

## Natural transformation in aquatic environments

JOHN H. PAUL and HAYDN G. WILLIAMS

*Department of Marine Science, University of South Florida, St Petersburg Campus, 140  
Seventh Avenue South, St. Petersburg, FL 33701, USA*

### Introduction

Transformation is a process in which competent cells take up DNA and incorporate it into their genome. It is one of the mechanisms by which genes may be spread through natural populations [27]. A wide variety of bacteria express competence (the physiological ability to take up DNA) during normal growth. Frischer et al. [9] found up to 16% of isolates taken from Tampa Bay were naturally competent. When competent cells come into contact with a source of DNA, this is bound to the cell and taken up. As well as DNA in free solution, competent bacteria can take up DNA associated with particulate matter [15,16,29], cellular debris [14], heat inactivated cells [23,30] and intact, live donor cells [1,23,24,28].

Transformation is generally most efficient with homologous chromosomal DNA. Once inside the cell it can be integrated into the host's chromosome by normal recombination processes [5,7]. However, in many cases almost any fragment of DNA may be taken up. Plasmid DNA without homology to the host may be recircularized by mismatch repair if multiple copies are taken up [6,10]. This would allow transformation between organisms of different genera. For example, Paul et al. [23] demonstrated transfer of a small non-conjugative plasmid from *E. coli* to a marine *Vibrio* by natural transformation.

Natural transformation may play an important role in the flow of genes through natural populations. One of the most commonly used approaches to study this type of gene transfer in nature has been to examine the behavior of model systems. Such systems often involve the transfer of an individual gene or group of linked genes to a predetermined recipient in a simple microcosm. For example Graham and Istock [8] examined transformation of *Bacillus subtilis* in soil microcosms, Stewart et al. [29] studied transfer of rifampicin resistance between strains of *Pseudomonas stutzeri* in sediment microcosms and Paul et al. [22] demonstrated the uptake of plasmid DNA by a marine *Vibrio* strain in marine water and sediment microcosms. Experiments usually compare the response of the system to various environmental conditions with idealized laboratory conditions. A variation on this approach is to perform open experiments in the field (*in situ*). Bale et al. [2,3] developed a method

to follow transfer of a conjugative plasmid in river epilithon. The technique was then adapted to study natural transformation [30].

### **Chromosomal transformation using *Acinetobacter calcoaceticus* in river epilithon**

#### *Experimental approach*

This article describes a method to demonstrate the transfer of the His<sup>+</sup> gene from *Acinetobacter calcoaceticus* BD413 [14] to a histidine auxotroph HGW1521(pQM17) [30] by natural transformation in both laboratory and open field experiments. The low transfer frequencies and/or target populations that often occur in these *in situ* experiments can result in very low numbers of transformants (<10 cells/ml) that are difficult to detect. Therefore it is advisable to perform a series of assays starting with simple laboratory experiments and progressing in complexity to *in situ* experiments with recipients growing as part of the natural population. Competent bacteria and a source of transforming DNA are inserted into an aquatic environment with minimal disturbance. After a period to allow gene transfer to take place the bacteria are extracted and enumerated on various selective media so that the transformation frequency (number of transformants per recipient) can be estimated. Whilst the method has been developed using *Acinetobacter* as a model organism, it may be possible to modify it for use with other naturally competent bacteria, genes or environments.

### **Procedures**

#### *Bacterial strains*

*A. calcoaceticus* strain BD413 is a prototrophic (His<sup>+</sup>) soil isolate expressing very high levels of competence [14]. *A. calcoaceticus* strain HGW1521(pQM17) is a histidine auxotroph (His<sup>−</sup>) derived from BD413 [30]. It is spontaneously resistant to 100 µg/ml rifampicin and 100 µg/ml spectinomycin and carries the plasmid pQM17 which encodes resistance to 27 µg/ml mercury [24].

#### *Source of transforming DNA*

Transforming DNA may be presented in a variety of forms such as cultures containing live bacteria, suspensions of heat inactivated cells, crude bacterial lysates, purified chromosomal DNA, purified plasmid

DNA or plasmid multimers. The type of transforming DNA used can affect the characteristics and frequency of transformation under various conditions. For example, purified DNA preparations generally give higher transformation frequencies whereas live donor cells may allow transformation to occur in the presence of nucleases [1].

#### *Whole cell preparations*

1. The donor organism (BD413) is grown overnight in 50 ml of Luria broth (LB).
2. Harvest the culture by centrifugation ( $10,000 \times g$ , 10 minutes) and wash twice in B22 salts solution. Resuspend the pellet in 50 ml of B22 salts solution.
3. The washed cell suspension can be used directly as a source of transforming DNA.

#### *Heat inactivated cell suspensions*

Pasteurize 1 ml of washed cell suspension in a sterile microfuge tube by heating to 72 °C for 2 hours. Heat inactivated cells may be stored at 5 °C until needed.

#### *Crude lysates*

Spin down 1 ml of washed cell suspension in a microfuge tube and resuspend the pellet in 1 ml of Juni lysis buffer. Pasteurize the suspension by heating to 72 °C for 2 hours and store at 5 °C until needed.

#### *Notes*

- Ad 1. Defined minimal media and/or antibiotic additions may be used instead of LB to maintain selective pressure for certain phenotypes.
- Ad 3. Whole cell preparations should be used immediately and cannot be stored.

#### *Solutions*

- B22 salts solution (pH 7.2) [2]
  - 3.89 g/l  $\text{KH}_2\text{PO}_4$ .
  - 12.5 g/l  $\text{K}_2\text{HPO}_4$ .
  - 0.19 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .
  - 1.09 g/l  $(\text{NH}_4)_2\text{SO}_4$ .

- Succinate-B22 minimal medium (pH 7.2) [2]
  - B22 salts solution.
  - 10 g/l sodium succinate.
  - If solid medium is required add 15 g/l agar.
- Juni lysis buffer (pH 7.0) [14]
  - 0.05% (w/v) SDS.
  - 0.15 M NaCl.
  - 0.015 M sodium citrate.
- LB(pH 7.2)
  - 10 g/l Tryptone.
  - 5 g/l yeast extract.
  - 10 g/l NaCl.

#### *Competent cells*

In the case of *A. calcoaceticus*, a 16 hours culture grown in LB should contain a large number of highly competent cells suitable for transformation assays. However, if the method is to be used with other organisms it is advisable to first determine the competence phase of that strain.

#### *Steps in the procedure*

1. Prepare an overnight culture of the recipient (e.g., HGW1521 (pQM17)) in 50 ml of LB.
2. Inoculate 500 ml of fresh LB in a 2 l flask to an optical density at 540 nm (A<sub>540</sub>) of 0.1 with the overnight culture and incubate with shaking at 20 °C.
3. Follow the optical density (A<sub>540</sub>). Remove enough culture to yield approximately 10<sup>7</sup> cells.
4. Filter the sample on to a 24 mm diam., 0.22 µm pore size nitrocellulose filter. Filter the source of DNA (e.g., 1 ml of a crude lysate of BD413) on to a 24 mm diam., 0.22 µm pore size nitrocellulose filter. Place the two filters together so that the DNA and cells are in contact.
5. Place the filters on an agar plate (e.g., PCA) and incubate at 20 °C for 15 minutes.
6. After the incubation, retrieve the filters and resuspend cells in 4 ml of B22 salts solution containing DNaseI (50 µg/ml) by vortexing for about 30 seconds. Prepare a decimal dilution series ranging from

1 to  $10^{-7}$  of the suspension in B22 salts solution containing DNaseI (50  $\mu\text{g/ml}$ ) and enumerate bacteria by plate counts on both media selective for recipients (PCA) and transformants (SE22). Incubate plates at 20 °C.

7. Repeat steps 3–6 at 1 hour intervals until the culture is in late stationary phase. Adjust the volume of culture filtered each time according to the optical density to give approximately the same number of recipients.
8. For each mating calculate the transformation frequency as the number of transformants (c.f.u./ml on SE22)/the number of recipients (c.f.u./ml on PCA). Determine the optical density and growth phase at which the highest transformation frequencies were achieved. Use cells grown to this point of their growth phase as a source of competent cells in future experiments.
9. Preparing competent cells: Take 50 ml of culture expressing maximum competence as determined above. Harvest the culture by centrifugation ( $10,000 \times g$ , 10 minutes) and wash twice in B22 salts solution. Resuspend the pellet in 50 ml of B22 salts solution.

#### *Notes*

- Ad 2. If more sample is needed for the experiment, replicate 500 ml cultures can be used.
- Ad 3. One ml of culture at an optical density of 1 should yield approximately  $10^7$  cells.
- Ad 5. If no transformants are formed within 15 minutes at any growth phase, try longer incubation periods (e.g., 1 hour).
- Ad 6. DNaseI should prevent any further transformation.
- Ad 9. Competent cells should not be frozen and stored but freshly prepared for each experiment.

#### *Solutions*

- DNaseI stock solution
  - 50 mg/ml DNaseI, filter sterilize.
- PCA (standard plate count agar) (pH 7)
  - 2.5 g/l yeast extract.
  - 5 g/l pancreatic digest of casein.
  - 1 g/l glucose.
  - 10 g/l agar.
- Rifampicin (Rif) stock solution
  - 40 mg/ml Rifampicin.
  - 0.2 M NaOH.

- PCA + Rif(pH 7)
  - PCA.
  - 100 µg/ml rifampicin.
  - pH 7.
- SE22 (pH 7.2)
  - B22 salts solution.
  - 10 g/l sodium succinate.
  - 0.3 g/l EDTA.
  - 15 g/l agar.
  - 100 µg/ml rifampicin.
  - 27 µg/ml mercury.
  - 0.05% spent culture of BD413 grown in succinate B22 medium, then filter sterilized to remove cells.

*Laboratory filter mating assay*

1. Filter  $10^7$ – $10^8$  competent cells (approximately 1 ml of an overnight culture) onto a 24 mm diam., 0.22 µm pore size nitrocellulose filter. Filter the source of DNA (equivalent to 1 ml of an overnight culture of donor) on to a 24 mm diam., 0.22 µm pore size nitrocellulose filter. Place the two filters together so that the DNA and cells are in contact.
2. Place the filters on an agar plate (e.g., PCA) and incubate at 20 °C for 24 hours.
3. After the incubation, retrieve the filters and resuspend cells in 4 ml of B22 salts solution containing DNaseI (50 µg/ml) by vortexing for about 30 seconds. Prepare a decimal dilution series ranging from 1 to  $10^{-7}$  of the suspension in B22 salts solution containing DNaseI (50 µg/ml) and enumerate bacteria by plate counts on selective media. Select recipient cells, HGW1521(pQM17) on PCA + Rif. If whole cell preparations of BD413 were used as donor, select donors on succinate-B22 minimal medium. Select prototrophic transformants of HGW1521(pQM17) on SE22.
4. Incubate the plates at 20 °C until individual colonies can be counted (typically about 2 days).

*Notes*

- Ad 3. If the culture forms a sticky mat of cells over the filter that is difficult to completely resuspend add 2 to 3 sterile glass beads (1–3 mm diam.) to the suspension prior to vortexing as this helps break up the biofilm.

- Ad 3. The DNaseI prevents further gene transfer from occurring whilst cells are plated out.
- Ad 3. Cell-to-cell transformation can occur on some selective media when live donors are used as a source of transforming DNA, resulting in overestimation of the transfer frequency [30]. The EDTA in SE22 prevents this.
- Ad 3. If low numbers of transformants are anticipated, filter 3 ml of the resuspended cell solution through a 0.25  $\mu\text{m}$ , 45 mm diam. nitrocellulose filter and place the filter cell side up on a SE22 plate.

#### *Transformation in beaker microcosms*

1. Collect 500 ml of river water in a sterile bottle and several stones from the river bed. Choose stones (approximately  $10 \times 15 \times 2$  cm) with at least one smooth flat surface.
2. Scrub the stones with a stiff bristled brush to remove native epilithon and rinse in distilled water. Wrap scrubbed stone in foil and sterilize (121 °C, 20 minutes).
3. Prepare donor and recipient filters as for laboratory filter matings (step 1).
4. Place the filters on the surface of the sterile scrubbed stone. Cover them with a larger sterile paper filter (Whatman No.1) and secure with elastic bands. Put the stone in a sterile 1 l beaker containing 400 ml of freshly collected river water and incubate at 20 °C for 24 hours.
5. After the incubation retrieve the filters and treat as for laboratory filter matings (steps 3–4).

#### *In situ transformation assay*

1. Transport cultures of competent cells and source of DNA to the river bank.
2. Prepare donor and recipient filters as for laboratory filter matings (step 1).
3. Place the filters on the surface of a sterile scrubbed stone. Cover them with a larger paper filter and secure with elastic bands. Put the stone in a large mesh size net bag which is then placed mid-stream on the river bed secured to a metal stake.
4. Attach a minimum/maximum thermometer to the stake to monitor the temperature of the river. Allow matings to proceed for 24 hours.
5. After the incubation use a sterile plastic bag to retrieve the stone. Remove the filters and treat them as for laboratory filter matings (steps 3–4).

#### *Notes*

- Ad 1. Pre-prepared competent cells can be transported to the river on ice. However, do not transport or store competent cells for long periods on ice as this will result in a loss of competence. An alternative method is to grow and prepare competent cells at the river site if laboratory facilities are available locally.
- Ad 3. To prevent contamination through handling, wear rubber gloves and wash them first in 70% ethanol and then in the river. Sterilize net bags and elastic bands by immersing in 70% ethanol and then wash thoroughly in the river.
- Ad 3. Additional stones collected locally and placed in the bag can help prevent the bags being moved by the current.
- Ad 5. Handle only the outside surfaces of the bag, use it as a sterile glove with which to pick up the stones. It is best to plate out cells as soon as they are removed from the river. If this is not possible transfer the suspension on ice to the laboratory.

#### *Transformation of bacteria incorporated into the epilithon*

Recipient and donor cultures are incorporated into growing epilithon (*in situ*) on separate stones, then placed together to allow gene transfer to occur. Transformants are directly enumerated from the biofilm.

#### *Steps in the procedure*

1. Collect smooth stones approximately  $10 \times 15 \times 2$  cm with at least one flat surface from the river bed and place aside on a sterile surface.
2. Prepare donor and recipient filters as for laboratory filter matings (step 1) but do not place filters together.
3. Place each filter, face down on a separate stone. Cover with a larger paper filter, secure with elastic bands and put each stone in a large mesh size net bag.
4. Secure bags to metal stakes in separate parts of the river, ensuring donors and recipients can not come into contact with each other. Incubate the stones in the river for 24 hours to allow development of a biofilm.
5. Retrieve the stones. Mark the area on the stone where each filter is with a diamond marker and remove the filters. Gently wash the stones by immersing in river water. Place the two stones together so that the area exposed to the source of DNA and the area exposed to the recipient are in contact. Hold the stones together with elastic bands.
6. Return the stone to the river and incubate for a further 24 hours.



7. Collect the stones in a sterile plastic bag. Separate the stones, place a rubber ring around the area of the stone harboring the recipient to form a well and add 3 ml of B22 salts solution containing DNaseI (50 µg/ml). Scrub the area within the well using a short, stiff bristled stencil brush for at least 3 minutes. Try to resuspend as much of the epilithon as possible. Transfer the suspension to a sterile bottle and vortex for 20 seconds.
8. Treat suspension as for laboratory matings. Calculate the transfer frequency as for laboratory filter matings.

#### *Notes*

- Ad 1. Both donor and recipient stones should have flat surfaces that can be placed together with as much contact as possible. If no suitable stones are available locally slate disks [25] can be used.
- Ad 7. Sterilize the brush and rubber ring by washing first in 70% ethanol, then in sterile B22 salts solution. If the suspension leaks away from the well, use a pipette to transfer liquid back into the well.

#### *Confirming putative transformants*

Colonies growing on the plates selective for transformants (SE22) must be shown to be transformants and not spontaneous mutants or indigenous organisms.

1. Test for known unselected characteristics. Pick 10–50 well isolated putative transformant colonies with a sterile toothpick and streak onto PCA + spectinomycin (100 µg/ml). Discard any isolates that are not resistant to spectinomycin.
2. Juni [14] suggested transformation assays could be used as taxonomic tests for certain organisms. True transformants should be able to re-transform other recipients. Prepare a crude lysate of the putative transformants as described above. Spread 100 µl of lysate over the surface of a succinate-B22-minimal medium plate and allow to dry. Spread 100 µl of a washed suspension of recipient (HGW1521(pQM17)) over the plate. To confirm that the lysate solution was sterile, spread 100 µl on to a PCA plate and to confirm the recipient was auxotrophic spread 100 µl of washed cell suspension on to a succinate-B22 minimal medium plate. Incubate the plates at 20 °C for 24 hours. If the putative transformant was an *Acinetobacter* it should have transformed the recipient to prototrophy,

forming a confluent lawn on the plate. The cells only and lysate only plates should be clear of growth.

3. If molecular probes are available to either the recipient or the gene transferred, it should be confirmed that the probe will hybridize to putative transformants.

#### *Control experiments*

1. To confirm transformants were produced by gene transfer and not spontaneous mutation repeat transformation assays substituting distilled water for the source of transforming DNA. For transformation to be considered to have occurred the transformation frequency must be significantly higher than the frequency obtained when no source of transforming DNA is added.
2. To confirm transformation only occurred during the incubation phase (e.g., in the river) repeat the assay omitting the incubation period, i.e., as soon as the source of DNA and recipients are placed in contact, immediately remove, resuspend and plate out them out.

#### *Calculating transfer frequencies*

The transformation frequency is expressed as transformants per recipient. Each experiment should be repeated in triplicate, using a separate overnight culture of both donor and recipient for each replicate. For each mating, calculate the c.f.u./ml of donors, recipients and transformants in the final suspension from the plate counts;

$$\text{Transformation frequency} = \frac{\text{number of transformants (c.f.u./ml on SE22)}}{\text{number of recipients (c.f.u./ml on PCA + Rif)}}$$

The overall transformation frequency is then calculated as the mean transformation frequency obtained from the replicate experiments. Mean transformation values can be compared by student t-tests or analysis of variance [11,26].

### **Plasmid transformation using marine high frequency of transformation recipients**

#### *Experimental approach*

Natural plasmid transformation is a less well-studied subelement of the field of natural transformation. Chromosomal transformation

involves transfer of genes between closely related species, and usually requires homologous recombination [27]. Plasmid transfer also requires some type of homology for recircularization of the plasmid, which is believed to enter the cell as a linear and possibly single-stranded molecule [4]. To ensure self-homology, we use plasmid multimers made in vitro as transforming DNA. The procedure described below involves the Inc Q/P4 plasmid pQSR50 [19], which is a Tn5 containing derivative of R1162 [18]. The plasmid encodes kanamycin and streptomycin resistance. The recipient used is the High Frequency of Transformation (HfT) *Vibrio* strain WJT-1C [9], but other HfT *Vibrio* strains such as MF1-C, MF4-C, and JT-1 can also be used. The strains are grown into stationary phase, and the DNA added either in seawater or marine sediment. The transformants are recovered by plating on selective media, and verified by molecular probing with a probe made to the neomycin phospho-transferase gene (*nptII*) of Tn5 [9].

### *Procedures*

#### *Strains*

*Vibrio* WJT-1C [9] grows on ASWJP+PY medium [20] at 28 °C, and can be maintained on plates stored at 4 °C for several months or stored in 50% glycerol/ASWJP+PY at –80 °C. *E.coli* RM1259 (pQSR50) was the source of the plasmid, and was grown on LB supplemented with 50 µg/ml kanamycin and 25 µg/ml streptomycin.

#### *Transforming DNA*

Large scale plasmid preparations were prepared from 4 × 500 ml cultures of *E. coli* RM1259 as previously described [9]. Plasmid multimers were produced by the protocol described below, which involves digestion at a unique restriction site followed by ligation under conditions which favor concatemerization. In our experience, the protocol cannot be successfully scaled up. The plasmid is digested in individual 10 µg quantities and then ligated. Typically we perform 10 such individual reactions at once.

#### *Steps in the procedure*

1. Digest pQSR50 by adding the following to a sterile 0.5 ml microfuge tube:

- A) 10 µg plasmid.
  - B) 4.5 µl 1% molecular biology grade bovine serum albumin.
  - C) 4.5 µl 10 × EcoR1 reaction buffer.
  - D) 5 µl EcoR1 (~15 U/µl) (as much as 8 µl may be used).
  - E) Sterile distilled water to bring total volume to 45 µl.
2. Vortex well. Digest at 37 °C for 3.5 hours. Remove 3 µl to assess digestion by 1% agarose gel electrophoresis, leave remainder at 37 °C until gel has finished running.
  3. If digestion is complete, set aside another 3 µl for the second gel.
  4. Denature the EcoR1 by heating at 70 °C for 20 minutes. Cool to 15 °C.
  5. Set up the ligation as follows:
    - A) 39 µl EcoR1 digested plasmid.
    - B) 20 µl sterile distilled water.
    - C) 15 µl T4 DNA Ligase Buffer, 5×.
    - D) 16 µl ligase.
  6. Incubate overnight at 15–16 °C.
  7. Assess degree of multimerization by running on a 0.4% agarose gel, being sure to run high molecular weight standards, and the digested but not ligated pQSR50.
  8. Add 5 µl sterile 0.5 M EDTA. Pasteurize multimers before use by heating at 70 °C for 2 hours.

#### *Preparation of recipient cells*

1. A culture of *Vibrio* WJT-1C (25 ml) is grown overnight at 28–30 °C on a gyrotatory shaking platform (150–200 r.p.m.).
2. Cells are harvested by centrifugation at 10,000 × g for 10 minutes at 20 °C.
3. The cells are resuspended in 20 ml of media lacking peptone and yeast extract (ASWJP) and used immediately.

#### *Water column transformation assay*

1. Collect seawater from the environment to be examined.
2. Add 0.5 ml (oceanic microcosm) or 1.0 ml (estuarine microcosm) of the competent cell suspension to 24.5 ml or 24.0 ml, respectively, of the seawater to be investigated in a sterile disposable 60 ml centrifuge tube.

3. If desired, add nutrients. These can be added as a solution of sterile filtered peptone and yeast extract, at a range of concentrations from 0.1 to 5 mg peptone/ml, and 0.02 to 1 mg yeast extract/ml.
4. Add 5 µg of transforming DNA (pQSR50 multimers as prepared above) or pasteurized calf thymus DNA (controls).
5. The mixture is incubated for the desired length of time on a gyro-tatory shaker set at 3–5 r.p.m. and at 25–30 °C.
6. The incubations are harvested by centrifugation when obvious growth has occurred (i.e., estuarine microcosms in the presence of nutrients) or by filtration onto sterile 47 mm 0.2 µm Nuclepore filters.
7. Cells on the filters are resuspended by placing the filters into 5 ml ASWJP in a 15 ml conical centrifuge tube and vortexing vigorously for 1 to 2 minutes. Cell pellets from centrifugation are resuspended in 5.0 ml ASWJP with vortexing.
8. Aliquots of this suspension are diluted and plated on ASWJP+PY for enumeration of total CPU, and on ASWJP+PY plus 500 µg/ml kanamycin, 1 mg/ml streptomycin, and  $5 \times 10^{-6}$  M amphotericin B to enumerate transformants.
9. Plates are incubated 24–48 hours to detect growth of transformants. The unique colony morphology of the HfT strains usually enables enumeration in the presence of the indigenous marine flora.
10. Presumptive transformants are verified by colony hybridization. Sterile MSI Magnagraph Nylon 66 filters (85 mm diameter) are used to lift colonies. The filters are then placed colony side up and grown on ASWJP+PY plus kanamycin and streptomycin for 48 hours at 28 °C.
11. Colonies on filters are lysed and the DNA immobilized on the filters [23] and probed with the *nptII* gene probe [9].

#### Notes

- Ad 1. Untreated seawater, filter sterilized seawater, or autoclaved, sterile filtered seawater may be used, depending upon the needs of the study. Autoclaved seawater should always be filtered to remove precipitates which will bind DNA.
- Ad 3. The use of nutrients results in higher transfer frequencies in most instances.
- Ad 5. The typical incubation time for a transformation assay is overnight (16 hours). Shorter times may be used (one to several hours). However, if very low frequencies are expected (as for experiments with the natural population present) addition of nutrients may enable detection of transfer.

### *Solutions*

#### ASWJP (Recipe for one liter)

1. To 900 ml distilled or deionized water add 22.05 g NaCl, 9.84 g MgSO<sub>4</sub>.
2. Add the following stock solutions;

Stock solution	Component(s)	Concentration (g/l)	Volume (ml)
#1	KCl	55.0	10.0
	NaHCO <sub>3</sub>	16.0	
#2	KBr	8.0	10.0
	SrCl <sub>2</sub>	3.4	
#3	Sodium silicate	4.0	1.0
#4	NH <sub>4</sub> NO <sub>3</sub>	1.6	1.0
#5	NaF	2.4	1.0
#6	Na <sub>2</sub> •HPO <sub>4</sub>	8.0	1.0
#7	CaCl <sub>2</sub> H <sub>2</sub> O	238.0	10.0
#44	Na <sub>2</sub> EDTA	3.0	10.0
	FeCl <sub>3</sub> •6H <sub>2</sub> O	0.384	
	MnCl <sub>2</sub> •H <sub>2</sub> O	0.432	
	CoCl <sub>2</sub> •6H <sub>2</sub> O	0.002	
	ZnCl <sub>2</sub>	0.0315	
	CuCl <sub>2</sub>	0.025	
	H <sub>3</sub> BO <sub>3</sub>	0.342	

All of the stock solutions are made in deionized water and stored in polypropylene bottles at 4 °C. Stock solution #44 is a trace metal solution made by dissolving all components separately. It should be a clear, light yellow solution. Upon refrigeration, precipitates form with time which do not affect the performance of the medium.

3. Bring volume to 1 liter with distilled water.
4. For ASWJP+PY, add 5 g peptone, 1 g yeast extract.
5. For agar, add 15 g agar.
6. Sterilize by autoclaving.

### *Sediment transformation assay*

Sediment transformation assays have been previously performed in flow-through columns [12,15,16] using 5 ml (or equivalent) syringes. Sands used in such columns have usually been autoclaved, washed, and “precharged” with transforming DNA. We have performed side-by-side comparisons of such columns with the much simpler, “plug” method described below and found comparable results.

#### *Steps in the procedure*

1. Add 3 cm<sup>3</sup> of sediment to a sterile 15 ml conical centrifuge tube.
2. Prepare HfT recipients as above ("preparation of recipient cells") except that 30 ml culture is required per sediment plug rather than 25 ml and that this volume of cells is resuspended in 100 µl ASWJP after the final wash. For example, if six sediment plugs are to be used, 180 ml of culture is required, resuspended in 600 µl ASWJP.
3. To each plug, 100 µl of the above recipient cell suspension and 15 µg DNA (plasmid multimers or control DNA) is added.
4. If nutrients are to be added, a concentrated stock is made (50 mg/ml peptone and 10 mg/ml yeast extract) and 100 µl is added.
5. The sediment mixture is stirred with a sterile pipette.
6. Plugs are incubated overnight (or the desired length of time in a time course study) at 28 °C (or the desired temperature if temperature is a factor being investigated).
7. Sediment is resuspended in 5 ml ASWJP with vigorous vortexing for 2 minutes.
8. Aliquots of the mixture are serially diluted and plated on ASWJP+PY for total CFU and on ASWJP+PY containing 500 µg/ml kanamycin, 1 mg/ml streptomycin, and  $5 \times 10^{-6}$  M amphotericin B for putative transformants.
9. Transformants are enumerated as above and verified by colony hybridization.

#### *Note*

- The sediment to be used can be autoclaved or non-sterile, depending on the purpose of the study. We have only observed transfer in autoclaved sediments. If non-sterile sediments are to be used, there will be a high level of resistance to kanamycin and streptomycin amongst the indigenous flora. Hopefully, colony hybridization would identify transformants.

#### *Plasmid transfer to the indigenous flora by natural transformation*

The transfer of plasmid DNA to the indigenous marine flora presents several challenges not encountered when using cultivated recipients. Natural plasmid transformation is an infrequently occurring process even in defined cultures where the physiology of competence development is understood. The proportion of the population which is naturally competent is unknown for natural samples, as well as the proportion in which an IncQ/P4 plasmid can replicate. Because natural

habitats rarely have more than  $10^6$  cells/ml, it is necessary to concentrate the microbial populations from water column samples. A second approach we have successfully used is to investigate environments where the microbial population has been concentrated by marine invertebrates, such as the tissues of filter feeders such as sponges, the guts of deposit-feeders such as holothurians (sea cucumbers), and the mucus of filter feeders such as scleratinian corals. Both approaches have yielded transfer to the indigenous flora in certain cases.

*Concentration of microbial populations from water column samples*

Microbial populations from 20 to 100 liters of seawater are concentrated using a Membrex Benchmark Rotary Biofiltration device fitted with a 400 cm<sup>2</sup> 100 kd filter set up in the recirculation configuration [13]. The concentrated cell suspension (termed the retentate) is typically 35 to 50 ml, with an efficiency of recovery of ~ 80%.

*Concentration of microbial populations from sponge tissues*

1. Sponge tissue samples (10 g wet wt.) are cut into 2 cm<sup>3</sup> pieces with a sterile scalpel.
2. The sponge tissue pieces are homogenized in 50 ml sterile ASWJP in a model 909-1 Beadbeater (230 ml capacity; Biospecs Products, Bartlesville, OK) in an ice bath jacket for 5 minutes.
3. Sponge tissue pieces are removed by centrifugation at 800 × g for 1 minute.

*Concentration of microbial populations from coral mucus*

1. Coral mucus is collected by SCUBA divers or snorkelers using 60 ml syringes with no needles. The mucus/seawater mixture is collected by drawing slowly on the plunger while moving the syringe orifice across the coral surface.
2. One liter of mucus is further concentrated to 35–50 ml by vortex flow filtration using a Benchmark Rotary Biofiltration device [13].

*Concentration of microbial populations from holothurian guts*

1. Holothurians (sea cucumbers) can be collected from shallow subtropical bays such as Florida Bay (check permit requirements with federal, state, and local authorities before collecting!).



2. Using a sterile scalpel, dissect cucumber longitudinally and remove gut intact. The gut contents (primarily sediments) are exuded into a sterile 60 ml centrifuge tube.
3. Extract 20 cm<sup>3</sup> of gut content material with an equal volume of ASWJP by vortexing vigorously.

*Transformation assays using indigenous marine bacteria as recipients*

1. Prepare plasmid multimers (pQSR50) as above.
2. Filter 5 ml of bacterial suspension (Membrex retentate, sponge extract, or gut content extract) through a sterile 47 mm 0.2 µm Nucleopore filter.
3. Place filter, cell spot side up, on an ASWJP+PY agar plate.
4. Add 4 µg plasmid multimers in 100 µl of 4.2 mM MgCl<sub>2</sub> by carefully spreading the DNA over the cell spot.
5. It is critical to have a control treatment consisting of five ml bacterial suspension filtered similarly and overlaid with calf thymus DNA.
6. Incubate the desired length of time (usually 16 to 20 hours) at 25–30 °C.
7. Resuspend cells by placing filter in 10 ml ASWJP+PY in a 125 ml sterile Erlenmeyer flask and shaking on a gyrotatory shaker at 150 r.p.m. for 1 hour at room temperature.
8. Serially dilute and plate on non-selective media to enumerate total CFU and on ASWJP+PY containing 500 µg/ml kanamycin, 1 mg/ml streptomycin, and  $5 \times 10^{-6}$  M amphotericin B to enumerate potential transformants.
9. Perform colony hybridization using the *nptII* probe. Pick hybridizing colonies, grow on selective media, and extract plasmid via a miniprep to verify plasmid acquisition [17].

*Notes*

There is usually a background level of resistance to kanamycin and streptomycin in most marine microbial communities. Therefore, it is imperative to verify plasmid acquisition, both by colony hybridization of controls and treatment plates and also by selecting individual hybridizing colonies for further study. Miniprep, restriction analysis, and Southern hybridization of these clones may still yield equivocal results. Restriction profiles of pQSR50 are often changed, either from marine bacterial methylation systems or by plasmid rearrangement [10].

**Table 1.** Plasmids and bacterial strains used in intergeneric, contact-dependent natural plasmid transformation

Strain or plasmid	Relevant characteristics	Source
<b>Plasmids</b>		
r1162	su <sup>r</sup> str <sup>r</sup> mob <sup>+</sup>	[18]
PQSR50	r1162::Tn5 km <sup>r</sup> str <sup>r</sup>	[19]
pLV1013	km <sup>r</sup> str <sup>r</sup> xylE c1857	[31]
<b><i>E. coli</i> donor strains</b>		
RM1259(pQSR50)	MV10 K12 C600 km <sup>r</sup> str <sup>r</sup>	[19]
RM1208(r1162)	MV10 K12 C600 str <sup>r</sup>	[19]
ED8564(pLV1013)	lac <sup>-</sup> met <sup>-</sup> thi <sup>-</sup> hsdR <sub>m</sub> <sup>-</sup> -r <sub>k</sub> <sup>-</sup> km <sup>r</sup> str <sup>r</sup> xylE	[31]
<b>Recipient strain</b>		
<i>Vibrio</i> JT-1	nal <sup>r</sup> rif <sup>r</sup>	[23]

### *Intergeneric plasmid transformation using E. coli donor cells and HfT recipients*

Most workers in molecular biology are familiar with artificial transformation of plasmid DNA using *E. coli* cells rendered competent by chemical or physical methods. Such cells are not naturally competent. We have demonstrated transfer of non-conjugative plasmids between *E. coli* donor cells and HfT *Vibrio* recipients by a contact dependant, DNase sensitive process [23]. We feel that such a process may be the major mechanism of transformation in marine and aquatic environments, because of the lability of dissolved ("free") DNA [21].

### *Strains and plasmids*

Table 1 shows the plasmids and *E. coli* strains used as plasmid donors. All *E. coli* strains are grown in LB medium [17] supplemented with the various antibiotics (usually kanamycin and streptomycin at 50 and 25 µg/ml, respectively). *Vibrio* JT-1 is a double antibiotic resistant chromosomal mutant of *Vibrio* WJT-1C and serves as plasmid recipient. It is grown on ASWJP+PY media at 28–30 °C in the presence of 500 µg/ml nalidixic acid and 150 µg/ml rifampicin.

### *Preparation of donor and recipient cells*

1. *E. coli* donor and recipient cells are grown under conditions described above overnight in gyrotatory shaking incubators (~150–200 r.p.m.).

2. Cells are harvested at  $10,000 \times g$  for 10 minutes at 20 °C and washed twice in growth media lacking antibiotics (ASWJP+PY for JT-1 and LB for *E. coli* strains).
3. Cells are resuspended in growth media lacking antibiotics and used immediately in transformation assays.

#### *Protocol for intergeneric transformation*

##### *Broth matings*

1. *E. coli* donor cells (1 ml) are mixed with 1 ml JT-1 recipient in a sterile 15 ml conical centrifuge tube.
2. A DNase control is set up identically, with the addition of 200 units of DNaseI.
3. The tubes are incubated at 30 °C statically for the desired length of time (up to 16 hours).
4. Aliquots of each DNase control and treatment tube are serially diluted and plated on the appropriate media. For example, for crosses involving *E. coli* RM1259 as donor and *Vibrio* JT-1 as recipient, aliquots are plated on LB plus kanamycin and streptomycin to enumerate donors, ASWJP+PY plus nalidixic acid and rifampicin to enumerate recipients, and ASWJP+PY plus kanamycin, streptomycin, nalidixic acid, and rifampicin (KSNR) to enumerate transformants.
5. Plasmid acquisition is verified by colony hybridization of at least one KSNR plate as described above.

##### *Filter matings*

1. One ml of donor cells is mixed with 1 ml of recipient cells and immediately filtered onto a sterile 47 mm, 0.2 µm Nuclepore filter.
2. A DNase control is set up similarly, except that after filtration, 200 units of DNaseI is dribbled over the cell spot.
3. The filter is incubated cell side up on an ASWJP+PY plate for the desired length of time (usually 16 hours) at 28–30 °C.
4. The filter is removed from the plate and added to 5–10 ml ASWJP+PY and vigorously vortexed for 2 minutes to resuspend cells. Aliquots are diluted and plated as for broth matings, above.

*Notes*

- Ad 1. It is recommended to pre-wet the filter by filtering 5 ml of medium prior to filtration of the cell mixture.
- Ad 2. The DNase added to the control plate completely inhibits transformation. If 'transformants' are detected in the DNase control they would result from spontaneous mutation or some other form of gene transfer such as conjugation.

*Matings in seawater*

1. One ml of donor and recipient cell suspensions prepared as described above are added to 4 to 25 ml of seawater in a sterile 60 ml disposable centrifuge tube.
2. If nutrients are to be added, concentrated peptone and yeast extract are added to a final concentration of 0.1 and 0.02 mg/ml, respectively.
3. Incubations are at room temperature or the desired temperature for the desired length of time (usually 16 hours).
4. Aliquots of the incubations are serially diluted and plated on the media described above.

*Notes*

Again, seawater to be used can be nonsterile, sterile filtered, or autoclaved, sterile filtered. The volumes can be scaled up and the experiments performed *in situ* using Fenwall Gas Permeable Tissue Culture Bags (Fenwall Scientific). It may be difficult to enumerate the donor population on LB containing kanamycin and streptomycin because of the high level of indigenous resistant organisms found in some environments. However, in our experience, there are usually no indigenous organisms that can grow on the KSNR plates.

*Matings in sediment*

1. Prepare donor cells and recipient cells as above but resuspend in 1/10 volume of grown media.
2. Add 3 cm<sup>3</sup> sediment to a sterile disposable 15 ml conical centrifuge tube.
3. Add an additional 1.5 ml ASWJP to cover the sediment.
4. Incubate for the desired time (usually 16 hours) at the desired temperature.
5. Add 3.5 ml ASWJP and vortex vigorously for 2 minutes.
6. Dilute and plate as for broth matings.

## References

1. Albritton WL, Setlow JK, Slaney, L (1982) Transfer of *Haemophilus influenzae* chromosomal genes by cell-to-cell contact. *J Bacteriol* 152: 1066–1070.
2. Bale MJ, Fry JC, Day MJ (1987) Plasmid transfer between strains of *Pseudomonas aeruginosa* on membrane filters attached to river stones. *J Gen Microbiol* 133: 3099–3107.
3. Bale MJ, Day MJ, Fry JC (1988) Novel method for studying plasmid transfer in undisturbed river epilithon. *Appl Environ Microbiol* 54: 2756–2758.
4. De Vos WM, Venema G (1981) Fate of plasmid DNA in transformation of *Bacillus subtilis* protoplasts. *Mol Gen Genet* 182: 39–43.
5. De Vos WM, Venema G (1982) Transformation of *Bacillus subtilis* competent cells: identification of a protein involved in recombination. *Mol Gen Genet* 187: 439–445.
6. Doran JL, Single WH, Roy KL, Hiratsuka K, Page WJ (1987) Plasmid transformation of *Azotobacter vinelandii* OP. *J Gen Microbiol* 113: 2059–2072.
7. Dubnau D (1991) Genetic competence in *Bacillus subtilis*. *Microbiol Rev* 55: 395–424.
8. Graham JB, Istock CA (1978) Genetic exchange in *Bacillus subtilis* in soil. *Mol Gen Genet* 166: 287–290.
9. Frischer ME, Thurmond JM, Paul JH (1990) Natural plasmid transformation in a high- frequency-of-transformation marine *Vibrio* strain. *Appl Environ Microbiol* 56: 3439–3444.
10. Frischer ME, Stewart GJ, Paul JH (1994) Plasmid transfer to indigenous marine bacterial populations by natural transformation. *FEMS Microb Ecol* 15: 127–136.
11. Fry JC (1989) Analysis of variance and regression in aquatic bacteriology. *Binary* 1: 83–88.
12. Jeffrey WH, Paul JH, Stewart GJ (1990) Natural transformation of a marine *Vibrio* species by plasmid DNA. *Microbial Ecol* 19: 259–269.
13. Jiang SC, Thurmond JM, Pichard SL, Paul JH (1992) Concentration of microbial populations from aquatic environments by vortex flow filtration. *Mar Ecol Progr Ser* 80: 101–107.
14. Juni E (1972) Interspecies transformation of *Acinetobacter*: Genetic evidence for a ubiquitous genus. *J Bacteriol* 112: 917–931.
15. Lorenz MG, Wackernagel W (1990) Natural genetic transformation of *Pseudomonas stutzeri* by sand-adsorbed DNA. *Arch Microbiol* 154: 380–385.
16. Lorenz MG, Aardema BW, Wackernagel W (1988) Highly efficient genetic transformation of *Bacillus subtilis* attached to sand grains. *J Gen Microbiol* 134: 107–112.
17. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
18. Meyer R, Hinds M, Brosch M (1982) Properties of R1162, a broad-host-range, high-copy-number plasmid. *J Bacteriol* 150: 552–562.
19. Meyer R, Laux R, Boch G, Hinds M, Bayly R, Shapiro JA (1982) Broad-host-range IncP-4 plasmid R1162: effects of deletions and insertions on plasmid maintenance and host range. *J Bacteriol* 152: 140–150.
20. Paul JH (1982) Use of Hoechst dyes 33258 and 33342 for enumeration of attached and planktonic bacteria. *Appl Environ Microbiol* 43: 939–944.
21. Paul JH, Jeffrey WH, David AW, DeFlaun MF, Cazares LH (1989) Turnover of extracellular DNA in eutrophic and oligotrophic freshwater environments of southwest Florida. *Appl Environ Microbiol* 55: 1823–1828.
22. Paul JH, Frischer ME, Thurmond JM (1991) Gene transfer in marine water column and sediment microcosms by natural plasmid transformation. *Appl Environ Microbiol* 57: 1509–1515.
23. Paul JH, Frischer ME, Thurmond JM (1992) Intergenic natural plasmid transformation between *E. coli* and a marine *Vibrio* species. *Mol Ecol* 1: 37–46.
24. Rochelle PA, Day MJ, Fry JC (1988) Occurrence, transfer and mobilization in epilithic strains of *Acinetobacter* of mercury-resistance plasmids capable of transformation. *J Gen Microbiol* 134: 2933–2941.

25. Rochelle PA, Fry JC, Day MJ (1989) Plasmid transfer between *Pseudomonas spp.* within epilithic films in a rotating disc microcosm. FEMS Microbiol Ecol 62: 127–136.
26. Sokal RR, Rohlf FJ (1981) Biometry. Freeman, San Francisco, USA.
27. Stewart GJ, Carlson CA (1986) The biology of natural transformation. Ann Rev Microbiol 40: 211–235.
28. Stewart GJ, Carlson CA, Ingraham JL (1983) Evidence for an active role of donor cells in natural transformation of *Pseudomonas stutzeri*. J Bacteriol 156: 30–35.
29. Stewart GJ, Sinigalliano CD, Garko KA (1991) Binding of exogenous DNA to marine sediments and the effect of DNA/sediment binding on natural transformation of *Pseudomonas stutzeri* strain ZoBell in sediment columns. FEMS Microbiol Ecol 85: 1–8.
30. Williams HG, Day MJ, Fry JC (1992) Natural transformation on agar and in river epilithon. In: Gauthier MJ (ed) Gene Transfers and Environment, pp. 69–76. Springer Verlag, Berlin Heidelberg.
31. Winstanley C, Morgan JAW, Pickup RW, Jones JG, Saunders JR. (1989) Differential regulation of lambda  $P_L$  and  $P_R$  promoters by a cI repressor in a broad-host-range thermoregulated plasmid marker system. Appl Environ Microbiol 55: 771–777.

<http://www.springer.com/978-1-4020-2176-3>

Molecular Microbial Ecology Manual

Kowalchuk, G.A.; de Bruijn, F.; Head, I.M.; Van der Zijpp, A.J.; van Elsas, J.D. (Eds.)

2004, XVI, 1778 p. In 2 volumes, not available separately., Hardcover

ISBN: 978-1-4020-2176-3