran 1997).

Studies pertaining transfer of IncP plasmids to marine bacteria are overall very scarce. Goodman et al. (1993) have shown transfer of RP1 between E. coli and strains of the marine Vibrio S14 under starvation conditions in artificial seawater. Furthermore, Sorensen (1993) demonstrated transfer of RP4 from E. coli to different marine bacterial isolates in filter crosses and in sterile seawater and to indigenous marine bacteria using an auxotrophic donor strain to select transconjugants on selective minimal media. In addition, Sandaa (1993) used Vibrio sp. S141, containing RP4, as a genetic donor in experiments to detect plasmid transfer and maintenance in marine sediments with marine bacterial isolates acting as genetic recipients. In this investigation,
no transfer was detected to the marine recipients, in spite of the fact that the plasmid was maintained for more than 67 days in the released host.

Within the latter context, in the study we hereby report we aimed at developing a selection based strategy for the facile investigation of the dispersal of IncP replicons from an *E. coli* donor to a wide range of natural marine bacteria, using a genetically tagged plasmid probe.

**Materials and methods**

**Bacteriological techniques**

The main bacterial strains and plasmids used in this work are listed in Table 1. Additional bacterial strains are described in Tables 2 and 3. *E. coli* strains were routinely grown at 37º C, unless otherwise indicated, in L medium (Robeson & Skarmeta, 1998). Marine bacteria were grown at 25º C in a marine medium prepared in 75% seawater that contained in g/l: Bactopeptone, 5 and Yeast extract, 1. For plates, agar was added at 1.5% (MA). Low Nutrient (LN) medium was as MA, but with Bactopeptone and Yeast Extract 100 fold less concentrated. McConkey agar base supplemented with 1% sugar was used as indicator medium.

Antibiotics were used in the following concentrations in µg/ml: Ampicillin (Ap), 50; Chloramphenicol (Cm), 20; Kanamycin (Km), 50; Nalidixic acid (Nal), 100; Tetracycline (Tc), 25 and Streptomycin (Sm), 200. Diaminopimelic acid (Dap) was used at 50 µg/ml. All media were from Difco (Detroit, Mi.) and supplements from Sigma (St. Louis, Mo.).

Characterization of marine bacterial isolates by biochemical tests and physiological features was according to Smibert & Krieg (1981) and by the API 20B identification system (Bio Mérieux, Montalieu, France).

**Genetical techniques**

Conjugative spin matings between donor and recipient strains in Eppendorf centrifuge tubes were as described (Robeson & Skarmeta, 1998). Surface matings between *E. coli* strains were performed spreading 2x10^9 cells of each donor and recipient on the surface of L agar plates, which were incubated for 6 h at 37º C prior to selection of recombinants.

Transfer of the plasmid probe pUCV2 (see below) from *E. coli* VAL1 (Table 1) to natural marine bacteria was detected by a plate transfer assay (Robeson et al., 1990) that consisted in spreading about 1x10^7 washed *E. coli* donor cells on the surface of an LN plate, which was then seeded with 50 µl of seawater to allow development of marine bacterial colonies. All potential recipient colonies could then be tested for acquisition of plasmid markers in MA supplemented with Cm and Km.

Table 1

**Bacterial strains and plasmids used in this study.**

<table>
<thead>
<tr>
<th><em>E. coli</em> Strains</th>
<th>Description</th>
<th>Plasmid and relevant markers</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>VAL 53</td>
<td>Prototroph F&lt;sup&gt;-&lt;/sup&gt; Contains transposon Tn9 (Cm-r) in Chromosome</td>
<td>Cm-r due to Tn9</td>
<td>This paper</td>
</tr>
<tr>
<td>VAL 2</td>
<td>Dap&lt;sup&gt;+&lt;/sup&gt; multiauxotroph Nal-r; sup E</td>
<td>pLM2 Ap (am) Tc (am) Km; Tra&lt;sup&gt;+&lt;/sup&gt; IncP</td>
<td>Mindich et al. (1976) and this paper</td>
</tr>
<tr>
<td>VAL 1</td>
<td>Same as VAL2 background</td>
<td>pUCV2 Ap (am), Tc (am) Km Cm Tra&lt;sup&gt;-&lt;/sup&gt; IncP</td>
<td>This paper</td>
</tr>
<tr>
<td>χ1849</td>
<td>Dap&lt;sup&gt;+&lt;/sup&gt; multiauxotroph Nal-r; sup E</td>
<td>None</td>
<td>R. Curtiss III</td>
</tr>
<tr>
<td>χ2605</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; prototroph Gal&lt;sup&gt;-&lt;/sup&gt;, Sm-r, Su&lt;sup&gt;+&lt;/sup&gt;</td>
<td>None</td>
<td>R. Curtiss III</td>
</tr>
</tbody>
</table>

For the construction of pUCV2, *E. coli* VAL2, containing the plasmid pLM2 was spin-mated with *E. coli* VAL53 that contains Tn9 in its chromosome. One of the VAL53 (pLM2) transconjugants obtained was then surface-mated with *E. coli* χ2605. The bacterial cell mixture was then replica-plated onto McConkey-galactose agar containing Sm, Cm and Km to select *E. coli* χ2605 (pLM2::Tn9 = pUCV2) exconjugants.
Results

To study transfer of IncP replicons to natural marine bacteria our first aim was to construct a suitable IncP plasmid probe that could be genetically recognizable and would allow easy selection of transconjugants. In plating non-polluted seawater samples in the presence of various antibiotics we found that the Cm-Km combination was particularly effective in eliminating all background growth of marine bacteria in MA medium. Therefore, we decided to derive an IncP probe containing Cm and Km resistance determinants. As a starting base we used plasmid pLM2, an IncP derivative that has amber mutated Ap and Tc resistance genes, which are only expressed in amber supressor (sup E) bacterial strains (Mindich et al., 1976).

We transposed Tn9 onto pLM2 and selected exconjugants of E. coli χ2605 as Gal- Sm-r colonies that became simultaneously resistant to Cm and Km. These exconjugants were Ap and Tc sensitive due the fact that E. coli χ2605 is not an amber supressor strain (Su0). We finally worked with a plasmid probe designated pUCV2, which conferred the expected phenotypes to sup E and Su0 E. coli strains; it rendered both E. coli strains χ2605 and χ1849 resistant to Cm and Km but only χ1849 expressed, in addition, Ap and Tc resistance. Furthermore, pUCV2 DNA could be detected as a single plasmid band with increased molecular mass in relation to pLM2 DNA, upon comparison of both molecules in a 0.5% agarose gel (data not shown). This difference in mass is expected due to insertion of Tn9 into pLM2.

We then tested transfer of pUCV2 from the E. coli VAL1 donor to known, typical marine bacteria. We chose this strain because of its inability to synthetize Dap, an essential cell wall component; consequently this donor can be selected against, even in a rich medium by omitting Dap. Results of these experiments are in Table 2.

In studying transfer of pUCV2 to natural marine bacteria in plate transfer assays we observed that E. coli VAL1, as expected, was unable to form colonies on LN agar plates. However, natural marine bacteria do form small colonies that could be picked and tested for acquisition of the Cm and Km resistance markers of pUCV2. When E. coli VAL1 was present in the LN plates, marine bacterial transconjugants could be recovered (Table 3). These bacteria were all Gram negative and most required NaCl for growth, both predominant features of marine prokaryotes. No Cm and Km resistant colonies were recovered from control LN plates without E. coli VAL1 cells.

### Table 2

<table>
<thead>
<tr>
<th>Recipient strains&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Transfer Frequency of transfer&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio harveyi BB7</td>
<td>+</td>
</tr>
<tr>
<td>Vibrio fischeri MJ-1</td>
<td>+</td>
</tr>
<tr>
<td>Deleya marina ATCC 25374</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas nautica DSM 50418</td>
<td>+</td>
</tr>
<tr>
<td>Alteromonas espejana BAL 31</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> The donor was E. coli VAL1 and the matings were done at 28°C. Vibrio strains were obtained from Dr. M. Silverman and A. espejiana from Dr. E. Canelo.

<sup>b</sup> Approximate values expressed as number of transconjugants divided by number of donor cells per ml. N.D. = not detected.

### Table 3

<table>
<thead>
<tr>
<th>Experiment N°</th>
<th>Mating Temperature</th>
<th>Transconjugants/ colonies tested in sample N°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>1</td>
<td>28°C</td>
<td>12/150 (8%)</td>
</tr>
<tr>
<td>2</td>
<td>20°C</td>
<td>3/80 (3.8%)</td>
</tr>
<tr>
<td>3</td>
<td>7°C</td>
<td>0/29 (0%)</td>
</tr>
</tbody>
</table>

Several Cm-Km resistant marine bacterial transconjugants, which were recovered in MA plates with Cm and Km and no Dap, to preclude growth of E. coli VAL1, were further purified and examined for plasmid content. In all of them we found plasmid DNA that migrated as pUCV2. Furthermore, they transferred Cm and Km resistance to sup E and Su0 E. coli strains and thus we could verify the Ap (am) Tc (am) phenotype conferred by pUCV2.

We should mention that we have also tested adding E. coli VAL1 cells to decomposing marine algal material and in one experiment, after incubation at...
room temperature (20-25 ºC) for 24 h, we recovered a pUCV2-containing marine bacterium which has not been further characterized.

Finally, some of the selected transconjugants obtained in plate transfer assays were picked at random and identified at the generic level. These results are shown in Table 4.

Table 4  
Bacterial genera of some transconjugants selected with pUCV2.  
Géneros bacterianos de algunos transconjugantes seleccionados con pUCV2.

<table>
<thead>
<tr>
<th>Bacterial genera</th>
<th>N° isolates identified (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter</td>
<td>2</td>
</tr>
<tr>
<td>Aeromonas</td>
<td>8</td>
</tr>
<tr>
<td>Alcaligenes</td>
<td>2</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>4</td>
</tr>
<tr>
<td>Vibrio</td>
<td>4</td>
</tr>
</tbody>
</table>

Discussion
In this work we sought to derive a transmissible plasmid probe that could serve as a model system to easily follow dispersal of IncP type plasmids to natural marine bacteria, based on the assumption that these plasmids are likely candidates for incrementing the pool of accessory DNA elements in marine prokaryotes. The probe pUCV2 seems suitable, in view of the fact that it confers resistance to both Cm and Km allowing a powerful selection of marine bacterial recipients of pUCV2. In addition, its use in a Dap- E. coli host greatly facilitates selection against the donor.

Our results, regarding transmission of pUCV2 to both laboratory and indigenous strains of marine bacteria, are compatible with the wide host range of an IncP replicon (Smith & Thomas, 1989). Furthermore, pUCV2 has proven useful as it is transferred to a significant fraction (3 to 8%) of marine bacterial colony forming units.

These observations suggest that wide host range plasmids such as those of the IncP group, could eventually be established in the gene pool of coastal marine bacteria that come in contact with allochthonous non-marine bacteria that enter the marine environment through terrestrial influxes and/or sewage discharges. This is possible since prolonged survival in seawater of non-marine bacteria like E. coli is feasible (Grimes et al. 1986, Ghoul et al. 1995); moreover, this bacterium displays ability to act as a genetic donor in marine microcosms (Sorensen, 1992, 1993; Goodman et al. 1993). Therefore, wide host range plasmids (R-factors) of “terrestrial” origin could contribute to the emergence of antibiotic resistance among marine bacteria many of which are Proteobacteria (Pinhassi et al. 1997) and thus, potential recipients of plasmids contained in allochthonous Gram negative bacteria. Evidence regarding this last point has been recently furnished by Chandrasekaran et al. (1998) who reported conjugal transfer of a multiresistance plasmid from Pseudomonas fluorescens to marine bacteria. Furthermore, plasmid flux from “terrestrial” to marine bacteria could also materialize via transformation (Williams et al. 1997) and transduction (Jiang & Paul 1998). The group Proteobacteria also contains many bacterial fish pathogens that are becoming increasingly drug resistant (Aoki, 1988) a fact that is compatible with the observations that were pointed out above. Consequently, it is by no means redundant to caution about the uncontrolled use of antibiotics to curtail bacterial infections in aquafarming.

Regarding the properties of the E. coli (Dap-)/pUCV2 probe delivery system, it has some advantages. In the first place, it allows selection of natural bacterial transconjugants in rich medium, a situation that favors the recovery of a wide range of culturable bacteria. This seems important insofar colony-forming bacteria, especially Proteobacteria, are dominant in marine bacterioplankton (Pinhassi et al. 1997). Secondly, the Dap- host could also be used to deliver other tagged plasmids to the non-culturable fraction of marine bacteria in a manner analogous to that described by Dahlberg et al. (1998) who studied horizontal transfer of a green fluorescent protein (GFP)-tagged plasmid in marine bacterial communities. Thirdly, pUCV2 also allows genetic detection which is less costly and easier to perform than molecular genetic methodology. This could prove to be an advantage in many laboratories. Finally, some concern springs from the fact that a severely debilitated strain such as E. coli VAL1 is still proficient as a genetic donor, even in adverse incubation conditions. This reinforces the idea that that laboratory bacterial strains containing natural plasmids or recombinant DNA material should be carefully handled. In summary, we have developed an IncP plasmid probe, contained in a suitable E. coli strain, that allows an efficient tracing of the fate of IncP replicons among bacteria in nature.

Acknowledgements
We thank the Universidad Católica de Valparaíso and FONDECYT for financial support and Ricardo Pefaur for critical review of the manuscript.
Literature Cited


