Molecular Microbial Ecology Manual, Second Edition **1.01**: 3–18, 2004.

Simplified protocols for the preparation of genomic DNA from bacterial cultures

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Introduction

The development of methodologies for the analysis of microorganisms and microbial ecology, at the molecular level (i.e., nucleic acids, proteins, lipids, and their genes), has progressed phenomenally in recent years. Each methodology has specific advantages and disadvantages, or complications. However, the advances in PCR, cloning, gene probing, sequencing and fingerprinting have enabled techniques exploiting nucleic acids to be utilised extensively for the analysis of microorganisms. Often, such protocols require, firstly, that the nucleic acids are extracted in a form which can be employed for the analyses. This may, in some cases, be more difficult than anticipated initially, since many bacteria are extremely resistant to cell disruption. Typically, these are Gram-positive bacteria (e.g., *Mycobacterium* spp., *Peptococcus* spp., *Rhodococcus* spp., etc.), as well as some Archae (e.g., methanogens), with thick cell walls of polysaccharide or pseudopeptidoglycan, and many species of fungi and algae.

General considerations

Several protocols have been developed and described for the preparation of genomic DNA from bacteria, beginning with the prototypal method of Marrnur [16], which involved: a) cell disruption by an enzyme-detergent lysis; b) extractions with organic solvents; and c) recovery of the DNA by alcohol precipitation. Subsequent protocols have usually involved some modification of one or more of these general steps.

Cell disruption

The most difficult and uncertain step in obtaining DNA from bacterial cultures is that of disrupting the cells. The difficulties derive, in part, from imposed limitations

in the handling of the preparations, which are necessary for obtaining genomic DNA of high molecular weight. Thus, in general, the most desirable means of disrupting bacterial cells for obtaining genomic DNA is through enzymatic digestion and detergent lysis. Such a strategy is enhanced by prior treatment of cells with a metal chelating agent, such as ethylenediamine-tetraacetic acid (EDTA). If the cell wall of the organism is susceptible to such treatments, relatively high molecular-weight genomic DNA can be obtained which is applicable for a number of analytical techniques. Further, the lysis should be carried out in a buffered (pH 8–9) medium containing EDTA. The alkaline pH reduces electrostatic interactions between DNA and basic proteins, assists in denaturing other cellular proteins and inhibits nuclease activities. EDTA binds divalent cations, particularly Mg^{2+} and Mn^{2+}, reducing the stabilities of the walls and membranes and also inhibits nucleases which have a requirement for metal cations.

Cell disruption by enzymatic treatments

Lysozyme, isolated commercially from chicken egg white, is a member of the broad class of muramidases which catalyse the hydrolysis of the β-1,4-glycosidic linkage between the N-acetylmuramic acid-N-acetylglucosamine repeating unit, comprising a major part of the peptidoglycan layer of the cell walls of most bacteria [18]. Lysozyme is especially effective in disrupting bacterial cells when used in combination with EDTA [15]. Lysozyme and related enyzmes are useful for disrupting the cells of a broad range of bacterial species, although many species are not particularly susceptible to muramidase treatment due, presumably, to layers of protein or capsular slime, which protect the peptidoglycan. Additionally, as their cell walls do not contain peptidoglycan, all described species of Archae are resistant to lysozyme activity.

Proteinase K, a serine protease produced by the fungus *Tritirachium album*, cleaves adjacent to the carboxyl groups of aliphatic and aromatic amino acids involved in peptide bonding [4], including those comprising the peptide cross-linking interbridges of the peptidoglycan layers of the cell walls of bacteria. The applicability of Proteinase K for disrupting bacterial cell walls is enhanced by its insensitivity to specific chelating agents, allowing it to be utilised in combination with EDTA and lysozyme. However, the peptide interbridges of the cell walls of different species, formed by different combinations of component amino acids, with inherently different susceptibilities to cleavage, may be more or less resistant to Proteinase K lysis.

While lysozyme and proteinase K are, probably, the enzymes most commonly used for the disruption of bacterial cells, additional bacterial cell-disrupting enzymes also have been reported with broad or narrow specificities. Other muramidases, mutanolysin and lysostaphin react, analogous to lysozyme, at the peptide linkages in the cell walls, although the species which are susceptible to these enzymes differ from those which are affected by lysozyme [2, 20, 26]. Subtilisins are extracellular proteases, produced by *Bacillus* spp., exhibiting a broad specificity in hydrolysing most peptide and ester bonds [24]. They are not inactivated

by chelating agents, which makes them applicable in combination with EDTA. The application of achromopeptidase has been limited to the disruption of Gram-positive cells, principally staphylococci [9], although applications with other bacteria have been reported.

Cell disruption by detergent treatments

Detergents provide effective, yet relatively gentle, means for disrupting cells, binding strongly to proteins and causing irreversible denaturation. Further, conditions which cause dissociation of protein (i.e., high pH, low and high ionic strength, etc.) tend to enhance, as well, the solubilisation efficiencies of detergents [7]. Detergents are particularly effective for disrupting bacteria when their cell walls have been damaged (e.g., through the actions of metal chelating agents, lysozyme and Proteinase K) prior to their addition to the cell suspension.

Sodium dodecyl sulfate (SDS) is an anionic detergent which reacts, at low concentrations, at protein hydrophobic sites, binding cellular proteins and lipoproteins, forming SDS-polypeptide micellar complexes, and effectively denaturing them and promoting the dissociation of nucleic acids [17]. Further, SDS inhibits nucleases and does not interact with the hydrophilic nucleic acids. Some proteins form SDS complexes only after they have been heated or treated with reagents (e.g., mercaptoethanol) to cleave intraprotein disulfide bonds.

N-lauroylsarcosine (Sarcosyl), empirically, may be more effective at denaturing cellular polysaccharide material and can be used, instead of SDS, for the disruption of bacterial cells (e.g., *Azotobacter, Beijerinckia, Klebsiella*, etc.) which produce copious amounts of capsule.

Cetyltrimethyl ammonium bromide (CTAB), a cationic detergent, has been used extensively in the preparation of nucleic acids from fungi and plants, when large amounts of polysaccharide materials tend to interfere with the extraction. However, CTAB also has been proven useful for DNA extractions from bacterial cells by denaturing and precipitating the cell wall lipopolysaccharides and proteins [12]. In the presence of monovalent cation (e.g., Na^+) concentrations above 0.5 M, DNA will remain soluble.

Nonpolar detergents, including the Triton X series, Tween series, Nonidet P-40, etc., are generally "milder" solubilising agents than the polar detergents and they seem to have a much more limited ability to initiate the disruption of bacterial cells.

Cell disruption by "physical" methods

Bacteria whose cell walls are not susceptible to enzymatic and detergent treatments may be disrupted using "harsher" (i.e., also on the DNA) methods which may be described, arbitrarily, as "physical" or "mechanical" [10,11,14,19]. Such methods generate DNA which is often sheared and usually not of the relatively uniform, large, molecular weight that can be attained using enzymatic and detergent disruption. Thus, such methods may not be appropriate for preparing DNA for specific analytical techniques. However, in instances wherein it has not been critical that

the DNA be of uniform high molecular weight, methods employing a French pressure cell or a sonicator have been used with success. The use of glass particles with the (mini)-bead beater is particularly effective for disrupting most bacteria and is the method of choice for the preparation of DNA from bacterial cells in problematic matrices (e.g., soils) [23]. Additionally, a method for the production of high molecular weight DNA from Gram-positive and acid-fast bacteria using a microwave oven has been described [1]. However, the efficacies of such methods, all of which require additional, specialised, equipment, have been limited, in most cases, in the range of bacteria for which a given method can be applied.

A further application which has been shown to be effective, particularly in combination with other steps, for disrupting extremely recalcitrant bacteria is the freeze (in liquid nitrogen) and fast thaw (at 95–98 °C) technique. This method is often used in procedures for extracting nucleic acids directly from environmental samples, such as soil and sediment [22]. Such a treatment enhances bacterial cell disruption (e.g., particularly species producing protective capsular slime and those involved in the formation of biofilms) by inducing phase changes in cell membranes through successive, rapid, extremes in temperature which render cells more susceptible to enzymatic and detergent lysis.

Nucleic acid extractions

The isolation of DNA from cells (i.e., selectively eliminating other cellular components except the DNA) is the most straightforward of the three general steps. The methods of choice for extractions, traditionally, have involved the application of organic solvents (e.g., phenol and chloroform) [13], which interact with hydrophobic components of protein and lipoprotein, causing denaturation. It is believed that forces maintaining the hydrophobic interiors of proteins, through their native conformations, are overcome by exposure to hydrophobic solvents, resulting in the unfolding and precipitation of the protein [6]. The precipitate of denatured cellular material remains within the organic phase, which is separated by centrifugation.

In general, phenol is an effective denaturing agent of protein, while chloroform will be more effective for polysaccharide materials. Thus, for the extraction of DNA from bacterial cells, mixtures of phenol/chloroform are more effective than either is, alone. Phenol of high purity (i.e., redistilled), saturated and equilibrated with buffer (pH 8) should be used for the extractions.

Recovery of DNA

The standard method for recovering DNA from cell lysates and suspensions is by the use of alcohol (i.e., ethanol or isopropanol) reversible denaturation (i.e., the helical structure is extensively destroyed) and subsequent precipitation [5], followed by centrifugation. It is recognised that DNA precipitates poorly in salt-free solutions and that alcohol precipitations should be performed in the presence of a monovalent cation with a concentration of, at least, 0.1 M. Precipitation of

DNA in suspensions is initiated by adding 0.1 suspension volume of 3 M sodium acetate (pH 5.2) and 2.0–3.0 suspension volumes (calculated after the addition of salt) of 100% ethanol (for DNA suspensions of low concentration, a higher ratio of ethanol to suspension volume will facilitate DNA precipitation) [21]. Alternatively, 0.5 volumes of 7.5 M ammonium acetate (pH 8) can be used instead of sodium acetate [3]. In this case, small nucleic acid fragments (approximately 150 nucleotides and smaller), will not be precipitated, which may be advantageous in some cases. Isopropanol (0.5–1.0 volumes) may be used, rather than ethanol, particularly when small volumes (e.g., less than 1.0 ml) are needed.

Although it has become an accepted practice to carry out DNA precipitations at extreme cold temperatures (e.g., −70 °C), data suggest that precipitations at such temperatures present no significant advantage over precipitations carried out in ice water (i.e., approximately 0 °C) and, in fact, may be counterproductive [27] (Fig. 1). Further, while the majority of DNA in concentrated suspensions is

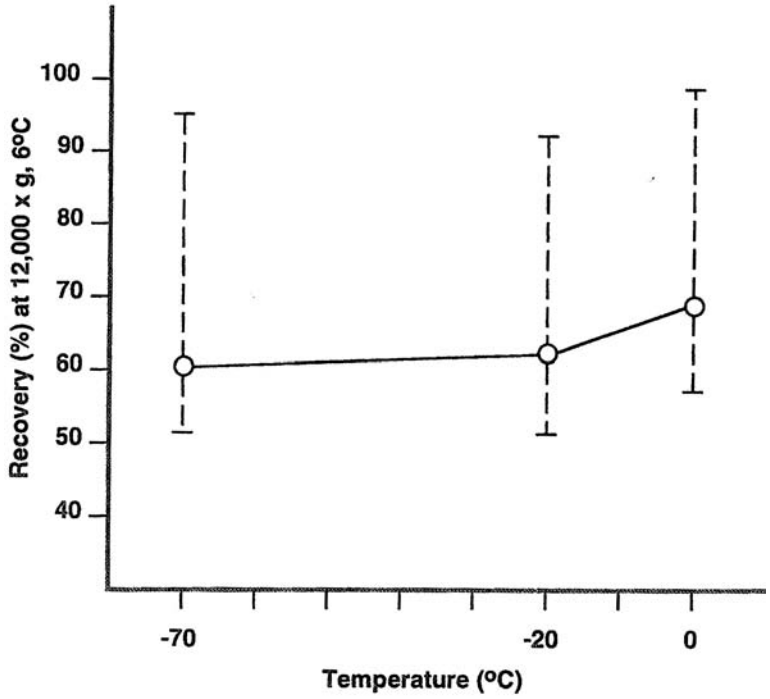

Figure 1. The recovery of DNA as a function of the precipitation temperature. Precipitations of varying amounts (0.6 ng–010 μg) of DNA at extremly low temperatures (i.e., −70 °C) are less efficient than at 0 °C. The efficiencies of recovery, by centrifugation (12,000 × g, 6 °C), were also observed to be dependent upon the amounts of DNA in suspension. The values indicated in the graph represent the means, calculated from the observed recoveries from suspension, of varying amounts of DNA. The ranges of observed recoveries are indicated, with the lowest and highest recoveries, at each temperature tested, and correspond to the lowest and highest concentrations of DNA, respectively. The graph was prepared from data taken from Zeugin and Hartley, 1985 [27].

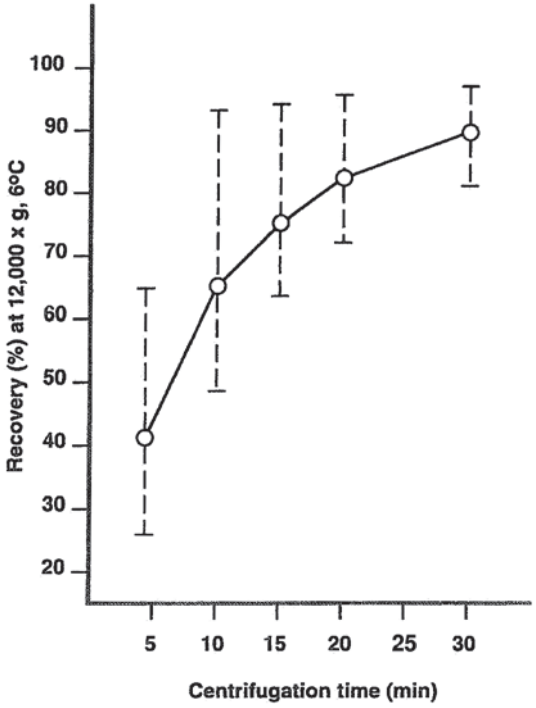

Figure 2. The recovery of DNA as a function of the centrifugation time. The recovery of varying amounts (0.6 ng–10 μg) of DNA is enhanced by increased centrifugation times. The efficiencies of recovery, by centrifugation (12,000 × g, 6 °C), were also observed to be dependent upon the amounts of DNA in suspension. The values indicated in the graph represent the means, calculated from the observed recoveries from suspension, of varying amounts of DNA. The ranges of observed recoveries are indicated, with the lowest and highest recoveries, at each centrifugation time tested, and correspond to.the lowest and highest concentrations of DNA, respectively. The graph was prepared from data taken from Zeugin and Hartley, 1985 [27].

recovered quickly (i.e., within 5 minutes) by centrifugation (12,000–15,000 × g), the recovery of DNA from dilute suspensions may require centrifugations for as long as 30 minutes (Fig. 2).

An important consideration to keep in mind throughout the extraction process is the relationship between the amount of DNA in suspension and the ability, ultimately, to recover it.

In studies to determine the optimal conditions for the recovery of DNA from suspensions by precipitation and centrifugation [27], the amount of DNA recovered was observed to be proportional to the concentration in suspension (Fig. 3). Thus, it is important to consider this relationship when deciding upon the extraction protocol to use and subsequent handling of the DNA.

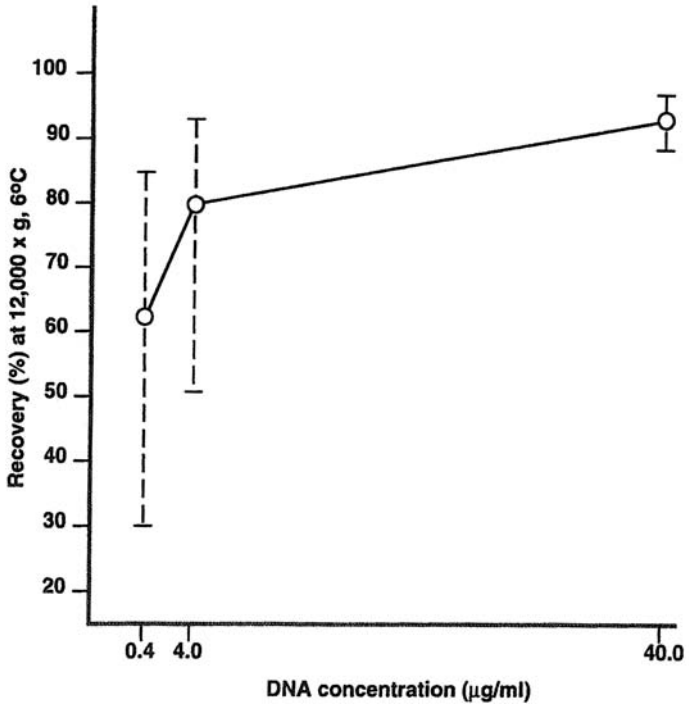

Figure 3. The recovery of DNA as a function of the amount of DNA in suspension. The recovery of DNA was observed to be dependent on the concentrations of the suspensions. The values indicated represent the means, calculated from the observed recoveries from suspension, after varying centrifugation times. The ranges of observed recoveries are indicated, with the lowest and highest recoveries, for each DNA concentration, corresponding to the shortest and longest centrifugation times (5–30 minutes). The graph was prepared from data taken from Zeugin and Hartley, 1985 [27].

Procedures

The specific methods described here are simplified, rapid, protocols observed to be effective for isolating genomic DNA, from a wide range of bacteria, of a quality applicable for PCR.

Protocol I – CTAB protocol for the extraction of bacterial genomic DNA

This protocol is derived from the "miniprep" method described by Wilson [25]. Broth cultures (2–5 ml) grown to mid-log growth phase are harvested in 2.0 ml Eppendorf tubes by centrifugation in

a microfuge at 10,000–15,000 × g for 10–15 minutes. In general, late-log growth phase cultures should not be used for preparing DMA, as nucleases tend to accumulate in older cultures. Alternatively, bacterial colonies grown on agar media may be washed off the agar and collected in an Eppendorf tube. The Bacteria Washing Buffer should not contain EDTA, as some bacteria (e.g., some Gram-negative species) will begin lysing upon exposure to chelating agents. After pelleting the cells, the medium is poured off and the rim of the tube is blotted with a paper towel to get rid of residual liquid. The bacterial pellet should weigh approximately 0.1 g (wet weight), which should provide 40–200 mg of DNA, depending upon the species of bacteria and the growth conditions. If there is more than 0.1 g per tube, the cell pellet should be resuspended with Bacteria Washing Buffer and redistributed accordingly into additional Eppendorf tubes. This is not unimportant, since the efficiency of the extraction decreases with increasing cell material. With experience, one can estimate reliably the mass of the cell pellet from its size.

Steps in the protocol

1. Resuspend the cell pellet (approximately 0.1 g) completely with 564 μl TE buffer (use a sterile toothpick to mix the pellet and ensure complete resuspension).
2. Add approximately 10 μg lysozyme (crystalline) to the cell suspension (from this point, do not vortex!). Mix thoroughly by inverting the Eppendorf tube several times. Incubate 10–60 minutes at 37 °C. Add 6 μl Proteinase K (10 mg/ml), and 30 μl SDS (10–20%). Mix thoroughly (do not vortex!). Incubate at 37 °C until the suspension becomes relatively clear and viscous.
3. Add 100 μl NaCl (5 M) and mix thoroughly (do not vortex!). Incubate suspension at 65 °C, 2 minutes. Add 80 μl CTAB/NaCl solution (preheated at 65 °C, use a pipette tip with the tip cut off to pipette the viscous CTAB/NaCl solution) and mix thoroughly (do not vortex!). Incubate suspension at 65 °C, 10 minutes.
4. Extract suspension with an equal volume (approximately 800 μl) chloroform/isoamyl alcohol (24:1) solution. Centrifuge (10,000 × g, 5 minutes) Transfer the upper (aqueous) phase (Supernatant 1), containing the nucleic acids, into a separate 2.0 ml Eppendorf tube.

5. Extract Supernatant 1 with an equal volume (approximately 800 μl) of phenol/chloroform/isoamyl alcohol (25:24:1) solution. Centrifuge (15,000 × g, 5 minutes). Transfer the upper (aqueous) phase (Supernatant 2), containing the nucleic acids, into a separate 2.0 ml Eppendorf tube.
6. Extract Supernatant 2 with an equal volume (approximately 800 μl) chloroform:isoamyl alcohol (24:1) solution. Centrifuge (10,000 × g, 5 minutes). Transfer the upper (aqueous) phase (Supernatant 3), containing the nucleic acids, into a separate 2.0 ml Eppendorf tube.
7. Add 0.7 volumes (approximately 560) isopropanol to precipitate nucleic acids. Mix gently by inverting the tube several times – the DNA should appear as a white, viscous, precipitate. Let sit at room temperature for 5 minutes to 1 hour. Centrifuge (12,000–15,000× g 15–30 minutes) at room temperature. The DNA should be visible as a pellet on the side of the Eppendorf tube. Remove the isopropanol carefully, so as to avoid disturbing the pellet.
8. Wash the pellet with 500 μl EtOH (70%) by inverting the tube several times. Centrifuge 12,000–15,000 × g, 15–30 minutes at room temperature. Carefully remove the EtOH and blot the rim of the tube with a paper towel to get rid of excess liquid.
9. Briefly (not more than 5 minutes) dry pellet in a speed-vac.
10. Resuspend each pellet in 50–60 μl TE Buffer. Let sit at 37 °C to allow the DNA to be resuspended completely.
11. Estimate the concentration of DNA in suspension by spectrophotometric measurement at 260 nm. For double-stranded DNA suspensions, at a wavelength of 260 nm and using a cuvette with a 1 cm light path, an OD of 1.0 is equal to a concentration of 50 Mg/ml. The quality of the DNA can be estimated by measure ments of the A_{260}/A_{280} and the A_{260}/A_{230} ratios. The size of the DNA can be estimated by agarose gel (0.5%, w/v) electrophoresis, subsequent staining with ethidium bromide and visualisation by U.V. illumination. DNA of uniform size (approximately 20 kb) indicates that the DNA has been extracted without excessive shearing. DNA which has been sheared or degraded by nucleases will appear as a broad smear, of smaller molecular weight products.

12. Adjust the DNA suspension to a final stock concentration (e.g., 1–10 μg/μl before using an aliquot for a PCR.

Notes

1. After adding TE buffer, some cells may begin to lyse and vortexing will induce shearing of released DNA. However, in the case of most bacteria, vortexing at this point will not produce noticeable shearing.
2. Many bacteria will lyse without using lysozyme. However, in many cases, lysozyme will facilitate lysis and, if it is used, it should be added before the Proteinase K and SDS. Many bacterial species will lyse quickly, but others may require longer incubation times. In some cases, overnight incubations, supplemented with additional Proteinase K and SDS, have proven successful in lysing the cells when shorter incubation times were not effective. K^+ should be excluded from all buffers when SDS is used, as the detergent will precipitate, except at elevated temperatures.
3. It is important that the NaCl solution be well mixed with the lysate before adding the CTAB/NaCl solution, as the nucleic acids will precipitate (at room temperature) with the CTAB if the total Na^+ concentration is below approximately 0.5 M.
4. A 1.0 ml micropipetter can be used, but the end of the pipette tip should be cut off to help prevent excessive shearing when pipetting the aqueous phase containing the DNA.
5. Older, oxidised, phenol solutions should not be used as they may cause "nicking" of the DNA. The phenol solution should contain an anti-oxidising agent (8-hydroxyquinoline) as an indicator (i.e., if the 8-hydroxyquinoline is oxidised, the phenol solution will turn a reddish color).
6. After successive extractions, eventually, a clear interface should be observed between the upper and lower phases. A white interface is an indication of the presence of protein and additional chloroform/isoamyl alcohol extractions may be necessary.
7. In some cases, a precipitate is not detected immediately after adding isopropanol. As the DNA pellet may sometimes be difficult to detect, it is important to note the orientation of the Eppendorf tube in the microfuge so that the position of the pellet will be known and not disturbed or aspirated inadvertently. The best way to remove the isopropanol without disturbing the DNA pellet is to use a Pasteur pipette with a very fine tip produced by drawing it out over a flame.
8. After washing with EtOH, the DNA pellet may appear translucent, due to the loss of salt.

11. Ideally, A_{260}/A_{280} should be 1.8–2.0. Ratios less than 1.8 indicate protein contamination, while ratios greater than 2.0 indicate the presence of RNA. The A_{230}/A_{260} ratio should be 0.3–0.9. Ratios greater than 0.9 indicate the presence of polysaccharide. All of these components may interfere with PCR. If the DNA suspension is contaminated with protein, it should be subjected to additional phenol/chloroform/ isoamyl alcohol extractions. If RNA is present, the DNA suspension should be treated with DNase-free RNase (added to a final concentration of 100 μg/ml). After incubation at 37 °C for 2 hours, the DNA suspension must be reextracted with phenol and chloroform: isoamyl alcohol and precipitated. If salt is present, the DNA suspension

should be reprecipitated by the addition of 0.7 volumes of isopropanol with a subsequent wash of 75% EtOH or, alternatively, the DNA suspension may be dialysed using a Microcon-100 (Amicon) spin-concentrator or a 0.025 mm (pore size) minifilter (Millipore).

Protocol II – Protocol for the extraction of genomic DNA from individual bacterial colonies

An additional protocol, an extension of a method described originally by Holmes and Quigley for the preparation of plasmid DNA (8), follows the rapid disruption of cells, from individual colonies picked from an agar medium, centrifugation to pellet cell debris, and the addition of an aliquot of the resulting supernatant directly (i.e., without additional purification steps) to the PCR. Besides being much more rapid than standard methods for preparing DNA from bacteria, this strategy possesses the added advantage that danger of contaminating the PCR with DNA from non-target organisms is decreased. A further advantage of this method is that the limited number of cells from an individual colony seem to be more susceptible, in comparison with the much larger number of cells of a cell pellet, to cell disruption methods. Obviously, such a protocol will yield only a limited amount of DNA, which makes it impractical for many subsequent analyses. However, for PCR, such a protocol is ideal for processing many samples rapidly.

Steps in the protocol

1. Individual colonies from an agar plate are picked (depending upon the size and age, 1–5 colonies are usually adequate for generating sufficient DNA) using a sterile toothpick or inoculating loop and resuspended in 100 μl sterile TE Buffer or sterile deionized H_2O. Following this:
   a) the cell suspension is placed in a water bath at 97 °C and "cooked" for 5–10 minutes, and/or
   b) the cell suspension is treated in a mini-bead beater (B. Braun Biotech Intl., GmbH) by shaking (approximately 2000 oscilla tions per minute) for 5 minutes with 0.5 g glass beads (0.17 – –0.18 mm) in the cell suspension.
2. Centrifuge the cell lysate (15,000 × g, 5–15 minutes).
3. Remove the supernatant containing the DNA and add an aliquot (1–5 μl) to a PCR reagent mix.

Notes

1. The cells may be treated before or after "cooking" in order to better facilitate disruption. For example, incubating the cell suspension with lysozyme and/or Proteinase K before and after a series of freezing (in liquid nitrogen) and fast thawing (at 95–98 °C), or using a (mini)-bead beater after "cooking", has improved the yields of DNA in some cases. The size-range of the beads used with the (mini)-bead beater is important, depending upon the type of microorganism intended to be disrupted. Beads of 300–500 μm in diameter are adequate for fungi and yeast, while beads of 100–200 μm should be used for bacteria. The beads are acid washed and baked or autoclaved before use. The principle and primary advantage of this protocol is to be able to add an aliquot of cell supernatant containing DNA directly (i.e., unpurified) to the PCR. Thus, the use of SDS or other detergents should be avoided.
2. The cell debris (i.e., most protein, lipids, etc.) will be pelleted by centrifugation, while DNA will remain in the supernatant. In order to facilitate the separation of the debris, protein-binding resins may be added (before cell disruption). Examples of such resins are: StrataClean™ Resin (Stratagene, Ltd.) and InstaGene Matrix (Biorad Laboratories).
3. Usually, there will be no problem amplifying the target. However, a complication may arise if too large a volume of the DNA supernatant is added to the PCR. The possibility exists that EDTA (from the TE Buffer used to resuspend the bacterial colonies) may cause inactivation of the *Taq* polymerase. Thus, in some cases, it may be worthwhile to resuspend the colonies in H_2O, rather than TE Buffer, before "cooking". Another option is to concentrate the final DNA supernatant using a Microcon-100 (Amicon) spin concentrator, effectively desalting the DNA supernatant. Additionally, an aliquot (1–5 μl) of the DNA supernatant can be used to load onto an agarose gel to estimate the quantity and quality of the DNA.

Solutions

- Bacteria Washing Buffer: 0.4 M NaCl, 50 mM Tris-HCl (pH 8.0), 50 mM $MgS0_4$, in sterile, deionized H_2O [sterilise by autoclaving]
- TE Buffer: 10 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA, in sterile, deionized H_2O [sterilise by autoclaving]
- Lysozyme: crystalline
- SDS: 10–20% (w/v) sodium dodecyl sulfate in deionized H_2O
- NaCl: 5 M NaCl in sterile, deionized H_2O [sterilise by autoclaving]
- NaCl: 0.7 M NaCl in sterile, deionized H_2O [sterilise by autoclaving]
- CTAB/NaCl: 10% (w/v) hexadecyltrimethyl ammonium bromide in sterile 0.7 M NaCl solution. [Heat solution to 65 °C before bringing to final volume]
- Chloroform-isoamyl alcohol: 24 volumes chloroform to 1 volume isoamyl alcohol

– Phenol: 250 ml redistilled, Tris-equilibrated, phenol in TE Buffer (pH 8.0) [250 ml redistilled phenol (melted at 65 °C) and 0.25 g 8-hydroxyquinoline is equilibrated twice with 250 ml 50 mM Tris-HCl (pH 9.0); a final equilibration is made with 50 mM Tris-HCl (pH 8.0) -the pH of the phenol should be approximately 8.0; add 125 ml TE buffer for storage (covered with aluminum foil) at 4 °C]
– Isopropanol (2-propanol): Molecular Biology Reagent grade
– Ethanol (EtOH): 70% (v/v) in sterile, deionized H_2O

Application of the method

Figure 4 shows the results of DNA extractions of two species of the genus *Rhodococcus* (Gram-positive), which is extremely resistant to cell lysis by

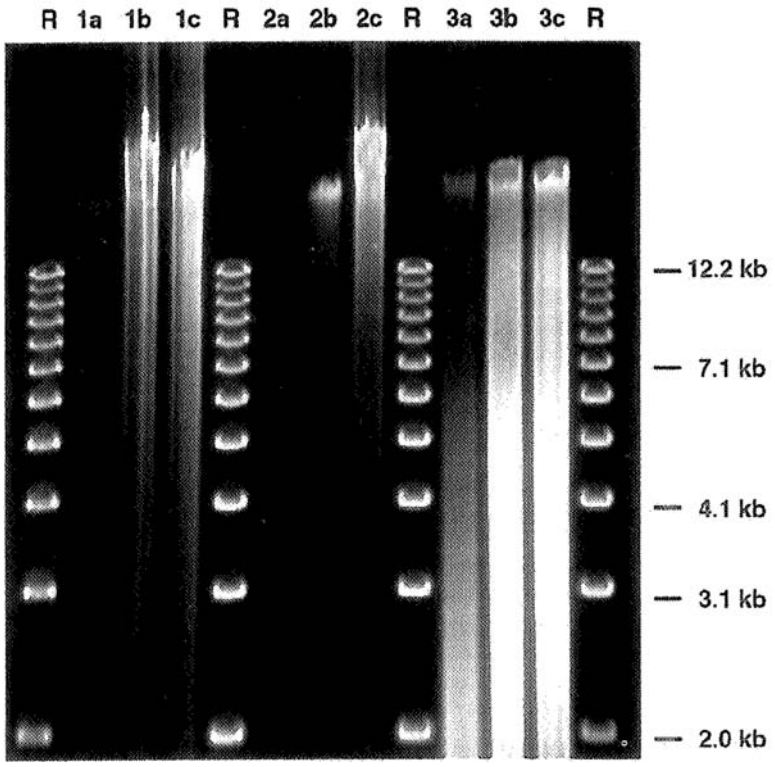

Figure 4. Agarose gel (1.0%, w/v) electrophoresis and ethidium bromide staining of genomic DNA prepared from: 1) *Rhodococcus rhodochrotis;* 2) *Rhodococcus globerulus*; and 3) *Pseudomonas aeruginosa.* Cells grown on agar media and picked from individual colonies were treated by: a) heating at 90 °C, 5 minutes; b) mini-bead beater, 5 minutes: or c) mini-bead beater, 10 minutes R: 1 kb ladder as reference (Gibco-BRL).

enzymatic and detergent treatments or by simple "cooking", and one species of *Pseudomonas* (Gram-negative), which is easily disrupted by enzymatic and detergent treatments or "cooking". The cell suspensions were prepared from colonies treated as described in Protocol II. "Cooking" the cells was effective for disrupting the cells and isolating genomic DNA from *P. aeruginosa*, but was much less effective for disrupting the cells of *R. rhodochrous* and was not effective, at all, for disrupting the cells of *R. globerulus.* An additional treatment of the cells for 5 minutes with the (mini)-bead beater was effective in disrupting the cells of all three species to enable the isolation of DNA for PCR. Treatment of the cells for 10 minutes with the (mini)-bead beater generated genomic DNA which was badly sheared.

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Molecular Microbial Ecology Manual, Second Edition **2.01**: 215–222, 2004.

Quantification of nucleic acids

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Abbreviations:

Bisbenzimide H 33258 (= Hoechst 33258)	–	2-(2-(4-hydroxyphenyl)-6-benzimidazolyl)-6-(1-methyl-4piperazyl)-benzimidazole, trihydrochloride pentahydrate.
DABA.2HCl	–	3,5-diamino-benzoic acid dihydrochloride;
DAPI	–	4,6-diamidino-2-phenylindole;
EDTA	–	sodium ethylene diamine tetraacetate;
PBS	–	phosphate buffered saline;
SSC	–	standard saline citrate;
Tris	–	Tris (hydroxymethyl)-aminomethane

Introduction

Methods for nucleic acids quantification in environmental samples are normally based on spectrofluorometry. When analyzing impure samples containing humic material, autofluorescence has to be measured and subtracted from the nucleic acid specific fluorescence. It is also recommended to use a known amount of nucleic acid as an internal standard, to correct for quenching. There are several methods which are specific for DNA and some which can be used for quantification of both DNA and RNA.

The following methods are more or less specific for DNA and can be used for low concentrations of DNA:

1) Reaction with 3,5-diamino-benzoic acid 2HCl (DABA.2HCl) [7]. This reagent is specific for primary aldehydes of the type R-CH_2-CHO. With material like soil, great care must be taken in the extraction procedure in order to ascertain that the measured fluorescence stems only from the DNA-DABA.2HCl complex.
2) Reaction with mithramycin. This antibiotic reacts with guanine in double stranded DNA, and the complex formed has a fluorescence which can be taken as a measure of the amount of DNA present. This method is absolutely specific

[4], but the binding efficiency is decreased in the presence of nucleoproteins, and it has a somewhat lower sensitivity than the DABA.2HCl-method.

3) Reaction with bisbenzimide H 33258 (Hoechst 33258). This is a very sensitive and specific reagent for fluorometric determination of DNA as it binds specifically to adenine plus thymidine base pairs [8].

A new fluorochrome which can replace Hoechst 33258 is the fluorochrome 4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylmethyl-edene]-1-[3′-trimethyammoniumpropyl]-quinolinum diiodide (YO-PRO™-1; Molecular Probes, Inc., Eugene, Oregon, USA). The fluorescence excitation and emission maxima of the DNA-YO-PRO-1 complex are 491 and 509, respectively. The sensitivity is over 400-fold greater than that of Hoechst 33258. The fluorochrome binds to RNA as well as DNA. The RNA and DNA content in a sample may be quantified by measuring the fluorescence before and after treatment with DNase-free RNase.

When applying the mithramycin or Bisbenzimide H 33258 method to intact bacteria, the cells first have to be lysed. The most efficient method is sonication, but the sonication conditions are very critical [2]. Sonication energy and length of time must be optimized in order to break as many bacteria as possible without degrading the DNA. Effective cooling during sonication will reduce the DNA degradation.

The first two methods gave similar results, when applied to bacteria extracted from soil, namely 8.4 fg DNA per microscopically counted cell [9]. This is in the upper range of what is normally found in bacteria [1]. Using bisbenzimide H 33258, Bakken and Olsen [2] found 2–9 fg per cell in bacterial isolates from soil, and 1.6–2.4 fg in directly extracted bacteria from the same soil. The DNA content of small cells passing through a 0.4 μm membrane filter was close to the average for the total bacterial flora. When the bacterial concentration is 10^{10} bacteria per g dry soil, this means that the DNA content is between 20 and 90 μg DNA per g dry weight of soil.

Procedures

Protocol 1

Reaction with DABA (diamino-benzoic acid hydrochloride)[7,9]

The cells are filtered onto a glass fiber filter with a layer of diatomite (diatomaceous earth Hyflo Supercel). The diatomite make it possible to filter a larger sample onto the filter before it get clogged. Some humic matter is removed by washing with sodium pyrophosphate and the cells are pre-extracted with trichloroacetic acid and an ethanol:ether solution to remove lipids, sucrose, etc., which will interfere with the assay. As ether will react with DABA it has to be

completely removed. DABA is allowed to react with deoxyribose at high temperature in the presence of mineral acid. Diatomite, and other particles are then removed by filtering through a membrane filter. It is important to use membrane filters which do not give high blank values. DABA yields a 1000-fold higher fluorescence with DNA than with RNA. The emission light of the DABA-DNA complex is measured in a spectrofluorometer at 500 nm with an excitation light set at 410 nm. Two parallel series are generated; one with an internal standard of calf thymus DNA added. The method is linear for DNA amounts ranging from 1 to 50 μg.

Steps in the procedure

1. Filter 2 ml of a 2% suspension of acid-washed diatomite onto a Whatman glass fiber filter type GF/F (20 mm diameter).
2. Filter a bacterial suspension (i.e. bacteria extracted from soil [3]) containing approximately 10^9 bacteria in 1 ml onto the filter. Take two replicates per sample.
3. Wash the filter with 2 × 5 ml 0.1 M sodium pyrophosphate, pH 7.0.
4. Extract the material on the filter with 2 × 5 ml cold 5% trichloroacetic acid, followed by 2 × 5 ml ethanol:ether solution (3:1).
5. Suck the filter dry, and dry it further in an oven at 60 °C for 1 hour.
6. Transfer the filter to a glass tube.
7. When using DNA as an internal standard, add 5 μg calf thymus DNA in 50 μl PBS to the dried filter of one of the parallel samples.
8. Add 400 μl 20% DABA.2HCl and incubate for 2 hours at 60 °C.
9. Add 4 ml 0.6 M perchloric acid.
10. Pass through a 20 mm Sartorius SM 11106 (0.45 μm pore size) filter to remove particles, diatomite and filter debris.
11. Measure the fluorescence in a spectrofluorometer with a 410 nm excitation light and a 500 nm emission light.
12. Use a sample of 2 ml of a 2% suspension of acid-washed diatomite filtered onto glass fiber filter and treated like the bacterial samples as a blank.
13. Prepare standard series from a stock solution of 1 mg/ml calf thymus DNA in PBS. To standards with 1–50 μg DNA in 50 μl PBS add 400 μl 20% DABA.2 HCl and incubate for 2 hours at 60 °C. Add 4 ml 0.6 M perchloric acid, cool to room temperature and measure the fluorescence.

Note

8. DABA.2HCl is prepared from 3,5 diamino-benzoic acid using the following protocol [5] (should be carried out in a hood):
   Dissolve 40 g of 3,5 diamino-benzoic acid in 400 ml boiling 6N HCl. Add 1–2 g charcoal, swirl the suspension briefly to mix well, and filter through a preheated (100 °C) 55 mm diameter Buchner funnel with Whatman glass fiber filter (type GF/C). Cool the filtrate in a refrigerator or cold room for 2–3 hours to crystallize the DABA.2HCl. Collect the crystals by filtering onto a sintered glass funnel, transfer them to a glass vial and dry them at 60 °C for 2 hours. DABA.2HCl can be stored in a closed glass vial in the refrigerator for 2 months. Prepare the DABA.2HCl solution immediately before use.

Equipment

- Ceramic or glass filter holders for 20 mm diameter filters
- Water suction equipment (glass)
- Whatman glass fiber filter, type GF/F, 20 mm diameter
- Sartorius SM 11106 filter, 20 mm diameter (0.45 μm pore size)
- Glass tubes
- Oven with thermostat

For preparation of DABA.2HCl:

- Whatman glass fiber filter, type GF/C, 55 mm diameter.
- Buchner funnel, 55 mm diameter
- Sintered glass funnel
- Oven with thermostat
- Fluorescence spectrophotometer

Chemicals and solutions

- 2% suspension of acid-washed diatomaceous earth (Hyflo Supercel, Johns-Manville Products, New York) in distilled water
- 0.1 M sodium pyrophosphate, pH 7.0
- 5% trichloroacetic acid
- Ethanol:ether solution (3:1)
- Calf thymus DNA
- 0.6 M perchloric acid
- Phosphate buffered saline (PBS); 0.8 g sodium chloride, 0.2 g potassium chloride, 1.44 g disodium hydrogen phosphate, 0.24 g potassium dihydrogen phosphate, 1000 ml sterile distilled water, pH 7.5
- 20% solution of DABA.2HCl in distilled water

For preparation of DABA.2HCl:

- 3,5 diamino-benzoic acid
- 6 N hydrochloride
- Charcoal, Norit A

Protocol 2

Reaction with mithramycin [9]
The DNA is measured in the extracts of cells after sonication. Two parallel series are generated; one with mithramycin added and one with buffer in order to measure the autofluorescence. In addition, fluorescence is measured in a standard series of calf thymus DNA. The fluorescence of the DNA-mithramycin complex is measured in a spectrofluorometer with an excitation light set at 410 nm, and the emission light measured at 515 nm. The method is linear with DNA amounts ranging from 5 to 50 μg.

Steps in the procedure
1. Take two series of aliquots from a suspension of soil bacteria containing approximately 2–5 $\times$ 10^9 bacteria, and pellet the bacteria by centrifugation at 10,000 $\times$ g for 15 minutes.
2. Wash the bacteria twice in 0.1 M sodium pyrophosphate and once in PBS by resuspending the cells and then pelleting them by centrifugation as above.
3. Add 3 ml PBS with 15 mM $MgCl_2$, pH 7.5 to the pellets of one of the series, and 3 ml mithramycin reagent to the other.
4. Place the samples in a cold bath (ethanol-dry ice, or -70 °C methanol) and sonicate them with a sonifier (Branson B-12 sonifier with a 1.25 cm titanium probe) at maximum output (100 W) for 5 minutes with 120 seconds pulses and 20 seconds pauses.
5. Centrifuge the samples for 10 minutes at 20,000 $\times$ g and transfer the supernatant to new glass tubes.
6. Measure the fluorescence of DNA-mitramycin complex in a fluorescence spectrophotometer at 515 nm with an excitation light set at 410 nm.
7. Add 20 μg calf thymus DNA in 50 μl PBS to the series with mithramycin, and measure the fluorescence again after 5 minutes to determine the quenching.

8. Measure the autofluorescence of the samples in the series with only PBS and $MgCl_2$ added.
9. Measure the fluorescence of the mithramycin reagent separately.
10. Prepare standard series from a stock solution of 1 mg/ml calf thymus DNA in PBS. To a series of standards with 1–50 μg DNA in 50 μl PBS add 3 ml mithramycin reagent and measure the fluorescence.

Equipment

- High-speed centrifuge with a rotor for 15–30 ml tubes (Sorvall SS-34 rotor)
- Branson B-12 sonifier (Branson Sonic Power SA, D-6055 Heusenstam) with a 1.25 cm titanium probe
- Fluorescence spectrophotometer

Chemicals and solutions

- Mithramycin solution (Mithracin; Pfizer Ltd); 200 μg/ml in 300 mM $MgCl_2$, stored frozen and diluted 1:20 with PBS, pH 7.5 before use
- 0.1 M sodium pyrophosphate, pH 7.0
- Phosphate buffered saline (PBS) with 15 mM $MgCl_2$; 0.8 g sodium chloride, 0.2 g potassium chloride, 1.44 g disodium hydrogen phosphate, 0.24 g potassium dihydrogen phosphate, 3.05 g magnesium chloride hexahydrate, 1000 ml sterile distilled water, pH 7.5
- Calf thymus DNA

Protocol 3

Reaction with bisbenzimide H 33258 [1, 8]

This method has become widely used for quantification of DNA. The DNA concentration is determined in crude cell extracts which are obtained by sonication in the presence of SSC and a detergent. DNA can be measured down to the nanogram range. The fluorescence is highly specific for DNA, fluorescence enhancement of RNA relative to DNA is below 1%. To correct for quenching by humic material an internal standard should be added to the samples. By using two different dye concentrations (0.15 μM and 1.5 μM), a linear response in the range from 0.05 to 10 μg DNA can be obtained.

Bisbenzimide H 33258 can be replaced by equimolar amounts of 4′,6-diamidino-2-phenylindole (DAPI) which is also an

adenine-thymine specific dye [6]. DAPI gives higher background fluorescence than bisbenzimide H 33258.

Steps in the procedure

1. Pellet the bacteria in samples containing 2×10^8 –10^9 cells by centrifugation at 10,000 × g for 15 minutes.
2. Resuspend the bacteria in 6 ml 1 × SSC and add 10 μl 5% Triton X-100.
3. Sonicate the bacteria in a cold bath (ethanol-dry ice, or −70 °C methanol) with an ultrasound sonifier (Branson B-12 sonifier with a 1.25 cm titanium probe) at maximum output (100 W) for 5 minutes with 120 seconds pulses and 20 seconds pauses.
4. Centrifuge the homogenate at 10,000 × g for 10 minutes at 5 °C.
5. Take 2 ml aliquots in duplicate from the cell extract. Mix one of the parallels with 1 ml 0.15 μM bisbenzimide solution (for samples containing 50 ng-1 μg DNA) or 1.5 μM bisbenzimide solution (for samples containing 1–10 μg DNA). Protect the samples with bisbenzimide from light.
6. Measure the fluorescence at 450 nm (emission) with an excitation light of 350 nm.
7. To determine the quenching add 0.1 or 1 μg calf thymus DNA in 50 μl 1 × SSC to the series with bisbenzimide, mix well and measure the fluorescence again.
8. Measure the autofluorescence of the samples without bisbenzimide.
9. Measure the fluorescence of the bisbenzimide reagent (diluted 1:2 with 1 × SSC) separately.
10. From a stock solution of 1 mg/ml calf thymus DNA in 1 × SSC prepare working stock solutions by diluting to 10 μg/ml (low range standards) and 100 μg/ml (high range standards) with 1 × SSC. Prepare series of low range standards containing 50 ng–1 μg DNA and high range standards containing 1–10 μg DNA, in 2 ml 1 × SSC. Add 1 ml 0.15 μM or 1.5 μM bisbenzimide reagent to the low and high range standards respectively.

Equipment

– Branson B-12 sonifier (Branson Sonic Power SA, D-6055 Heusenstam) with a 1.25 cm titanium probe

– High-speed centrifuge with rotor for 10–15 ml tubes
– Fluorescence spectrophotometer

Chemicals and solutions

– Bisbenzimide H 33258 solution (Hoechst 33258, Calbiochem or Boehringer Mannheim). A stock solution is prepared by dissolving 1 mg/ml of bisbenzimide H 33258 in ultrapure distilled water. Can be stored at 4 °C in the dark for a week.
– Working solution of bisbenzimide; dilute 10 μl of the stock solution in 100 ml TEN buffer (10 mM Tris, 1 mM sodium ethylene diamine tetraacetate (EDTA), 0.1 M sodium chloride, pH 7.4).
– Standard Saline Citrate (SSC); 1 × SSC is 0.15 M sodium chloride, 0.15 M trisodium citrate in distilled water, pH adjusted to 7.0. Prepare as a 10 × SSC stock solution and dilute as needed.
– 5% Triton X-100
– Calf thymus DNA

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Molecular Microbial Ecology Manual, Second Edition **3.01**: 509–522, 2004.

Amplification of ribosomal RNA sequences

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Introduction

Comparisons of rRNA sequences, pioneered by Woese and his colleagues, defined the main lineages in the evolution of microorganisms [42]. An advantage of rRNA sequence comparisons is the generation of an increasingly expanding data base against which newly determined sequences may be compared [5,23]. Nearly 60,000 16S rRNA sequences are currently available in the Ribosomal Database Project II [23]. Initially, sequences were obtained from well described pure cultures for phylogenetic research. Pace et al. [31] recognized that as the rRNA tree filled in, the data base would serve not only for continued comparison of sequences obtained from pure cultures, but also for comparison of sequences obtained directly from natural microbial communities without needing to grow the representative members in the laboratory. The concept of comparing gene sequences from microbial communities revolutionized microbial ecology. Subsequently, a suite of molecular methods was developed that employ rRNA sequences [1,11,37].

Divergence of the primary lines of bacterial descent occurred early in biotic history so highly conserved molecular chronometers are best suited to the task of reconstructing bacterial phylogeny [42]. Ribosomal RNAs are integral elements of the protein synthesizing apparatus, the basic components of which are present in all primary kingdoms, and are among the most highly conserved cellular molecules. Yet, rRNAs also contain sufficient sequence variability so that relationships between closely related groups can be determined. The abundance of rRNAs in actively growing cells made them readily obtained in the purified form needed for the earliest methodologies which determined their sequences directly. At first, rRNAs were compared by oligonucleotide cataloguing [42]. This entailed the digestion of purified rRNA with a ribonuclease to generate fragments that were then electrophorectically separated and individually sequenced. Comparison of rRNA oligonucleotide catalogs, and contiguous sequences determined from cloned genes, led to identification of highly conserved nucleotide tracts in dispersed regions of the 16S rRNA. The conserved tracts, serving as priming sites, made it possible to rapidly determine nearly complete sequences using rRNA as

the template for reverse transcriptase in dideoxynucleotide terminated sequencing reactions [18,19]. Highly conserved nucleotide tracts have also now been identified in the 23S rRNA [18].

The advent of PCR [35] made rRNA genes even more accessible for sequencing. In any PCR it is necessary to first have knowledge of sequence at the distal and proximal ends of the DNA to be amplified. The conserved nature of 16S rRNA enabled the design of primers that amplify nearly full length 16S rRNA sequences. As the template is the rRNA gene, the amplification target or product is sometimes referred to as rRNA. Medlin et al. [25] first described amplification of 16S-like rRNA from algae, fungi, and protozoa, and reports using 16S rRNA of bacteria and other eukaryotes soon followed [9,10,39,40]. The small amount of DNA needed for a PCR enables amplification from minute amounts of material to obtain phylogenetic information when only a few cells are available. The amplifications provide a simple, rapid approach (without the need to screen a large genomic DNA shotgun library) to obtain rRNA that may either be sequenced directly when working with pure cultures [6,7,9,11], or cloned and sorted through a recombinant library when working with natural communities.

Experimental Approach

Precautions

The exceptional sensitivity of PCR makes the possibility of amplifying contaminating DNA a real concern [38]. Care should be taken to avoid the introduction of extraneous DNA into PCR reagents or reaction mixtures. This is particularly necessary when performing amplification of rRNA genes; rRNA genes are ubiquitous, and the primers used in rRNA gene amplifications are often designed for broad phylogenetic groups. Therefore, appropriate negative control reactions must be employed. The potential to amplify contaminant DNA is even greater when using broad specificity primers in nested PCRs. Products from contaminant DNA in a negative control not visible during the first round of amplification could become apparent after the second round of amplification.

Contaminant DNA can arise from the carry over of previous amplifications of homologous targets, from DNA purifications carried out in the laboratory and from microbial contamination of reagents. DNA contamination can be effectively avoided with careful laboratory practice. Guidelines include physically separating areas where PCR reagents are used from areas where finished reactions are used, autoclaving solutions and PCR tubes, briefly centrifuging reaction tubes to avoid splashes when they are opened, changing gloves frequently, always using positive (previously verified template) and negative (no template) controls, and using positive displacement pipettes [16,17]. Aerosol resistant tips are a convenient alternative to positive displacement pipettes. Pipette tips that have a shaft guard (Rainin; Oakland, CA; #GPS-10G) are available to protect PCR reagents or reactions against contamination from the pipette shaft. Additionally, never use

the same pipette tip to withdraw from a tube more than once. Small aliquots of reagents can be stored at −20 °C until needed, with a "working PCR Box" kept at 4 °C that contains one aliquot of each reagent. DNA contamination in reagents, including that introduced during their manufacture, can be actively controlled by UV or DNase treatment [30, 33]. It is important to work in an area with limited air disturbance, such as might occur near a fume hood or beneath an air duct, when preparing reactions. DNase and RNase free tubes, tips and reagents are widely available and are highly recommended. The investigator should become familiar with these guidelines and use them habitually when performing PCRs.

Template

The first section of this manual (see Section 1) describes procedures for the isolation of microbial nucleic acids from pure cultures and environmental samples. The sensitivity of PCR also makes it possible to amplify rRNA genes directly from small amounts of cells [12,36], a portion of a lyophilized cell pellet [41], or with DNA obtained using rapid nucleic acid purification protocols [11].

DNA templates must be of high-molecular weight. The size and quality DNA is assessed by electrophoresis on a 0.8% agarose gel. Often nucleic acid samples purified from the environment are highly degraded and sheared which can lead to the formation of chimeric rRNA gene PCR products. During the annealing cycle, amplified rRNA genes from different templates may re-associate leading to heterogeneous products in proceeding cycles [20,32].

DNA purified from an environmental sample often has contaminating inhibitors in the final preparation. DNA purity is assayed by determining the ratio between wavelength measurements at 260 nm and 280 nm. Pure DNA has an A260/A280 ratio between 1.8 and 2.0. An absorbance ratio of less than 1.8 will indicate the presence of potential inhibitors such as humic acid, protein or phenol. These inhibitors may be eliminated by re-extraction, ethanol precipitation and/or centrifical ultrafiltration through a purification cartridge (such as available from Mo Bio Laboratories, Inc.; Carlsbad, CA). However, it is unlikely that DNA extracted from an environmental sample will be purified to an A260/A280 ratio of 1.8. Typically one can expect the DNA to have an A260/A280 ratio of around 1.6 with a brownish color due to humic substances. The presence of inhibitors in the final DNA preparation, which may lead to false negative results, can be assessed by preparing control reactions spiked with template DNA known to amplify under the PCR conditions being used.

It is also possible to perform amplification beginning with a rRNA template. In the first step of the process a cDNA is generated through reverse transcription (RT) [28]. However, secondary structures and modified nucleotides in an rRNA template can interfere with polymerization causing early termination of the cDNA. This could skew conclusions of studies on *in situ* microbial diversity. Interference from secondary structure can sometimes be overcome by using a DNA polymerase such as *Tth* (*Thermus thermophilus*) that can transcribe RNA at a higher temperature in the presence of Mn^{2+}. Next, the RT product is amplified in a PCR (*Tth* in

Table 1. Web Sites Useful for Primer Design.

Software	Sponsor	Web address
CODEHOP	Fred Hutchinson Cancer Research Center	http://www.blocks.fhcrc.org/codehop.html
Primer3	Whitehead Institute for Biomedical Research	http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
NetPrimer	PREMIER Biosoft International	http://bioinf.bmi.ac.cn/mirror/NetPrimer/netprimer.html
Primer Premier 5	PREMIER Biosoft International	http://www.PremierBiosoft.com/primerdesign/primerdesign.html
GeneWalker	CyberGene	http://www.cybergene.se/primerdesign/
Web Primer	Saccharomyces Genome Database	http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer
PRIMROSE	School of Biosciences, Cardiff University	http://www.cf.ac.uk/biosi/research/biosoft/

the presence of Mg^{2+} will accomplish this as well). Examination of the RT-PCR product on an agarose gel often reveals a very faint band or no band at all. It is often necessary to do a second round of PCR here, using the RT-PCR product as the second round template.

Primers

The goal in designing primers is developing a pair that will effectively hybridize to the target DNA and achieve the desired amplification. Beyond that, a well designed primer can enhance the efficiency and yield of the reaction. The finer points of primer design have been detailed by Innis and Gelfand [14] and by Saiki [34]. General primer characteristics are: a length of 18–24 bases, noncomplementary 3′ ends to avoid primer-dimer formation, no internal secondary structure and a 40–60% G-C content. The calculated T_m of the primers should be closely matched and in the range of 55 to 65 °C. There are many software programs available on the internet that will aid in primer design and primer analysis. Some of the programs available are listed in Table 1.

Primers of broad specificity that amplify nearly full-length 16S-like, small subunit rRNA genes are listed in Table 2. The primers in Table 2 generally target the same region of the gene with minor variations in length or sequence used by different investigators. Restriction endonuclease sites engineered at the 5′ ends are recognized by enzymes that rarely cut within a small subunit rRNA gene. A primer pair, one forward and one reverse, selected for the broadest specificity among the organisms of interest (i.e., *Bacteria*, *Archaea*, or *Eukarya*) should enable amplification of *most* small subunit rRNA genes. It has become evident in recent years that

domain level primers are *not* all encompassing and this should be considered when selecting a primer set and interpreting the results [13,24,29]. Additional primers of broad specificity that will amplify shorter lengths can be adapted from those used for rRNA sequencing [7,18].

Primers can also be used that selectively amplify rRNA genes of phylogenetically defined groups [2]. Selection of primers can often be guided by comparison of sequences in a data base, or by what has proven successful in previous amplifications from closely related groups or strains. Many selective rRNA gene primers have been described in the literature and it is beyond the scope of this chapter to tabulate them. PRIMROSE is a program that uses sequences from the Ribosomal Database Project to identify and determine the phylogenetic range of oligonucleotides that may be used as rRNA probes or primers [3]. The PRIMROSE website is provided in Table 1. ProbeBase [22] is a database of published rRNA probes with information on target site and specificity. The Ribosomal Database Project II [23] and the ARB website (Strunk,O. and Ludwig,W., 1993–2002, ARB—a software environment for sequence data, http://www.arb-home.de) have programs for checking intended specificities of probes and primers, including those for large subunit 23S-like rRNAs, against rRNA sequences. It is highly recommended that any primer pair, whether newly designed or previously published, be evaluated for its intended specificity prior to beginning any study.

Reaction conditions

"Basic PCR" in the procedures section below describes a good starting reaction. It is always advisable to optimize around a standardized reaction and begin by varying annealing temperatures and Mg^{2+} concentrations [15]. In general, a reaction component at too high a concentration can promote misincorporation of nucleotides and non-specific priming. A less than optimal concentration can reduce the yield of the reaction. The products obtained from a reaction are analyzed on an agarose gel (0.8–2 %, depending on expected product size) where under the optimal conditions a single, bright band of the expected size will be observed.

Thermally stable polymerases from different suppliers may vary in definition of units and optimum magnesium concentration. Reaction conditions may therefore vary depending on the particular enzyme used. Enzyme storage buffers may contain gelatin, bovine serum albumin or nonionic detergents to stabilize the polymerase.

In standard 50 µl reactions, 2.5 units of *Taq* polymerase are routinely used, with a recommended range of 0.5–2.5 units. It is important to choose the DNA polymerase based on the application and need for accuracy [15,32]. Characteristics of DNA polymerases that can be used in PCRs are provided in Table 3.

Stock dNTP solutions come already prepared as part of a PCR kit or may be obtained from suppliers of molecular biology reagents. It is highly recommended that dNTP solutions are of the highest quality available and obtained from suppliers specializing in molecular biology. dNTPs are present in equimolor amounts of 20 to 200 µM each. Magnesium, critical to primer annealing and extension, should be optimized and present in the range of 0.5–2.5 mM. KCl promotes primer annealing

Table 2. PCR primers useful for the amplification of nearly full length small subunit rRNA genes.

Primer[a]	Sequence[b]	Priming site[c]	Designed Specificity	Reference and comments[d]
63f	CAGGCCTAACACATGCAAGTC	43–63	most *Bacteria*	[24]
T63F	CAGGCCTAACACATGCAAGTT	43–63	most *Bacteria*	[13]
1387r	GGGCGGWGTGTACAAGGC	1404–1387	most *Bacteria*	[24]
1389r	ACGGGCGGTGTGTACAAG	1406–1389	most *Bacteria*	[29]
fD1	ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG	8–27	most *Bacteria*	[39] for, *Eco*RI, *Sal*I
fD2	ccgaattcgtcgacaacAGAGTTTGATCATGGCTCAG	8–27	enterics and relatives	[39] for, *Eco*RI, *Sal*I
fD3	ccgaattcgtcgacaacAGAGTTTGATCCTGGCTTAG	8–27	*Borrelia* spirochetes	[39] for, *Eco*RI, *Sal*I
fD4	ccgaattcgtcgacaacAGAATTTGATCCTGGCTTAG	8–27	Chlamydiae	[39] for, *Eco*RI, *Sal*I
rD1	cccgggatccaagcttAAGGAGGTGATCCAGCC	1541–1525	many *Bacteria*	[39] rev, *Hind*III, *Bam*HI, *Xma*I
rP1	cccgggatccaagcttACGGTTACCTTGTTACGACTT	1512–1492	enterics and most *Bacteria*	[39] rev, *Hind*III, *Bam*HI, *Xma*I
rP2	cccgggatccaagcttACGGCTACCTTGTTACGACTT	1512–1492	most *Bacteria*	[39] rev, *Hind*III, *Bam*HI, *Xma*I
rP3	cccgggatccaagcttACGGATACCTTGTTACGACTT	1512–1492	Fusobacteria and most *Bacteria*	[39] rev, *Hind*III, *Bam*HI, *Xma*I
5′	gcgggatccGAGTTTGATCCTGGCTCAG	9–27	most *Bacteria*	[20] for, *Bam*HI
3′	cgcggatccAGAAAGGAGGTGATCCAGCC	1542–1525	most *Bacteria*	[20] rev, *Bam*HI
pA	AGAGTTTGATCCTGGCTCAG	8–27	most *Bacteria*	[8,9] for
pH	AAGGAGGTGATCCAGCCGCA	1541–1522	most *Bacteria*	[9] rev

reverse	GGTTACCTTGTTACGACTT	1510–1492	*Bacteria*	[8] rev
Primer A	ccgaattcgtcgacAACCTGGTTGACCTGCCAGT	1–21	*Eukarya*	[25] for, *Eco*RI, *Sal*I
Primer B	cccgggatccaagctTGATCCTTCTGCAGGTTCACCTAC	1795–1772	*Eukarya*	[25] rev, *Sma*I, *Bam*HI, *Hind*III, *Pst*I
Primer A	ccgtcgacgagctcAGAGTTTGATCMTGGCTCAG	8–27	most *Bacteria*	[11] for, *Sal*I, *Sac*I
Primer B	cccgggtaccaagcttAAGGAGGTGATCCANCCRCA	1541–1518	most *Bacteria*	[11] rev, *Sma*I, *Kpn*I, *Hind*III
forward	CTCCGGTTGATCCTGCC	7–23	used with a hyperthemophilic methanogen	[4] for
reverse	GGAGGTGATCCAGCCG	1539–1524	used with a hyperthemophilic methanogen	[4] rev
27f	AGAGTTTGATCMTGGCTCAG	9–27	most *Bacteria*	[18] for, adapted from a sequencing primer
1492r	TACGGYTACCTTGTTACGACTT	1513–1492	most *Bacteria*, *Archaea*	[18] rev, sequencing primer adaptable to PCR
1525r	AGGAGGTGWTCCARCC	1541–1525	most *Bacteria*, *Archaea*	[18] rev, sequencing primer adaptable to PCR

[a]Some primers were not specifically named by the authors. Note the names “Primer A” and “Primer B” were used by different authors for different primers.
[b] Sequences written in the 5′ to 3′ direction. Lower case indicates linker sequence that contains restriction endonuclease recognition sites, upper case indicates region hybridizing with the rRNA gene. The *Pst*I site in eukaryotic Primer B isn within the region hybridizing to the rRNA gene. M = A and C; R = G and A; Y = C and T; W = A and T; N = C,A,T and G; positions where the synthesized primer contains equimolar amounts of more than one nucleotide.
[c] Priming sites indicated by E. coli 16S rRNA numbering for Bacterial- and Archaeal-specific primers, and by the numbering of Saccharomyces cerevisiae 18S rRNA for the eukaryote primers.
[d] for; forward primer with the same sequence as the rRNA site, with extension towards the 3′ end of the rRNA. rev; primer is complementary to the rRNA sequence, with extension towards the 5′ end of the rRNA.

Table 3. Thermally Stable DNA Polymerases.

Thermophilic DNA Polymerase	Features and Applications	Exonuclease Activity	Optimum Extension Temp.
Taq (*Thermus aquaticus*)	PCR for amplifying, cloning or labeling. Has a relatively high error rate.	5′–3′	70–75 °C
Tth (*Thermus thermophilus)*	Efficiently reverse transcribe RNA in presence of Mn^{2+} at higher temp. and synthesize DNA from DNA template in presence of Mg^{2+}. Primer extension.	5′–3′	70–75 °C
Pfu (*Pyrococcus furiosus*)	High fidelity enzyme, low error rate. Results in blunt ended PCR products. Use in PCR, primer extension, cloning, DNA expression, mutation analysis.	3′–5′	72–74 °C
Tli (*Thermococcus litoralis)*	High fidelity PCR, Primer extension.	3′–5′	70–75 °C
Tfl (*Thermus flavus*)	PCR, RT-PCR, 3′ A-tailing of blunt ends, Primer extension, DNA sequencing	5′–3′	70–75 °C

and should not exceed 50 mM. The reactions are buffered with Tris-HCl (10–50 mM, pH 9.0). Primer concentrations used in rRNA gene amplifications range from 0.1 to 0.6 μM with 0.1 ng to 10.0 ng DNA template added to a 50 μl reaction.

Enhancers

It is often beneficial to add additional components to the PCR to "enhance" the specificity and efficiency of the amplification reaction [15]. Enhancer benefit should be determined experimentally during the PCR optimization of each specific template/primer combination. Some commonly used enhancers are described in Table 4.

Post amplification

The products of rRNA gene amplification are often further analyzed. Direct analysis, as might occur during a study of microbial community composition, can be accomplished with denaturing gradient gel electrophoresis (DGGE) [27] or identification of terminal restriction fragment length polymorphisms (T-RFLP) [21]. Clone libraries can be conveniently constructed with commercially available kits designed for PCR products (e.g., TA Cloning System; Invitrogen Corp., San Diego, CA). rRNA inserts in clone libraries are readily screened by digestion with restriction enzymes that recognize four base pairs [26]. Conserved rRNA sequencing primers [7,18] enable rapid sequencing of plasmid inserts or PCR products. These procedures are described in detail elsewhere in this manual.

Table 4. Common PCR Enhancers.

Enhancer	Function	Concentration range
DMSO (dimethyl-sulfoxide)	ΔT_m of primer-template hybridization reaction to enchance specificity.	1–10% (v/v)
Formamide	ΔT_m of primer-template hybridization reaction to enchance specificity.	1.25–10%
Glycerol	ΔT_m of primer-template hybridization reaction to enchance specificity. Stabilizes polymerase.	5–20% (v/v)
BSA (bovine serum albumin)	Binds deleterious factors that might otherwise bind to the polymerase.	10–100μg/ml, 0.01–0.1% (w/v)
Non-ionic detergents	neutralizes charges of ionic detergents used in template preparation and other inhibitors	Tween 20: 0.05% (v/v) Triton-X-100: 0.01% (v/v) Nonidet P40 0.5% (v/v)

Procedures

Work at a clean area covered with fresh bench paper and observe the precautions given above. Include the appropriate positive and negative controls. DNA is added next to last to limit the potential for carry over and the DNA polymerase is added last.

Template DNA

High-molecular weight DNA (around 25 kb) of the highest purity obtainable should be used. We use about 100 ng of pure culture DNA in a 50 μl reaction. Cells from pure cultures may also serve to provide template (see Note).

Note:
Suspend a small amount of cells from a plate colony (about 1 μl) in 1.0 ml 1/10 TE, vortex, and alternatively freeze (−70 °C) and thaw (65 °C) the suspension three times. About 2 μl of this lysate is added to the reaction. This may also be adapted to small amounts of cells in liquid cultures.

Basic PCR

Add the following components to a sterile, DNAse/RNase free, thin walled, 0.2 ml tube:

PCR Reagent	Voulme for standard 50μl reaction	Final concentration	Optimization range	Commonly used range
10X PCR buffer	5.0 μl	1 X	1 X	1 X
25mM $MgCl_2$	5.0 μl	2.5 mM	0.5–5.0 mM	1.5–2.5 mM
10mM (each) dNTP	1.0 μl	200 μM	200 μM each	200 μM each
Forward primer (25μM)	0.8 μl	0.4 μM	0.1–0.6 μM	0.1–0.6 μM
Reverse primer (25μM)	0.8 μl	0.4 μM	0.1–0.6 μM	0.1–0.6 μM
DNA template	varies depending on DNA concentration	0.1–10 ng		1–10ng bacterial DNA 0.1–1ng plasmid DNA
H_2O	bring to 50μl volume			
5 Units/μl DNA polymerase	0.5μl	2.5 Units		0.5–2.5 Units/50μl reaction

Mix gently, quick spin in a microcentrifuge to bring the solution to the bottom of the tube and place in the temperature controller.

Notes:
The 10 X PCR buffer indicated does not contain Mg^{2+}. The final concentration of magnesium used in this reaction is 2.5 mM. Alternatively, a PCR buffer which already contains magnesium may be purchased and the magnesium concentration optimized if needed.

When performing multiple PCRs with the same primer pair a "master mix" that combines components can be prepared and aliquoted to the reaction tubes.

A mineral oil overlay is often placed on the reaction mixture if the thermal cycler used does not have a top heater.

Thermal cycle parameters:

Cycle	Temperature	Temp Range	Time	# of Cycles
Initial Denaturation	94 °C	94–95 °C	2 minutes	1
Denaturation	94 °C	94–95 °C	30 seconds–1 minute	25–30
Annealing	5 °C below primer T_m	55–72 °C	1 minute	25–30
Extension	72 °C	72 °C	30 seconds–1 minute	25–30
Final Extension	72 °C	72 °C	15 minutes	1
Soak and Hold	4 °C	4 °C	99 hours	99

Note:
Lowering the annealing temperature diminishes primer specificity and allows for mismatches in the template/primer hybrid. This is useful when the primer

sequence may vary from the target site, but it could also allow for amplification of non-specific sequences.

Enzyme addition

Start the thermal cycler temperature program. When the initial denaturing temperature has been reached, pause the machine and add:

2.5 units of DNA polymerase to each reaction tube

Complete the thermal cycle program.

Note:
This "hot start" increases specificity of priming by thoroughly denaturing the template.

Agarose gel analysis

Analyze 15 μl of the completed reaction on an agarose gel with size markers (e.g., 1 kb ladder; Bethesda Research Laboratories, Gaithersburg, MD).

Solutions (Stored in small aliquots at −20 °C.)

TE
- 10 mM Tris-HCl, pH 8.0
- 1.0 mM EDTA.

10X PCR buffer for Taq DNA polymerase (supplied with enzyme)
- 100 mM Tris-HCl, pH 9.0.
- 500 mM KCl.

25 mM $MgCl_2$ supplied with enzyme

dNTP mixture (with a final concentration of 10 mM each dNTP)
- Available commercially

Primers are diluted in sterile distilled water to a stock concentration of 25 μM.

Double distilled water, autoclave to sterilize and aliquot through a 0.2 μm syringe filter.

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Molecular Microbial Ecology Manual, Second Edition **4.01**: 853–860, 2004.

Use of Biolog® for the Community Level Physiological Profiling (CLPP) of environmental samples

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Introduction

Carbon is a key factor governing microbial growth in soil, and functional aspects related to substrate utilisation can provide important information beyond that afforded by taxonomic level investigations or structural investigations based on rRNA or rRNA analysis [11]. The functional diversity of microorganisms, particularly as defined by the substrates used for energy metabolism, is integral to our understanding of biogeochemistry [16]. Indeed, it has been argued that it is diversity at the functional level rather than at the taxonomic level that is crucial for the long-term stability of an ecosystem [24].

The method involves direct inoculation of environmental samples into Biolog® microtiter plates (containing different C sources, nutrients, and a redox dye), incubation, and spectrometric detection of heterotrophic microbial activity. The method was originally developed [3] for medical strain identification, and has only later been adapted for use with inocula from extracted microorganisms from environmental samples [7]. Its simplicity and speed of analysis are attractive to the microbial ecologist, but the technique requires careful data acquisition, analysis, and interpretation.

Several approaches have been used to account for biases related to inoculum density, incubation time, and microenvironment [6]. For instance, standardisation of initial inoculum density is commonly used [7], although it is laborious and choice of cell enumeration method remains a subject of debate. Normalisation of optical density (OD) readings by dividing by average well colour development (AWCD) is restricted to a number of conditions [13, 14, 21]. Single time point readings [7] and integration of the OD over time are still the most widely used measuring strategies, but can be compromised by the effect of inoculum density. Use of continuous plate reading for analysing the kinetics, rather than the degree of colour development at a given time [13, 6], has led to the development of a sigmoidal type of growth model [22]. Nevertheless, the kinetic parameters are not independent of inoculum density [22, 9] and need to be normalised prior to statistical analysis [6]. Recently,

a normalisation procedure that employs integrated OD values derived from four dilutions of the same sample rather than a single dilution level has been developed as a cell density- and time-independent method of analysis [4]. Most of these approaches, however, have yet to be applied often enough to allow robust critical comparison.

Sampling, extraction of microbes and microplate incubation

As for most biological investigations, fresh samples are superior to any stored samples. If storage is necessary, samples may be stored up to 10 days at 4 °C, for longer periods freezing is recommended, which, however may cause a certain bias towards frost tolerant organisms. Before extraction, we usually let the samples equilibrate at room temperature for 2 days, and sieve samples with a 2 mm mesh sieve.

The literature contains many methods for bacterial extraction from soil, sediment and compost samples [see chapter 1.10]. The extraction procedures vary widely among each other but they all include two main steps: 1) aggregate dispersion and 2) separation between cells and organic and inorganic particles. Dispersion techniques can be physical, chemical or both. Separation is carried out by centrifugation, according to sedimentation velocities (low-speed centrifugation), buoyant density (high-speed centrifugation), or both [1].

Two microplate options are possible, either use of Biolog EcoPlates [18] or GN plates [3], containing 31 or 95 different C sources, respectively, plus a water control. The EcoPlates contain 3 replications of the C and control wells. The EcoPlates contain substrates that are known to be plant root exudates [5] or that have previously been found to have a high discriminatory power among soil communities [15]. The plates are inoculated with 130 µl suspension, diluted (in 1/4 strength Ringer solution) to obtain a cell density of approximately 1×10^8 cells ml^{-1} (acridine orange direct count, AODC) [2]. The plates are then incubated at 20 °C in the dark (other incubation temperatures may also be selected), and subsequent colour development is measured every 12 hours for 5 d (592nm) using an automated plate reader. Readings may be terminated if the average well colour density reaches an optical density of 2. To avoid problems with different inoculum densities among soil samples, the determination of microbial biomass (e.g. with substrate induced respiration or a fumigation-extraction procedure) may be used for an initial comparison, and subsequent dilution of the extracts so as to obtain similar microbial biomass for each sample inoculum.

Data Management

Raw OD data are corrected by blanking each response well against its own first reading (immediately after inoculation). This blanking not only avoids the intrinsic absorbance of the carbon sources but also the negative values when compared to subtracting the control well from the response well. The AWCD is calculated from each plate at each reading time. For each plate, those time points of reading are

selected that have an AWCD closest to 0.6. Alternatively, also other AWCDs (e.g. 0.30, 1.00) may be chosen. To diminish biases by different inoculum densities, data may be normalised by dividing each well OD by the AWCD [8]. This is particularly important when inoculum densities are not standardised prior to inoculation.

Data analysis may be further elaborated by calculating the area under the curve for each well OD for the entire period of incubation [12], or by estimation of kinetic parameters (K, r, s) by fitting the curve of OD versus time to a density dependent logistic growth equation [22],

$$Y = OD_{592} = \frac{K}{1 + e^{-r(t-s)}}$$

where K is the asymptote (or carrying capacity), r determines the exponential rate of OD change, t is the time following inoculation of the microplates, and s is the time when the mid point of the exponential portion of the curve (i.e., when y = K/2) is reached.

Diversity parameters, such as Shannon's diversity index (H) and Evenness (E) [25], can also be calculated as a means of evaluating microbial community functional diversity [27], using the following equations:

$$H = -\sum p_i(\ln p_i) \quad and \quad E = \frac{H}{H_{\max}} = \frac{H}{\log S},$$

where p_i is the ratio of the corrected absorbance value of each well to the sum of absorbance value of all wells, S (substrate richness) is the number of different substrates used by the community (counting all positive OD readings). H encompasses both the richness and evenness of substrate utilisation, E (substrate evenness) is a measure of the uniformity of activities across all substrates.

Procedures

The following procedure is the basic protocol for CLPP analysis. According to specific needs, extraction procedures may be modified, inoculation densities and temperature may be altered, or single-point data reading may be replaced by continuous readings (see above).

Cell Extraction

We suggest two alternatives for the extraction procedure. The first one combines physical and chemical agents for soil dispersion and a low-speed centrifugation for separating bacterial cells from soil particles according to their size. The second one includes a physical dispersion and a high-speed centrifugation step, using a non-ionic substance (Nycodenz®) to generate a density barrier between cells

and soil colloids. The latter extraction method is more expensive and time-consuming than the former, but ensures the maintenance of the CLPP of the extracted and purified bacterial community [23].

Extraction protocol 1 (modified from [17])

1. Blend 5 g fresh soil/sediment/compost with 20 ml of 0.1% (w/v) sodium cholate solution, 8.5 g cation exchange resin (Dowex 50WX8, 20–50 mesh, Sigma) and 30 glass beads.
2. Shake the suspension on a head-over-head shaker (2 hours, 4 °C).
3. Centrifuge at 800 $\times g$ for 2 minutes.
4. Decant the supernatant into a sterilised flask.
5. Re-suspend the pellet in 10ml Tris buffer (pH 7.4) and shake for 1 hour.
6. Centrifuge as above and add the supernatant to the first step extract.
7. (optional). If the extract is turbid or dark (due to clay or humus particles) centrifuge the resulting supernatant another time.

Extraction protocol 2 (as described in [1])

1. Add 20 ml of Milli-Q water to 2 g soil wet weight in 40 ml centrifuge tubes.
2. Disperse the suspension by using a Waring blender for 1 minute (3 times, 4 °C).
3. Pipette carefully 6–7 ml of Nycodenz® (60% w/v) below the soil suspension.
4. Centrifuge at 10,000 $\times g$ for 30 minutes (4 °C) with a swing-out rotor, for establishing the density barrier.
5. Transfer 10 ml of the supernatant to a 250-ml centrifuge bottle containing 100 ml of Milli-Q water.
6. Centrifuge at 16,000 $\times g$ for 60 minutes (4 °C).
7. Resuspend the bacterial pellet in 1/4 strength Ringer solution (Merck, Darmstadt, Germany).

Inoculation and incubation

1. If you want to perform statistical testing of single substrate utilisation with MANOVA, inoculate a sufficient number of replicates (one EcoPlate contains 3 replications) according to $\mathbf{n_i} \times \mathbf{q} = \mathbf{n} \geq \mathbf{31} + \mathbf{q} + \mathbf{2}$, where q is the number of groups to be compared, n_i is

the replicate number required per group (sample sizes are equal in each group). For example, if two groups are compared, an $n_i > 17$ is required (i.e. 6 EcoPlates) [20].
2. Dilute the samples with 1/4 strength Ringer solution 10 (sediment)- to 1000 (composts)- fold and check the cell density by acridine orange direct counting (AODC).
3. Dilute your samples appropriately to obtain a cell density of approximately 1×10^8 cells ml^{-1}.
   Note: In case of background colouration of the extract, further dilutions are recommended.
4. For environmental investigations the use of Biolog® EcoPlates™ [18] is suggested. The plates are inoculated with 130µl of the diluted cell suspension.
5. Cover the plates with a lid and incubate at 20 °C in the dark (other incubation temperatures may also be used; if you chose >30 °C, more frequent readings are suggested).
6. Measure colour development (592nm) every 12 hours for 5 d using an automated plate reader. Readings may be terminated if the average well colour density reaches an optical density of 2.

Note

If curve parameters or the area under the curve are to be determined make sure you always have the same reading intervals and the same number of readings (*e.g.* [11]). In case of a long lag-time, or low incubation temperature, incubation may be prolonged (reading interval up to 24 hours). Make sure that desiccation is avoided by placing the plates in polyethylene bags if you incubate at temperatures >30 °C.

Data Management

1. Raw OD data are corrected by blanking each response well against its own first reading. This blanking not only avoids the intrinsic absorbance of the carbon sources but also the negative values when compared to subtracting the control well from the response well.
2. Two data management alternatives are suggested:
   a) Single-point absorbance readings. There are three possibilities:
      – Select a single reading time (e.g. 24 hours, 48 hours, 72 hours). This is only feasible when inoculum densities are previously standardised.
      – Calculate the AWCD from each plate at each reading time. For each plate, the time point where the AWCD is closest to 0.6

is chosen. Alternatively, other AWCDs (e.g. 0.30, 1.00) may also be chosen.
   - Normalise data by dividing each well OD by AWCD [8]. This is particularly important when inoculum densities are not standardised prior to inoculation.

b) Kinetic approach: This requires continuous readings. It yields more information, but is more dependent on inoculum density than the approach using the AWCD normalised data [9]

3. Use discriminant analysis (DA) or principal component analysis (PCA) for exploratory data analysis, and MANOVA for statistical testing (applied on DA or PCA factors)
4. From the data, functional diversity parameters may be deduced from single substrates or substrate groups (like carbohydrates, amino acids, carboxylic acids, etc.). Use discriminant analysis (DA) or principal component analysis (PCA) for exploratory data analysis, and MANOVA for statistical testing (applied on DA or PCA factors)
5. Data analysis may be further elaborated by calculating the area under the curve for each well OD for the entire period of incubation [12], by the estimation of kinetic parameters [22] or by studying different dilution levels as outlined above.

Applications of the method, limitations and final remarks

Community level physiological profiling is a fast screening method to detect differences among treatments. Since first published, *in vitro* community level physiological profiles (CLPPs) have been used widely to characterise microbial communities of different habitats, ranging from sediments to seawater, and from oligotrophic groundwater to soils and composts [7, 10, 11, 19].

However, it is not possible to draw conclusions on cause-effect relationships because the utilisation of a certain substrate is not necessarily coupled to a change in that substrate's availability. Also, links to structural changes of a community may not be made, since changes in CLPPs can either be due to an adaptation of the prevalent microflora, or to a change in community composition. The researcher must always keep in mind that it is not the utilisation of single substrates, but

the change in the substrate utilisation pattern that is important. It also must be emphasized that CLPP is not a culture-independent method, but rather biases towards fast-growing, easily culturable species [26]. Thus, CLPP should not be seen as a stand-alone method, but it can be highly complementary to other approaches (e.g. classical and molecular) in the polyphasic analysis of microbial communities.

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Molecular Microbial Ecology Manual, Second Edition **5.01**: 1047–1068, 2004.

Natural transformation in aquatic environments

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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+ 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 tranformation 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+) soil isolate expressing very high levels of competence [14]. *A. calcoaceticus* strain HGW1521(pQM17) is a histidine auxotroph (His−) 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 used immediately and cannot be stored.

Solutions

– B22 salts solution (pH 7.2) [2]
  – 3.89 g/l KH_2PO_4.
  – 12.5 g/l K_2HPO_4.
  – 0.19 g/l $MgSO_47H_2O$.
  – 1.09 g/l $(NH_4)_2SO_4$.

- 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 (A540) of 0.1 with the overnight culture and incubate with shaking at 20 °C.
3. Follow the optical density (A540). Remove enough culture to yield approximately 10^7 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 DNasel (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 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 × 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 DNasel 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 μm, 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 × 15 × 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 DNasel (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;

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

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 μ g 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 gyrotatory 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 5×10^{-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 $MgSO_4$.
2. Add the following stock solutions;

Stock solution	Component(s)	Concentration (g/l)	Volume (ml)
#1	KCl	55.0	10.0
	$NaHCO_3$3	16.0	
#2	KBr	8.0	10.0
	$SrCl_2$	3.4	
#3	Sodium silicate	4.0	1.0
#4	NH_4NO_3	1.6	1.0
#5	NaF	2.4	1.0
#6	Na_2• HPO_4	8.0	1.0
#7	$CaCl_2H_2O$	238.0	10.0
#44	Na_2EDTA	3.0	10.0
	$FeCl_3$• $6H_2O$	0.384	
	$MnCl_2$• H_2O	0.432	
	$CoCl_2$• $6H_2O$	0.002	
	$ZnCl_2$	0.0315	
	$CuCl_2$	0.025	
	H_3BO_3	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^3 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 5×10^{-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^2 100 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 $\sim$ 80%.

Concentration of microbial populations from sponge tissues

1. Sponge tissue samples (10 g wet wt.) are cut into 2 cm^3 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 $\times$ 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^3 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 Nuclepore 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 $MgCl_2$ 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 $^\circ$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 5×10^{-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
Plasmids		
r1162	$su^r str^r mob^+$	[18]
PQSR50	r1162::Tn5 $km^r str^r$	[19]
pLV1013	$km^r str^r$ *xylE* c1857	[31]
E. coli donor strains		
RM1259(pQSR50)	MV10 K12 C600 km^r str^r	[19]
RM1208(r1162)	MV10 K12 C600 str^r	[19]
ED8564(pLV1013)	lac^- met^- thi^- $hsdr^-_m$ $-r^-_k$ km^r str^r xylE	[31]
Recipient strain		
Vibrio JT-1	$nal^r rif^r$	[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 × 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 DNasel.
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 DNasel 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^3 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.

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Molecular Microbial Ecology Manual, Second Edition **6.01**: 1187–1200, 2004.

Lac as a marker gene to track microbes in the environment

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Introduction

The environmental use of microorganisms as a means to combat pests and diseases, to remediate contaminated land and act as bio-fertilisers and plant growth promoters has been a focus for research for many years. However, the successful application of living microbial inocula is dependent on a thorough understanding of the behaviour of the organisms involved in relation to the environment into which they are released [8]. In order to gain this type of understanding, a large variety of selective and semi-selective media have been developed that allow selective recovery of organisms from environmental samples. Such media make use of specific characteristics that are inherent to the organism under study. Such characteristics include, growth conditions (temperature, pH, etc), resistance to antibiotics and heavy metals, tolerance to osmotic pressure and/or expression of phenotypic characteristics such as fluorescent pigments, coloration and morphology. In this way, media have been designed for the isolation and quantification of many ecologically important bacteria [27]

Recent advances in recombinant technology have allowed the identification, characterisation and isolation of genes that encode for useful characteristics in relation to environmental applications. After isolation, and introduction into suitable vector systems, such genes can subsequently be introduced into a single recipient organism, allowing the creation of a genetically modified microorganism (GMM) with potentially 'improved' characteristics for environmental use. However, public concerns over the possible (negative) consequences of releasing functionally 'improved' organisms into the environment has increased the need for the development of sensitive detection techniques for microorganisms in environmental samples. The need to detect GMMs in the environment has created an impetus for the development of detection methods employing molecular techniques. Besides genes that encode for functional characteristics that are aimed to improve the environmental performance of a GMM, a variety of genes have been isolated from several organisms to facilitate detection of a particular GMM in the environment. Depending on the type of genetic marker employed, a GMM may be tracked phenotypically, relying on the expression of the marker gene by the GMM. Alternatively,

the presence of a marker gene can be identified by methods that do not rely on its expression [19]. In general, maker genes can be divided into three groups:

1. Short but unique oligonucleotide sequences that act as a genetic signature.
2. Genes that provide a selectable characteristic, such as resistance to an antibiotic, a heavy metal or ability to metabolise an unusual chemical.
3. Chromogenic markers that provide a colour change.

Chromogenic markers contain one or more genes whose presence in a microbial cell may be detected by an ability to produce a colour change in a substrate. Isolation of microorganisms on media supplemented with substrates that are capable of being transformed into coloured products has been used successfully to distinguish bacteria expressing the *xylE* gene, which encodes for catechol 2,3-dioxygenase [30] and those that carry the *lacZY* genes from *Escherichia coli*. The latter encode ß-galactosidase (*lacZ*) and lactose permease (*lacY*) [14]. Other systems in this category are GUS (expression of ß-glucoronidase), the *lux* operon from *Vibrio fischeri*, in which organisms are identified by their ability to bio-luminesce [28] and accumulation of GFP (Green Fluorescent protein) within cells that express the GFP genes from the jelly fish *Aequorea victoria* [5].

The *lac* operon

The *lac* operon of *E. coli* spans approximately 5300 base pairs and includes the *lacZ*, *lacY* and *lacA* genes in addition to the operator, promoter and transcription terminator regions. Since its description [18], the lactose operon has been a model system of great usefulness in biology. Study of this operon has touched on some significant questions in biology. For example, fundamental questions of the mechanisms involved in expressing genes were first studied in this system [18]. The discovery of the *lac* repressor and its binding to an operon site on the DNA was one of the first problems concerning protein-DNA interactions to be examined [25]. Studies of ß-galactosidase in relation to *lacZ* mutants have been important in defining many aspects of gene-protein relationships. Studies with fragments of ß-galactosidase also have served as a model system for investigating protein-protein interactions. The lactose permease, the product of the second structural gene, *lacY*, was the first membrane transport protein that was studied extensively. Many fundamental concepts of the transport of molecules into the cell were derived from these studies. It is therefore not surprising that structures of the lactose operon have been investigated intensively [3]. For example, Fowler and Zabin [15] reported the amino-acid sequence of ß-galactosidase in 1978. When methods for determining DNA sequences became available, the DNA sequence of both the *lac* repressor and the control elements of the lactose operon was determined [3,16]. After the DNA sequence of *lacY* was confirmed [4], the DNA sequence of *lacZ* was determined [20] with the amino acid sequence of ß-galactosidase as confirmation. The third component of the *lac* operon, *lacA*, was sequenced in 1985 and its amino-acid sequence determined [17]. The *lacA* gene encodes for

thiogalactosidetransacetylase and is thought to be involved in detoxification of thiogalactosides by the cell.

Procedures

Genetic marking of fluorescent Pseudomonas with lacZY

To be of use for tracking microbes in the environment, marker genes need to be rare in the release environment to allow distinction between the marked organism and indigenous populations on non-selective media. Alternatively, the isolation medium should be selective, not allowing growth of indigenous bacteria that express the phenotypic features encoded by the marker gene. All fluorescent pseudomonads isolated from the environment are unable to use lactose as a carbon source. This feature makes the genes that make up the *lac* operon ideal as genetic markers for this group of bacteria. Not only do these genes enable a recipient organism to utilise lactose as the sole carbon source, but the *lacZ* gene, which encodes for ß-galactosidase, allows cleavage of the substrate X-gal (5-chloro-4-bromo-3-indolyl-ß-D-galactopyranoside) into a bright blue product that is easily recognised [14]. To determine whether *lacZ* alone was sufficient for lactose utilisation, *lacY* was eliminated on broad host-range plasmids (pMON5002 and pMON5013) which were constructed to carry genes into recipient bacteria. Plasmid pMON5002 was restricted with EcoRI at the unique site in the *lacZ* coding sequence and with BglII, which cuts uniquely down stream from *lacY*. The incorporation of the *lacZ* gene alone did not confer the ability of transformed *Pseudomonas* cells to efficiently utilise lactose as a sole carbon source. The inclusion of *lacY* (lactose permease) enabled growth at a rate comparable to that observed on glucose. This implies that in *Pseudomonas*, as in *E.coli*, ß-galactosidase remains a cytoplasmic protein, which does not gain access to sufficient lactose, without an active lactose transport mechanism. The *lacY* product, lactose permease, provides this transport mechanism [14].

Although different strategies can be employed to generate *lacZY* marked bacteria, the following might serve as an example of procedures employed by Barry [2] to obtain a *lacZY* marked *P. aureofaciens* that was used for release in the field [12]. Although the *E. coli lacZY*

genes expressed from different promoters on broad host-range plasmids are highly effective selectable markers for *Pseudomonas* [14], these genes are equally effective when delivered by a transposon Tn*7*-*lac* element into the bacterial chromosome. The advantage of Tn*7* is that the transposition gene products function in *trans* and only 160 base pairs at each terminus are required to allow this transposition to occur. In addition, Tn*7* inserts with high specificity integrate at high frequency into the chromosomes of many Gram-negative bacteria. Tn*7* typically has only one insertion site per bacterial chromosome and is relatively rare in the environment [24]. Therefore, using the Tn*7* based delivery system eliminates the need for screening through unwanted transposon mutants [22]. Originally the Tn*7*-*lac* element was composed of two unstable plasmids of different incompatibilities. Although the method is useful for generating *lac*-marked *Pseudomonas*, features that make this method unsuitable for common use are that any manipulation of the Tn*7* element has to be done on a low copy plasmid not amenable to fast multiplication [2]. Also the Tn*7*-*lac* element itself contains a number of unknown regions of DNA that might originate from *E. coli* or from φ80 or λ at the end of the *lacA* gene. The first improvement involved the use of the smaller IncQ plasmids in the cloning and delivery system. Because of the decrease in size and the apparent broader host-range of IncQ plasmids, the double IncQ system can be used effectively in the cloning of genes and in the introduction of Tn*7*-*lac* elements into *Pseudomonas*. To further facilitate the cloning steps, and in particular the construction of more versatile and more widely applicable Tn*7*-*lac* elements, a small replicon was made by making a deletion of pUC8. A 500 bp fragment was cloned in this from into the *E. coli* chromosome containing the Tn*7* insertion site. Into this *E. coli* strain the Tn*7*-*lac* element had been transposed and the bacterium contained a helper plasmid. From the progeny of this bacterium, a replicon of 2 kb with an 11 kb transposon was isolated. In this form the Tn*7*-*lac* element was easier to manipulate. Smaller cloning Tn*7*-*lac* elements were then constructed and their effectiveness for the expression of the *lac* genes determined. Mono-component Tn*7*-*lac* delivery systems were developed to expand the range of bacteria that may be marked with the *lac* genes. These 'suicide' delivery vectors, based on unstable IncQ replicons or on pBRS22, eliminate the need for antibiotic sensitivities in the target bacteria and for the replication

of the delivery replicon. The IncQ-based mono-component delivery system was used to mark a fluorescent pseudomonad (Ps. 3732RN) to create Ps 3732RNL11. The Tn*7*-*lac* element in Ps 3732RNL11 is composed of around 1700 bp of the termini of Tn*7* and the *lacZY* genes (and a truncated *lacA* gene) promoted by the *iuc* operon promoter. The element used (Tn*7*-*lac*7117) contains a number of restriction sites to allow ease of cloning of additional genes, promoter replacements or substitution of *lac* with other selectable markers.

For applications in the environment, clearly stable integration of recombinant materials into the genome is desirable. Bailey et al. [1] successfully inserted two gene marker cassettes into the chromosome of a *P. fluorescens* isolate (SBW25). Given the potential for random and deleterious insertion, a strategy was adopted to facilitate detection whilst minimising the possibility of gene exchange and metabolic disruption. Two distinct chromosomal sites were therefore selected, approximately 1Mb apart on the chromosome, namely sites Ee and site -6-.

Site Ee was isolated from an EcoRI fragment from SBW25. The marker *lacZY* genes were inserted into a unique BglII site within the Ee site under the control of the iucA promoter isolated from pMON7117 [2]. This fragment was transferred onto a mobilisable suicide delivery plasmid and integrated into the chromosome of SBW25 at the Ee site by homologous recombination. Site -6- was also isolated on an EcoRI chromosomal fragment. A marker gene cassette containing *KanR* and *XylE* genes were inserted into the unique BglII site within the -6- site, with the integrating fragment being delivered by electroporation.

The transformation of *Pseudomonas* spp. using mobilisable suicide vectors as above or electroporation as in our laboratories using pMC1871 (Pharmacia) is often difficult and inefficient, requiring large quantities of transforming DNA and much subsequent screening. *Pseudomonas fluorescens* SBW25 appears to be more difficult in this regard than most other fluorescent *Pseudomonas* but even here methodologies have been developed for successful integration of genes.

The following might serve as a guide:

1. To provide biomass suitable for transformation, careful growth of bacteria to mid exponential phase is required, using cells at OD = 0.6 (550 nm).

2. After chilling on ice for 30 minutes, cells are pelleted by centrifugation in Falcon tubes (3K rpm for 3 minutes) followed by resuspension in 15% glycerol (V/V).
3. Cells from an initial culture volume of 250 ml are finally resuspended in 250|l of glycerol solution and stored on ice.
4. Purified plasmid DNA is obtained at a concentration of 1 mg/ml. Forty five ml of chilled SBW25 suspension is mixed with 5ml (5mg) of plasmid DNA in a pre-chilled microfuge tube and stored on ice for 1 minute before being transferred to a pre-chilled electroporation cuvette (2 mm gap, Biorad).
5. Electroporation is carried out using a Biorad Gene Pulsar apparatus (settings: 2.5KV, 200 (25(F)).
6. Within one minute of applying the potential, the cell suspension is mixed with 500 ml of SOB broth and gently mixed.
7. The contents of the cuvette is incubated at 30 °C for 4 hours before plating onto tetracycline-containing LB plates followed by incubation at 30 °C for 48 hours.
8. An initial screen of recombinants is made by selecting for a *TetR, KanR, lacZ*+ phenotypes. Recombinants carrying the newly introduced DNA are identified by using PCR analysis of DNA from isolates using primers homologous to regions of DNA that should be present within the newly inserted DNA.
9. After genotypic identification, functional assays for insertion of desired sequences are normally carried out. For example, using hplc or bioassay plates where the inserted genes are responsible antibiotic biosynthesis.

Integration of DNA of up to 7kb in length has been successfully achieved using this method, with predicted genotype and functionality observed.

Recovery of fluorescent pseudomonads expressing *lacZY* from environmental samples

Selective agar based media

Bacteria expressing the *lacZY* marker genes will be able to grow on mineral media such as M9 [26] amended with 1% (w/v) lactose [14].

Intrinsic resistance to antibiotics, such as rifampicin, might achieve further selection from the native microbial populations. This approach allows sensitive selection of up to 1–10 colony forming units (cfu) per g of non-sterile soil [13]. Problems arise when the natural soil populations have a significant proportion of bacteria that can use lactose as a carbon source and/or is resistant to the antibiotics used to select for the recombinant strain. For example, 1% of the culturable microbial community in a silty-loam field soil (Hamble series) taken from Littlehampton (W. Sussex, UK) was able to utilise lactose as the sole carbon source. A further 3.5% of the community was resistant to kanamycin incorporated at a level of 100mg/l, while 0.03% of the culturable bacterial community could utilise lactose and expressed resistance to kanamycin [11]. Clearly, minimal media such as M9 [26], amended with lactose and/or antibiotics are of little use for the selective recovery of recombinants in such situations. In this case, it was estimated that the detection limit of a triple marked *P. fluorescens* strain (SBW25EeZY-6KX, expressing the *lacZY* genes for lactose utilisation, the *aph1* gene for kanamycin resistance and the *xylE* gene encoding for catechol 2,3 dioxygenase), on minimal medium [26] amended with 1% (w/v) lactose, 50 mg/l X-gal and 100 mg/l kanamycin, was around 10^3 cfu/g soil [11]. On roots, where bacterial numbers are one to two log units higher than in soil, detection would only be possible if the recombinant was present in concentrations $>10^4$ cfu/g rhizosphere soil. Consequently, a more sensitive method was required to isolate *lacZY* marked *Pseudomonas* cells from environmental samples. A growth medium, called P-1, developed for the selective recovery of *Pseudomonas* strains producing fluorescent pigment [21] provided the solution. This medium is made up as follows:

KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.2 g
$NaNO_3$	5.0 g
Desoxycholate	1.0 g
Betaine	5.0 g
Agar	15 g
Distilled water	1 litre
pH	7.2–7.4

To obtain a clear medium, the ingredients are mixed thoroughly in a dry flask, before water is added. After mixing with water, the pH is adjusted using 1N NaOH. In our case, per flask, 5ml 1N NaOH is pipetted into a litre of medium to obtain the required pH. The medium is then autoclaved. When the medium has cooled sufficiently (40–45 °C), 50mg Xgal is added (1 ml stock solution of 50mg Xgal per ml DSMO). This medium will allow growth of both *lacZY* positive fluorescent *Pseudomonas* and indigenous *lacZY* deficient fluorescent *Pseudomonas*. The former will produce dark blue fluorescent colonies, while the latter will form white fluorescent colonies when viewed under long wave UV light. However, it has to be noted that the production of the blue pigment that results from the cleavage of X-gal will to some extent mask the fluorescence of the *lacZY* positive bacteria on agar media. The medium thus created enables phenotypic selection of the genetically marked *Pseudomonas*, from a background of indigenous fluorescent *Pseudomonas*. As indigenous fluorescent *Pseudomonas* are common (typically between 10^5–10^6 cfu/g soil), further selective agents have to be added to obtain a medium that is more selective. In the case of the triple marked *P. fluorescens* described before, addition of 100 mg kanamycin per litre medium, will only allow recombinants to grow. No doubt the same result can be obtained using other antibiotics to which the strain under investigation is resistant.

Surprisingly, good selective recovery can be obtained using P-1 medium from mashed root and leaf material that is colonised by fluorescent *Pseudomonas*, even though this material contains relative large quantities of carbohydrates and other substances that can be utilised by a variety of microorganisms. This indicates that the betaine used in the medium as a carbon source is probably not crucial for the observed selectivity for fluorescent pseudomonads. The more likely chemical in the medium that is responsible for *Pseudomonas* selectivity is desoxycholate. In fact, incorporation of desoxycholate into TSA (triptic soy agar) at a rate of 1g/l seems to induce selectivity for *Pseudomonas* (De Leij, unpublished results). Therefore, the use of selective media that obtain their selectivity independent of the carbon source allows recovery of recombinant cells from environmental samples that are relatively rich in nutrients. Examples include compost, sewage sludge, plant material and foodstuff. This also means that selective media for *lacZY* marked bacteria that are based on the

incorporation of lactose as the sole carbon source are unlikely to remain selective when recovery is attempted from samples that contain alternative carbon sources.

Most Probable Number techniques involving lacZY

The extreme selectivity of P-1 medium amended with antibiotics also allows the use of enrichment methods involving larger quantities of soil. Instead of using a selective agar medium, a broth can be created using the same recipe without the agar. A 0.1% soil suspension in P-1 broth amended with kanamycin (100 mg/l) and X-gal (50 mg/l) will provide a clear medium, that will turn blue after 3–7 days incubation in shake culture at 25 °C when at least 1 recombinant cell is present [9]. In theory, recombinant cells can be detected in this medium up to any required sensitivity (for example, using one litre broth, 1 cell/10g of soil can be detected). In practice, dilution series are prepared in 10ml selective broth and after incubation in shake culture those dilutions that turn blue are scored [9]. Whereas it is difficult to see fluorescence of *lacZY* positive colonies on agar, exposure of broth that contains *lacZY* positive fluorescent *Pseudomonas* will fluoresce intensely when exposed to long wave UV light. Most probable number estimates are subsequently used to estimate the number of cells in the original sample. Most probable number estimates, however, are inherently variable (unless large numbers of replicates and small dilution steps are used). So unless the number of recombinants in the sample are below the detection limit of agar plating (ca 50 cfu/g sample) dilution plating will be the method of choice to estimate the number of recombinant cells in a sample. In the case of qualitative detection of recombinant cells in environmental samples, enrichment is the more sensitive approach.

Application of *lacZY* marker genes in ecological studies

Environmental fitness of LacZY marked organism

The use of marker genes, such as *lacZY*, offers clear advantages in providing an extra tool allowing selective recovery of marked organisms from environmental samples. However, marker genes offer no

advantage to the modified organism in terms of its competitive ability. It seems therefore logical to assume that the extra metabolic load of expressing these genes will put the organism at a distinct disadvantage compared to the non-modified parental strain. For example, Compeau et al. [7] found that rifampicin resistant mutants of *P. putida* were recovered from soil at lower rates compared to their non-modified parents. After only two days in soil there was more than a 10-fold difference in recovery of the two strains. This problem is claimed not to occur with fluorescent *Pseudomonas* that are marked using the Tn7-*lac* system [23]. Co-inoculation of the rhizosphere of maize with a Tn7-*lac* modified *P. aureofaciens* (strain L11) and its non-modified parent (strain RN) at different ratios did not give an significant difference in recovery of the two strains 2, 3, 4, 8 or 11 days later [23]. Similarly, Bailey et al. [1] found that no difference in the competitive ability of the triple marked *P. fluorescens* SBW25EeZY-6KX and its non-modified parent in the phytosphere of sugar beet. However, recent investigations indicate that effects on environmental fitness of *lacZY* modified strains depend on the environmental conditions into which the recombinant is released. Environments, such as the rhizoshere of pea, provide enough nutrients to mask any difference in metabolic load. In environments with a certain degree of nutrient stress, such as in soil and the rhizosphere of wheat, the extra metabolic load conferred by expressing *lacZY* reduces the environmental fitness of the marked strain significantly [10]. Other factors, such as disruption of existing genes due to insertion of the marker genes seem to play no significant role [10]. In summary it can be stated that the use of *lacZY* as a marker gene, is likely to result in a lower recovery rate compared with the parental strain when conditions are adverse. In situations where there is little nutrient stress, it is unlikely that detectable differences between parental and the recombinant strain will occur.

Relevance of cfu counts

The use of the *lacZY* marker lies in the fact that these genes allow easy identification of microbial colonies that express ß-galactosidase activity on selective media into which X-gal is incorporated. This method of estimating viable recombinant cells in an environmental sample assumes therefore that:

- All recombinant cells present in a sample can be extracted
- Each viable cell that is extracted will result in a distinct blue colony
- Incubation conditions, determined by nutrients, time and temperature, will activate all the recombinant cells in a given sample to multiply and form a colony.

Clearly none of these assumptions can be taken for granted. For example, when microbial cells are incorporated into soil, invariably only 10% of the cells added to the soil can be recovered. This phenomenon is difficult to explain, as there are several factors that influence recovery. First of all it is possible that a large proportion of the cells added to a soil system will die and are therefore lost. Secondly, some cells might become firmly absorbed to soil particles. Thirdly, it is possible that the extraction procedure (soil mixing, whirly-mixing, etc.) will damage cells beyond recovery. And fourthly, cells might clump together, forming apparently a single colony, while in fact this colony has originated from more than one cell. The last factor touches on the second assumption that each viable cell will form a distinct blue colony. Clearly, a colony might originate from several cells, in which case the estimate of the recombinant population in soil will be an underestimation. Furthermore, cfu counts on agar media rely on the ability of cells to multiply. However, this might not always the case. It is now generally accepted that many bacteria can enter a viable but non-culturable state [6]. Cells that have entered this state are not recoverable using traditional plating techniques. There is some evidence that *Pseudomonas* cells could enter such a state [29]. It was found that one year after introduction into agricultural drainage water *P. fluorescens* counts were consistently higher using immuno fluorescens (IF) than using cfu counts on agar. This indicated that a proportion of the *P. fluorescens* added became non-culturable. It was however not clear if these cell cells were simply not viable or that they were viable but non-culturable as the IF technique can not distinguish between the two states. Our own investigations with the triple marked *P. fluorescens* described above, indicate that colonies formed on P-1 medium amended with kanamycin and X-gal can be phenotypically very different. During times of environmental stress, instead of forming loosely packed colonies after approx. 2–3 days incubation at 25 °C, colonies appeared after more than 10 days incubation. The morphology of those colonies was entirely different from those formed by

non-stressed cells. The colonies were small, very dark blue and the cells in the colony were very tightly packed (De Leij, unpublished observations). These observations indicate that prevailing environmental conditions can induce physiological changes in *Pseudomonas* cells that will affect their recovery on agar media.

Conclusions

In summary it can be stated that:

1. *LacZY* provides a convenient, safe, well-described marker system for bacterial species that do not posses these genes.
2. Incorporation of *lacZY* into the bacterial genome might lead to a slight reduction of environmental fitness when cells are moderately stressed.
3. Because members of the indigenous microbial community commonly express *lacZY* themselves, selection procedures based on the *lacZY* should be combined with suitable selective media.
4. Results obtained from cfu estimates almost certainly under-estimates the true viable recombinant population in the environment.

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Molecular Microbial Ecology Manual, Second Edition **8.01**: 1563–1580, 2004.

Assessment of the membrane potential, intracellular pH and respiration of bacteria employing fluorescence techniques

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Introduction

All living cells require energy to grow and multiply, for synthesis of enzymes, nucleic acids, polysaccharides, and other cell components, for cell maintenance and repair of damage, for motility, and for numerous other processes. In microorganisms, there are basically two forms of metabolic energy: energy-rich phosphate bonds such as ATP, and electrochemical energy provided by ion gradients. Fermentative microorganisms, for example, can produce ATP by substrate level phosphorylation. In this process, ATP is formed by transfer of a phosphate group from a chemical compound to ADP. Additionally, many microorganisms have developed another strategy to obtain metabolic energy based on conversion of chemical, light or redox energy to energy stored in ion gradients. The generation of such gradients is generally accomplished by primary transport systems, such as the respiratory chain, anaerobic electron transfer systems, or H^+-ATPases. In most microorganisms, the ions used to generate gradients are protons. This form of metabolic energy is termed the proton-motive force (pmf). The pmf can be the driving force for the uptake of various compounds by secondary transport systems, but can also be applied to synthesize ATP via the F_0F_1-ATPase. The pmf consists of the electrical potential ($\Delta\Psi$) and the chemical proton potential ($-Z\ \Delta pH$, where $Z = 2.3\ RT/F$) according to equation 1.

$$pmf = \Delta\Psi - \frac{2.3RT}{F}\Delta pH \tag{1}$$

where $\Delta\Psi$ is the membrane potential (in Volt), R is the gas constant (in J K^{-1}), F is the Faraday constant (in C mol^{-1}), T is temperature (in degrees Kelvin), and ΔpH is the pH gradient (pH_{in} – pH_{out}).

This chapter discusses and describes methods for the measurement of each of these parameters.

Membrane potential

The $\Delta\Psi$ in cells can be determined by the distribution of lipophilic ionic molecules between the cells and the suspending medium according to the Nernst equation [21, 24, 55]:

$$\Delta\Psi = -\frac{RT}{nF} ln \frac{[X]_i}{[X]_o} \tag{2}$$

where n is the number of electrons per mole, $[X]_i$ is the concentration of the indicator inside the cell (in mol m^{-3}), and $[X]_o$ is the concentration outside the cell (in mol m^{-3}). The properties of the ideal membrane potential probe are discussed by Lolkema [35] and summarized here: **(i)** the probe should pass the membrane rapidly, **(ii)** it should not bind to the membrane or other cellular constituents, **(iii)** it should be detectable at very low concentrations, and **(iv)** it should be biologically inert. Generally, small lipophilic charged molecules, such as tetraphenylphosphonium (TPP^+) ions are used for membrane potential measurements. Recently, the use of fluorescent distributional probes has become popular. The choice of probes include rhodamine 123, positively charged carbocyanines such as 3,3-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), 3,3-diethyloxacarbocyanine iodide ($DiOC_2(3)$), and 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$, and the negatively charged bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) [3, 4, 20, 28, 31, 37, 38, 64]. Cells that have a membrane potential (negative inside) accumulate the cationic rhodamine 123 and also cyanines, whereas oxonols are excluded. In eukaryotic cells, rhodamine 123 accumulates preferentially in the mitochondria, due to the high membrane potential present in this organelle [51], but also the less hydrophobic cyanines are expected to accumulate in mitochondria [46]. Furthermore, a high intramitochondrial carboxycyanine concentration is likely to be toxic to the cells, and the fluorescence is likely to be quenched [55]. Other problems associated with lipophilic membrane potential probes are their potentially strong binding to cell constituents such as membranes, and formation of non-fluorescent aggregates at higher concentrations [21, 24]. To avoid these problems, Krasznai et al. [33] developed a calibration procedure based on the assumption that a direct relation exists between the total cell-related fluorescence and the free intracellular dye concentration, which is in (Nernstian) equilibrium with the extracellular fluorescence. Using this procedure, the membrane potential in rat thymocytes and human lymphocytes could be measured with the oxonol $DiBAC_4(3)$. The obtained results were in good agreement with those obtained by the patch clamp method.

Flow cytometry is a popular technique for the analysis of individual cells, but as pointed out clearly by Shapiro [56], the flow cytometer measures the amount of fluorescent dye rather than the concentration. The amount of probe, however, is not a good parameter for the measurement of the membrane potential because the fluorescence may fluctuate depending on *e.g.* the size of the cells. This led to the development of a ratiometric method to measure the membrane potential of bacteria using the oxocarbocyanine dye $DiOC_2(3)$ [43, 44]. This dye supposedly

forms aggregates emitting red fluorescence, dependent on the membrane potential and the size of the cells. The normal green fluorescence of the dye is dependent on the size, but not on the membrane potential. The ratio between the green and the red fluorescence should eliminate the dependence of the fluorescence on the size of the cells [43].

In Gram-negative bacteria, a potential problem for measurement of the membrane potential is that proper distribution of the membrane potential probes is sometimes hindered by the low permeability of the outer membrane. The addition of EDTA or EGTA can help to permeabilize the outer membrane, but such treatments may obviously influence cell viability.

Intracellular pH

The chemical proton potential can be determined by measuring the pH gradient, *i.e.* the pH difference between the intracellular pH (pH_{in}) and extracellular pH (pH_{out}). Several techniques have been developed to determine the pH_{in} of microbial cells including distribution of weak acids [13, 16], the ^{31}P nuclear magnetic resonance technique [22, 47, 52], and the application of pH-dependent fluorescent probes [11, 39]. Fluorescent probes that have been exploited to measure the pH_{in} in microbial cells include fluorescein, 5 (and 6-)-carboxyfluorescein [cF], 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), 5 (and 6-)-carboxyfluorescein succinimidyl ester (cFSE), 5 (and 6-)-carboxy-10-dimethylamino-3-hydroxy-spiro[7*H*-benzo[c]xanthene-7,1′(3′*H*)-isobenzofuran]-3′-one[cSNARF-1], and 8-hydroxy-1,3,6-pyrene-trisulfonic acid [Pyranine]. Fluorescein and cF are easily incorporated into cells by incubation with their diacetate esters [10]. Furthermore, these probes can be excited at the popular 488 nm excitation line, and are highly fluorescent (*i.e.* they have a high extinction coefficient and quantum yield). However, fluorescein and, to a lesser extent, cF are poorly retained in the cytoplasm. A strategy to circumvent this problem is to employ more polar fluorescent compounds, such as BCECF, which is generally retained much better in the cells. Furthermore, to minimize resulting background problems, chemical elimination of extracellular probe [42] and mathematical correction of the fluorescent signal for efflux [39] have been exploited. On the other hand, in energized yeast cells, there are strong indications that fluorescent probes such as cF and BCECF are actively extruded to the extracellular environment and/or accumulated in intracellular compartments, in particular the vacuoles [9, 14, 40]. To avoid problems with translocation of fluorescent dye, the fluorescent probe cFSE can be used for determination of the pH_{in} of bacteria [11]. Bacteria can easily take up cFSE by incubation with its diacetate ester cFDASE. Once it is incorporated, it is thought that its succinimidyl group forms conjugates with aliphatic amines [11, 27]. The fluorescence can be detected after hydrolysis of the diacetate form by intracellular esterase activity. This approach avoids problems due to leakage or active efflux of the probe and allows accurate calibration of the fluorescence signal.

A recent development is the application of green fluorescent protein (GFP) as a pH probe. It has been found that the fluorescence spectrum of some mutant GFP's respond to changes in pH [34]. Because GFP is small, very stable, does not perturb the metabolism of the cells, and can easily be targeted to specific intracellular compartments, it is quickly becoming a popular tool for pH_{in} measurements. Some examples include the measurement of the pH_{in} of peroxisomes in fungi [69], and *Lactococcus lactis* [45]

Respiration

In microbial cells, energy can be generated via electron tranfer systems. Such systems are composed of a series of electron carriers, which are usually membrane bound, such as NADH dehydrogenases, flavoproteins, cytochromes and quinones that can accept electrons from an electron donor and can transfer them to an electron acceptor. The energy released during this process is used to extrude protons to the external environment. The net result is the generation of a proton motive force, which can *e.g.* be used to generate ATP. If O_2 is used as the external electron acceptor, the process is called aerobic respiration. Traditionally, the respiration of bacteria is therefore determined by measurement of the oxygen consumption. Alternatively, tetrazolium dyes may be applied to measure respiration. These dyes are thought to act as an alternative electron acceptor [1], and, consequently, respiring cells can reduce tetrazolium dyes to their respective formazan products. In 1984, Stellmach [61] described the synthesis of the non-fluorescent redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), and Rodriquez et al. [50] demonstrated in 1992 that respiring bacterial cells can reduce CTC to the red fluorescent, water-insoluble formazan product 3-cyan-1,5-di-tolyl-formazan (CTF). CTF has an absorption peak at 450 nm, and the fluorescence of the crystals is in the red region (approx. 570–650 nm).

The procedure commonly used for tetrazolium reduction assays involves incubation of the cells (20 minutes to several hours) in the presence of the tetrazolium dye followed by fixation using formaldehyde, paraformaldehyde or formalin, and mounting with paraffin oil or immersion oil on a microscope slide for examination. This is typically combined with counterstaining with DAPI for determination of the total cell count. Formazan deposition may be enhanced by the addition of substrates such as succinate, glucose, and intermediate electron carriers such as phenazine methasulfate [23, 60, 66, 70]. On the other hand, tetrazolium reduction may be reduced by extracellular inorganic phosphate, at concentrations above 10 mM [60]. Depending on the lipophilicity, some formazan products can diffuse out of the cells and form extracellular deposits. This could, in some cases, be suppressed by addition of cobalt ions, which supposedly form a complex with the formazan [66].

The use of CTC also has its limitations. In several cases, addition of CTC proved to be lethal to the microbial cells at concentrations as low as approx. 5 mM [25, 53, 67]. Furthermore, CTC reduction by yeast cells is not very reproducible, most likely

because CTC does not easily enter respiring yeast cells, due to its negative charge [8]. Another important issue is that the exact mechanism of formazan formation is still not completely clear. Smith and McFeters [61] concluded from their study with *Escherichia coli* that CTC was reduced prior to ubiquinone in the electron transfer chain, but could not satisfactorily explain the inhibition of CTC reduction by potassium cyanide, an inhibitor of the terminal oxidase. In this respect, Seidler [58] proposed that under aerobic conditions tetrazolium salts may be reduced by superoxide radicals, *i.e.* if the actual redox potential of the O_2/O_2 radical couple ($E'_{1/2} \sim -330$ mV) is more negative than that of the tetrazolium/formazan couple. The reduction potential ($E'_{1/2}$) of CTC is approx. -200 mV, indicating that the formazan formation may well be due to superoxide radicals instead of the enzymes from the electron transfer chain.

Procedures

In the following section, detailed protocols are described for measurement of the membrane potential of *Listeria monocytogenes* (based on the method described by Bennik et al. [3] with some modifications), the intracellular pH of *Lactococcus lactis* (based on the method described by Breeuwer et al. [9] with some modifications), and the analysis of the respiration of *Listeria monocytogenes* using CTC and flow cytometry.

Membrane potential ($\Delta\Psi$) measurements

Materials:

- 50 mM potassium phosphate buffer (KP_i) pH 7.
- Cell culture: *L. monocytogenes*, grown in BHI in a shaking water bath at 30 °C to optical density at 620 nm of 0.6.
- Fluorescent probe: $DiSC_3(5)$ (Molecular Probes Europe B.V., Leiden, The Netherlands), stock solution 3 mM in DMSO, stored in freezer.
- Water bath, 30 °C.
- Eppendorf tubes 1.5 ml.
- Eppendorf centrifuge (Biofuge fresco, Heraeus Instruments, Osterode, Germany).
- Spectrofluorimeter (Perkin-Elmer LS 50B, Nieuwerkerk a/d IJssel, The Netherlands).
- Glucose (1 M).
- Valinomycin (stock solution 3 mM in ethanol).

– Nigericin (stock solution 3 mM in ethanol).
– 3 ml fluorescence glass cuvette (4 sides transparent).
– Small magnetic stirrer bar for in cuvette.
– Waste container.
– Ethanol (70 %) for rinsing.

Measurement:

1. Grow *L. monocytogenes* (BHI, 30 °C, shaking) until $O.D._{620\ nm}$:0.6 (approximately: 5.10^8 cells per ml).
2. Harvest 30 ml cells by centrifugation (10 minutes at 2800 × *g*) and wash twice in 50 mM KP_i pH 7 buffer.
3. Resuspend in 30 ml 50 mM KP_i pH 7 buffer and store on ice until use.
4. Turn on the spectrofluorimeter and computer.
   Excitation wavelength 643 nm, slit width 10 nm.
   Emission wavelength 666 nm, slit width 10 nm.
   Measurement time 900 seconds at intervals of 0.5 seconds.
   The water bath connected to cuvette holder is set at 30 °C.
5. Clean the cuvette with water (3 times) and 70 % ethanol (3 times), and blow dry with air.
6. Add 3 ml cell suspension to the cuvette (the concentration of cells in the cuvette should be approximately $5{\cdot}10^8$ cells per ml buffer), use small magnetic stirrer to mix cells[1].
7. Add 5 μl DiSC3(5) to a final concentration of 5 μM and wait until the signal is stable (after ±400 seconds).
8. Add test compound, *e.g.* 10 mM glucose, and wait again until the signal is stable.
9. To prevent generation of a transmembrane pH gradient, nigericin (10 μl; final concentration 1 μM) may be added.
10. At the end of the assay, valinomycin (10 μl; final concentration 1 μM) is added. The $\Delta\Psi$ is dissipated and the signal will increase. The signal value obtained after dissipation is generally lower than the start value due to binding of the probe to the cells[2].

An example of a typical experiment is shown in Figure 1. First, the cell suspension is added to the cuvette. Subsequently, the $DiSC_3(5)$ probe is added and the signal rapidly decreases due to (Nernstian) re-distribution of the probe (inside the cells the fluorescence of the probe is quenched which results in decrease of the total signal). Addition of

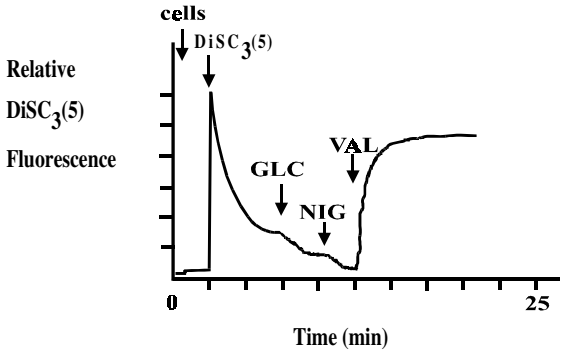

Figure 1. The membrane potential of *Listeria monocytogenes* measured by $DiSC_3(5)$.

glucose induces a further decrease of the signal due to an increase in the membrane potential. Addition of nigericin results in dissipation of the pH gradient, which may be compensated for by increasing the membrane potential. Addition of valinomycin results in dissipation of the pmf and the signal increases again.

Notes

1. *Depending on the bacterial species the optimum cell concentration may vary. With L. monocytogenes, a relatively high concentration of cells is required to obtain sufficient fluorescence signal.*
2. *The fluorescence signal after addition of valinomycin corresponds to a* $\Delta\Psi$ *of zero, but the described method does not include calibration of the membrane potential. It only gives qualitative changes in membrane potential.*

Internal pH (pH_{in}) measurements

Materials:

- 50 mM potassium phosphate buffer (KP_i) pH 7.
- Cell culture:
  Lactococcus lactis ML3, grown at 30 °C in M17 + 5 g/l glucose to optical density at 620 nm of 0.6.
- cFDASE stock solution in acetone (3.8 mg/ml). This solution should be kept at −20 °C in acetone resistant vials, and is stable for at least half a year. To avoid evaporation of the acetone, the solution should be returned to the freezer immediately after use.
- Acetone (reagent grade).
- Water bath, 30 °C
- Eppendorf tubes 1.5 ml.

– Eppendorf centrifuge (Biofuge fresco, Heraeus Instruments, Osterode, Germany).
– Spectrofluorimeter with ratio option, *i.e.* the emission is measured at two different excitation wavelengths at very short time intervals and the ratio of the intensities is calculated and displayed.
– Glucose (1 M).
– Valinomycin (stock solution 3 mM in ethanol).
– Nigericin (stock solution 3 mM in ethanol).
– 3 ml fluorescence glass cuvette (4 sides transparent).
– Citric acid monohydrate (50 mM).
– Potassium chloride (4 M).
– Disodium hydrogen phosphate dihydrate (50 mM).
– Glycine (50 mM).
– NaOH (3 M).
– HCl (4 M).

Fluorescent labeling:

1. Harvest 1 ml cell suspension by centrifugation in an Eppendorf tube (11300 × *g*, 2 minutes), wash once in 50 mM potassium phosphate buffer pH 7.0, and resuspend the cells again in 1 ml KP_i pH 7 buffer.
2. Add 10 µl cFDASE previously diluted 10× in acetone (20 µl cFDASE + 180 µl acetone) to 1 ml sample and incubate 15 minutes at 30 °C[3].
3. Wash 2× in KP_i pH 7, resuspend the cells again in 1 ml KP_i pH 7 buffer, and add 10 µl glucose (1 M), and incubate 15 minutes at 30 °C[4].
4. Wash 1× in KP_i pH 7 to remove extruded cFSE, resuspend the cells in 1 ml KP_i pH 7 buffer, and add again 10 µl glucose (1 M), and incubate 15 minutes at 30 °C.
5. Wash 1× in KP_i pH 7, resuspend the cells again in 1 ml KP_i pH 7 buffer, and place Eppendorf tube on ice[5].

Measuring:

6. Measure the pH_{in} using the spectrofluorimeter. A glass cuvette filled with 3 ml buffer of desired pH is placed in the stirred and thermostated cuvette holder of the spectrofluorimeter. After 1 minute cells (diluted to a concentration of approx. 10^7 cells per ml) are added, and after time x, y, z, etc. glucose or other reagents are added[6]. The fluorescence intensities are measured at excitation wavelengths of 500 and 440 nm by rapidly altering the

monochromator (ratio option) between both wavelengths (<2 seconds). The emission wavelength is 530 nm, and the excitation and emission slit widths are 5 and 10 nm, respectively.

The incubation temperature is 30 °C. It is a good habit to equilibrate the pH_{in} and pH_{out} at the end of each assay by addition of valinomycin (1 μM final concentration) and nigericin (1 μM final concentration) in the cuvette. Additionally, the extracellular fluorescence signal (background) is determined by filtration (do this very gently!) of the cell suspension through a 0.22 μm pore-size membrane filter into a clean cuvette and measurement of the filtrate (take out the cuvette during the measurement)[7].

Calibration:

7. Prepare a series of citrate phosphate glycine buffers ranging from pH 3 to 9 at 0.5 pH unit intervals.
   Preparation: take 75 ml $Na_2HPO_4 \cdot 2H_2O$ (50 mM) and add 2.5 ml KCl (4 M) and 75 ml glycine (50 mM).
   a. buffers between 5 and 7: add citric acid monohydrate (50 mM) until desired pH and add water to total volume of 200 ml.
   b. buffers higher than pH 7: add citric acid monohydrate (50 mM) until pH 7, and add NaOH until desired pH. Add water to total volume of 200 ml.
   c. buffers lower than pH 5: add citric acid monohydrate (50 mM) until pH 5, and add HCl until desired pH. Add water to total volume of 200 ml.
8. Add 3 ml buffer pH (3 to 9) to the cuvette and after 1 minute add cells. After 5 minutes, the pH_{in} and pH_{out} are equilibrated by addition of valinomycin (1 μM final concentration) and nigericin (1 μM final concentration) in the cuvette. After 10 minutes, the extracellular fluorescence signal (background) is determined by gentle filtration of the cell suspension through a 0.22 μm pore-size membrane filter by use of a 5 ml syringe into a clean cuvette and measurement of the filtrate (take out the cuvette during the measurement).

A typical example of a pH_{in} measurement of *Lactococcus lactis* is shown in Figure 2. First, buffer (50 mM KP_i buffer pH 6.5) is added to the cuvette. The signals at 490 nm and 440 nm excitation of the buffer are very low (because there is no fluorescent probe present), and the 490-to-440 ratio signal should be close to 1. When the fluorescent cells

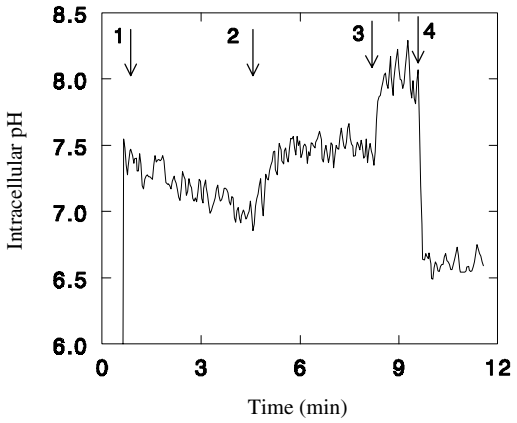

Figure 2. The 490-to-440 nm ratio of cFSE labeled *L. lactis* cells measured at excitations of 490 nm and 440 nm at an emission wavelength of 525 nm. Measurements in the cuvette were performed at 30 °C in 3 ml of 50 mM potassium phosphate buffer, pH 6.5. The following additions were made at the times indicated by the arrows: 1, cell suspension (100 μl); 2, lactose (10 mM); 3, valinomycin (1 μM); and 4, nigericin (1 μM). At the end of the assay (not shown) the cuvette was removed for a short time from the spectrofluorometer and the filtrate was measured after filtration of the cell suspension through a disposable disc filter (0.22 μm pore size).

are added to the cuvette, the 490-to-440 ratio signal increased to about 3. Glucose addition resulted in a clear increase of the ratio. Addition of valinomycin further increased the ratio, as the cells try to compensate for the dissipation of the membrane potential by exporting protons. Upon addition of nigericin the pH gradient is dissipated.

Notes

3. *In the cells the cFDASE is converted into cFSE and 2 acetate molecules. The cFSE gives a bright green fluorescence when excited at 460 to 490 nm. It is common practice to check the labeling by use of a fluorescence microscope with a standard FITC filter set. A part of the cFSE will bind to aliphatic amines, but most of the cFSE remains unbound.*
4. *In this step the cFSE, which is not bound, is actively extruded to the extracellular environment. This is necessary to minimize leakage or extrusion of probe during the measurement.*
5. *It is a good habit to check the cFSE labeling of the cells using a fluorescence microscope equipped with a standard FITC filter set. cFSE-labeled cells appear as green to yellow labeled cells.*
6. *The number of cells to be added to the cuvette depends on the signal-to-noise ratio of the 490/530 nm intensity signals and 440/530 nm intensity signals. In our spectrofluorimeter the 440/530 nm signal of KP_i buffer pH 7 without any addition is about 0.9 (on a scale from 0 to 1000). The 440/530 signal of the cells should be higher than*

15 to obtain a stable ratio signal. This signal can be increased obviously by adding more cells, but it should be realized that at a certain moment the optical density will become too high and other effects will occur such as concentration quenching, which may disturb the measurement.

7. *At the end of the experiment, the background fluorescence is determined by filtration through the 0.22 μm disposable disc filters. The fluorescence in the filtrate is generally 10 to 20 % of the total signal before filtration, and may result from (unbound) cFSE, which may leak from the cells during the measurement. It was not possible to determine the impact of the filtration itself on the leakage of cFSE from the cells, but using centrifugation instead of filtration does not generally yield better results.*

Assessment of respiration

Materials:

- PBS : 0.2 g KCl, 0.2 g KH_2PO_4, 1.5 g Na_2HPO_4 et 8 g NaCl per liter, adjusted to pH 7.2 with concentrated HCl.
- 50 mM potassium phosphate buffer (KP_i) pH 7, filter sterilized through 0.2 μm membrane filter.
- Cell culture: *L. monocytogenes*, grown in Brain Heart Infusion broth (BHI) at 30 °C to optical density at 620 nm of 0.6.
- Eppendorf tubes 1.5 ml.
- Eppendorf centrifuge (Biofuge fresco, Heraeus Instruments, Osterode, Germany).
- Water bath 30 °C.
- Fresh CTC solution: weigh 0.015 g and solubilize in 10 ml filter sterilized milliQ water (5 mM), add 0.47 ml glucose (20 % solution in water) to give a final concentration of 50 mM. This solution is not very stable, and should be used the same day. CTC was obtained from Polysciences, Warrington, USA.
- Flow Cytometer (FACSCalibur, Becton Dickinson, Erembodegem-Aalst, Belgium).
- Sheath fluid (FACSFlow).
- Cleaning solutions (FACSSave and FACSRinse).

Procedure:

Labeling of cells with CTC

1. Harvest 1 ml cell suspension by centrifugation in an Eppendorf tube (11300 × *g*, 4 minutes), wash in PBS, and resuspend again in 1,5 ml PBS. The suspension can be stored on ice.

2. Take 0.15 ml cell suspension and centrifuge (11300 $\times$ *g*, 4 minutes), and resuspend the cells in 1.5 ml CTC solution + glucose (50 mM)[8].
3. Incubate the cell suspension for 45 minutes at 30 °C in the dark.
4. Check the labeling of the cells using fluorescence microscopy with a standard FITC filter (excitation 460–490). The CTF should be visible as red crystals inside the cells.
5. Store cells on ice before analysis.

Flow cytometric analysis

6. Turn on the flow cytometer using the standard start up procedure.
7. In the acquisition menu, set the number of events to be acquired to 5000 or max. 2 minutes.
8. Set the amplifiers [forward scatter (FSC), side scatter (SSC), FL1, FL2, and FL3] to logarithmic mode. The settings for the gain should be E01, 380, 600, 550, and 690 for FSC, SSC, FL1, FL2, and FL3, respectively.
9. In the threshold window, set SSC as the primary threshold with a cut off value (channel number) of 108. Only signals with an intensity greater than or equal to this threshold channel number will be processed by the flow cytometer.
10. Before measurement dilute the cells to approx. 10^5 cells per ml in milliQ water.
11. CTC positive cells are detected by the FL3 (red) fluorescence parameter.

Note

8. *Cell aggregation as result of the CTC labeling may be a problem with some bacteria [65]. This can be avoided by using low cell concentrations (<10^7 cells/ml). In some cases, sonication at low intensity for short time periods (which should not harm the cells) may help.*

Application of the Methods

The membrane potential, the pH gradient, and the electron transfer chain activity of microorganisms can be useful as indicators for cell viability in addition to traditional methods such as plate counting. Membrane potential probes such as $DiBAC_4(3)$, for instance, have been applied to evaluate the antimicrobial effects of bacteriocins or

antibiotics [4, 28, 64]. The cFSE method has been widely applied for the measurement of the pH_{in} in Gram-positive bacteria [4, 15, 26, 36, 41, 48, 54, 59, 68] and yeasts [7, 12]. In Gram-negative bacteria such as *E. coli,* the use of the cFSE method is complicated by the difficulty of the prefluorochrome cFDASE (molecular weight 557) to pass the outer membrane in the Gram negative cell wall. Nevertheless, Riondet et al [49] showed that a short incubation with EDTA is perhaps an approach to overcome this problem.

The Tetrazolium dyes may be used to measure respiration. CTC is the most popular redox dye and has e.g. been used to determine the number of respiring *Micrococcus luteus*, *Listeria monocytogenes* and *Pseudomonas fluorescens* in pure cultures [6, 29, 32], respiring bacteria in water [2, 30, 50, 57, 60], soil [71], microbial communities in bioreactors [17], biofilms [19, 63, 72], and as indicator of viability in anaerobic bacteria [5].

Acknowledgements

We would like to thank Suzanne Luppens and Benoit Barbaras for their information concerning the CTC procedure, and Patrick Verbaarschot for critically reading the manuscript.

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