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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

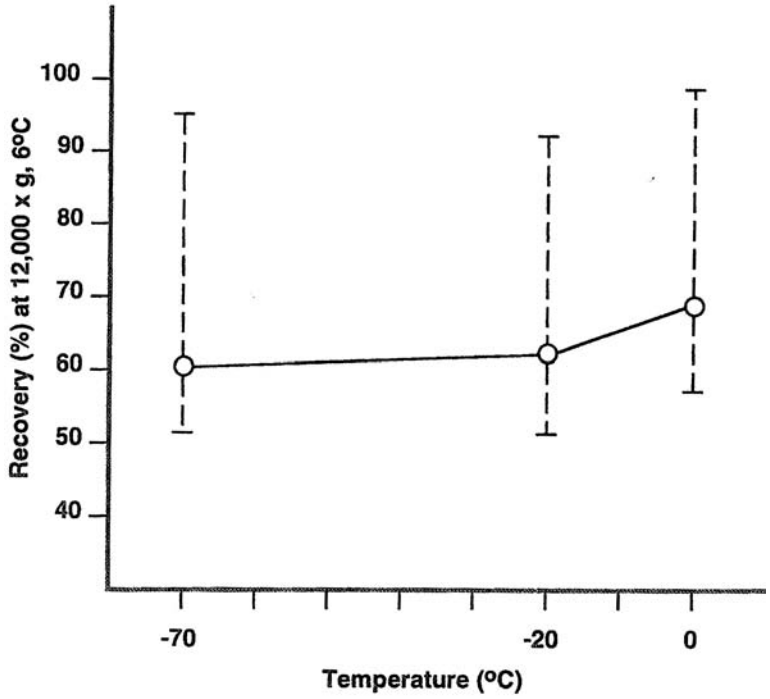

Figure 1. The recovery of DNA as a function of the precipitation temperature. Precipitations of varying amounts (0.6 ng–010 μg) of DNA at extremly low temperatures (i.e., −70 °C) are less efficient than at 0 °C. The efficiencies of recovery, by centrifugation (12,000 × g, 6 °C), were also observed to 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].

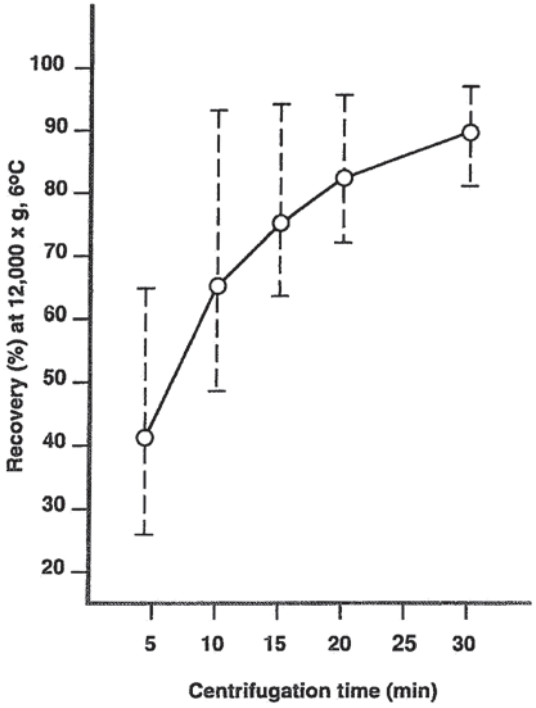

Figure 2. The recovery of DNA as a function of the centrifugation time. The recovery of varying amounts (0.6 ng–10 μg) of DNA is enhanced by increased centrifugation times. The efficiencies of recovery, by centrifugation (12,000 × g, 6 °C), were also observed to be dependent upon the amounts of DNA in 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.

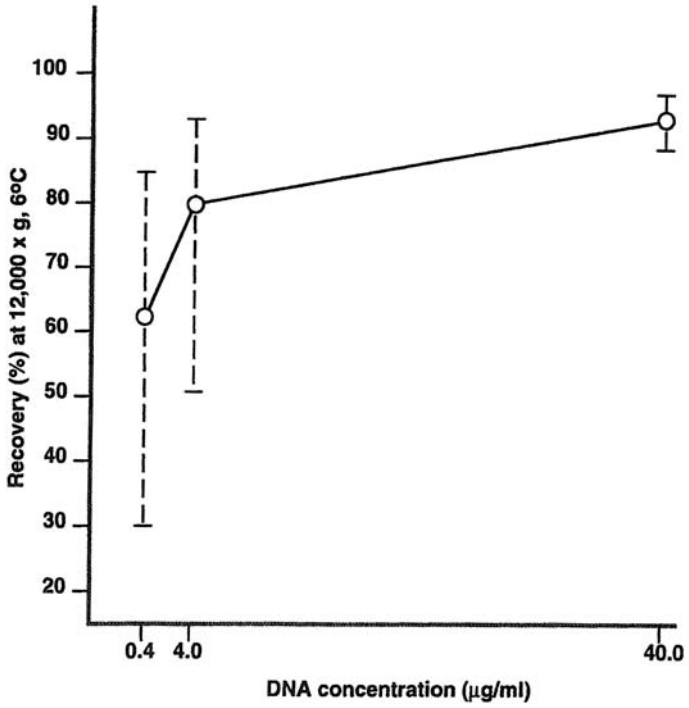

Figure 3. The recovery of DNA as a function of the amount of DNA in suspension. The recovery of DNA was observed to be dependent on the concentrations of the suspensions. The values indicated represent the means, calculated from the observed recoveries from suspension, after varying centrifugation times. 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

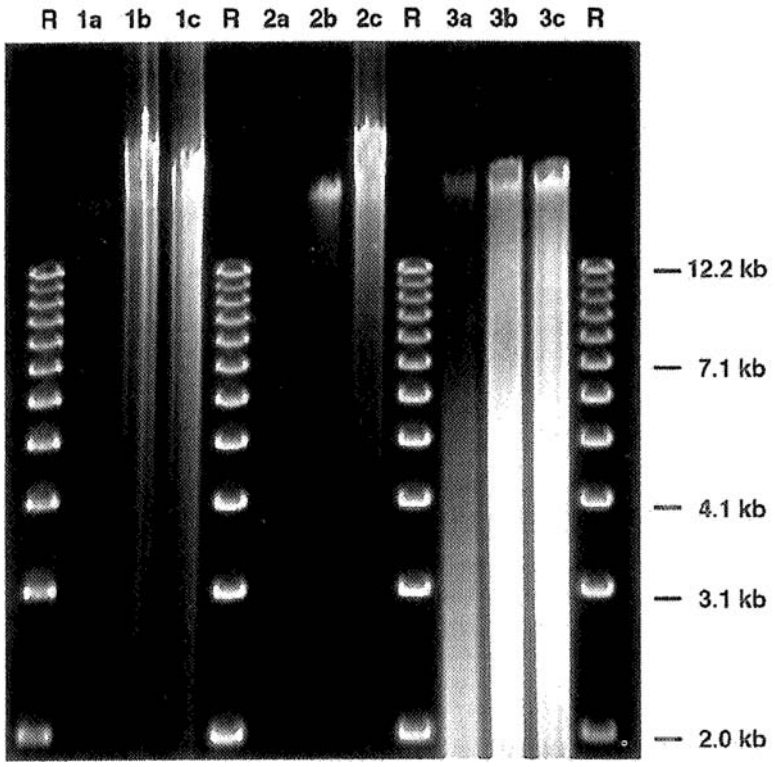

Figure 4. Agarose gel (1.0%, w/v) electrophoresis and ethidium bromide staining of genomic DNA prepared from: 1) *Rhodococcus rhodochrotis;* 2) *Rhodococcus globerulus*; and 3) *Pseudomonas aeruginosa.* Cells grown on agar media and picked from individual colonies were treated by: a) heating at 90 °C, 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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