

Isolation of Intact High Molecular Weight Chromosomal DNA from *Desulfovibrio* spp.

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A simple, reproducible method to extract high quality DNA from sulphate-reducing bacteria (SRB) is described. SRB isolated from patients with ulcerative colitis, and from environmental samples, were used for purification of chromosomal DNA. DNA yield purified by this method was more than one order of magnitude higher compared with the Marmur method, phenol-chloroform or QIAGEN procedures. A 10ml culture was enough for isolation of sufficient DNA to perform hundreds of PCR-based reactions, and can be used in other DNA manipulation techniques such as restriction digestion and DNA cloning despite a low yield of cells (1×10^7 - 1×10^8 cells/ml).

Results and Discussion

Sulphate-reducing bacteria (SRB) are a group of phylogenetically diverse anaerobic microorganisms that were first discovered by Beijerinck in 1895. At present, 14 genera have been identified, the two most established genera of SRB being *Desulfovibrio* and *Desulfotomaculum* (Barton, 1995; Postgate, 1984). The SRB are broadly distributed on earth and they play a significant role in nature by virtue of their potential for numerous interactions such as geochemical transformations, fuel production, food spoilage, biocorrosion, bioremediation, environmental nutrient cycles and associations with animal diseases (Barton, 1995). The involvement of SRB in human disease such as ulcerative colitis (UC) has also been suggested (Gibson, *et al.*, 1991). Therefore, the SRB attract the attention of many scientists around the world, especially now when modern molecular biological techniques can be applied for the investigation of these microorganisms. Purification of nucleic acids is a first step in most molecular biology studies and in all recombinant DNA techniques. High quality DNA is required for these studies. Nevertheless, the purification of chromosomal DNA from SRB is not always a straightforward procedure. A number of existing DNA purification methods have been tested, including the use of the standard approach of Marmur (1961), the phenol-chloroform procedure (Ausubel *et al.*, 1995) and a commercially available kit (QIAGEN). However, using these methods, it was impossible to obtain sufficient amounts of intact chromosomal DNA from 10ml of

SRB culture. Therefore, a guanidine isothiocyanate (GI) procedure for isolation of RNA from eukaryotic cells and extraction of chromosomal DNA from *Bacteroides intermedius* and *Bacteroides gingivalis* bacterial cells was modified and adapted to isolate DNA from clinical and environmental strains of SRB (Chirgwin *et al.*, 1979; Lipke *et al.*, 1987).

The environmental strain of SRB, *Desulfovibrio indonensis* (NCIMB 13468) (Feio *et al.*, 1998; Zinkevich *et al.*, 1996) and several SRB strains (2R, 4R and 7R) isolated from patients with UC, were used for the comparative test of DNA isolation procedures. All isolation procedures were carried out with duplicate samples. DNA was extracted from aliquots taken from each cell pellet sample employing the phenol-chloroform (Ausubel *et al.*, 1995), the Marmur (1961) technique and using the Genomic-tip 20/G (QIAGEN) method. Another aliquot from each sample was used to recover DNA, applying the modified GI procedure as adapted for SRB (Table 1). Compared to the original methods (Chirgwin *et al.*, 1979; Lippke *et al.*, 1987), the following simplification and modifications were made: (i) ethidium bromide (EtBr) was included during the centrifugation, (ii) the concentration of CsCl and time for spinning were increased and (iii) the centrifuge tubes used were of a smaller size. The presence of EtBr allowed direct DNA visualisation, instead of fractionation, of the tube contents and detection DNA in each sample. The increasing of CsCl concentration and spinning time improved the quality of DNA. The DNA purity, determined from the A260/A280 ratio averaged 1.9 for all samples compared with a ratio of 1.54 for DNA obtained without these modifications. The diminishing of the tube size enabled the efficient recovery of DNA from 10ml of culture. The quantity of DNA was enough to be visible after centrifugation without UV light.

The yield and quality of DNA were analysed by spectrophotometer and electrophoretically (Table 2; Figure 1A, B). The DNA obtained using the method of Marmur (1961), phenol-chloroform procedure (Ausubel *et al.*, 1995) and Genomic-tip 20/G (QIAGEN) had a smaller average length and showed a heterogeneous size distribution of

Table 1. Modified DNA isolation protocol

1. A 10ml culture of *Desulfovibrio indonensis* and strains 2R, 4R and 7R were grown at 37C for seven days in Postgate medium C (Postgate, 1984).
 2. The cells were harvested using centrifugation at 2,600g for 30 min (IEC Centra-CLD).
 3. Each pellet was suspended by 3ml of lysis buffer [5M guanidine isothiocyanate, 50mM Tris-HCl (pH 7.5), 10mM EDTA, 140mM 2-mercaptoethanol, 2% of N-lauroylsarcosine].
 4. 1.5g of CsCl was added to each homogenate and dissolved by inversion.
 5. 30µl of Ethidium bromide (EtBr, 5mg/ml solution) was layered over a 1.5ml cushion of 5.7M water solution of CsCl in a 4.5ml centrifuge tube (Beckman) and the homogenate was layered over. This stage is critical and EtBr should not be mixed with homogenate.
 6. Samples were centrifuged for 24h in a Beckman centrifuge L8-55 at 42,000rpm (208,532g) at +20C in a swinging bucket SW 50.1 rotor.
 7. After ultracentrifugation, the visible DNA-containing fraction was removed and dialysed at +4C against 10mM Tris-HCl (pH 8.0), 1mM EDTA (TE) buffer for 24 h.
 8. EtBr from DNA was removed by extraction with isoamyl alcohol and DNA was recovered by adding 0.6 volume of isopropanol at room temperature.
 9. The pellets were washed twice with 70% ethanol, dried at room temperature and then dissolved in 200µl of TE buffer.
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fragments, indicating degradation (Figure 1 A, lanes 1-3; B, lane 2). DNA recovered after applying the GI method yielded intact high molecular weight chromosomal DNA. There was no RNA contamination, nor any sign of degraded DNA (Figure 1 A, lanes 1; B, lane 1). The yield of DNA using this method was also more than one order of magnitude higher compared with other procedures (Table 2). This is another advantage for anaerobic microorganisms such as SRB, which grow very slowly, and produce a low yield of cells (1×10^7 - 1×10^8 cells/ml) in comparison to bacteria like *E. coli* (1×10^{10} cells/ml). In control experiments, when we used Genomic-tip 20/G (QIAGEN) for isolation of chromosomal DNA from *E. coli* JM109 cells, the yield and quality of DNA corresponded to the manufacturer's descriptions (Figure 1 B, lane 3). The difficulties with isolation of genomic DNA of SRB can be explained by the high content of intracellular nucleases, (which were efficiently inactivated by the guanidine isothiocyanate during the lysis procedure) as well as the high level of production of extracellular polymeric substances, which surround the cells.

DNA obtained by the guanidine isothiocyanate procedure was then digested with restriction enzymes and analysed electrophoretically. Figure 1 C (lanes 1 - 3) shows an example of an agarose gel of a *Hind*III, *Bam*HI and *Pst*I restriction digestion of *Desulfovibrio indonensis* DNA extracted by this method. *Eco*RI, *Eco*RV and *Bg*III restriction enzymes (NE BioLabs) were also used (data not shown). DNA was completely digested with restriction enzymes and there was no evidence of the presence of nuclease(s) in any sample. The quality of digest indicated that this DNA is suitable for cloning procedures. The ability of the isolated DNA to be used for PCR was also determined. Primers for PCR (Figure 1 D) were designed as described elsewhere (Zinkevich & Beech, 1999). With all isolated chromosomal DNA, PCR products of the expected size were

Table 2. The yield and purity of DNA isolated by different methods

| Isolation Procedure | Microbial strain | Ratio of absorbance at 260 nm / 280 nm | Yield of DNA ($\mu\text{g ml}^{-1}$ culture) |
|--------------------------|---------------------------------|--|---|
| Marmur | <i>Desulfovibrio indonensis</i> | 1.89 | 0.81 |
| | 2R | 1.86 | 0.76 |
| | 4R | 1.87 | 0.91 |
| | 7R | 1.91 | 0.72 |
| Guanidine Isothiocyanate | <i>Desulfovibrio indonensis</i> | 1.92 | 9.76 |
| | 2R | 1.89 | 9.28 |
| | 4R | 1.88 | 10.2 |
| | 7R | 1.91 | 9.97 |
| Phenol-Chloroform | <i>Desulfovibrio indonensis</i> | 1.83 | 0.21 |
| | 2R | 1.85 | 0.33 |
| | 4R | 1.87 | 0.29 |
| | 7R | 1.77 | 0.19 |
| QIAGEN | <i>Desulfovibrio indonensis</i> | 1.82 | 0.53 |
| | 2R | 1.86 | 0.47 |
| | 4R | 1.79 | 0.5 |
| | 7R | 1.81 | 0.51 |

obtained and all DNA produced a clear, sharp and reproducible pattern (Figure 1 D, lanes 1-4). PCR experiments were repeated over a period of 6 months and the same banding patterns were obtained which indicates the reproducibility of the results and the integrity of DNA.

The guanidine isothiocyanate procedure for the isolation of DNA proved to be rapid, as well as quantitative without involving labour-intensive standard methodology. The procedure was also successfully used for the isolation of high molecular weight DNA

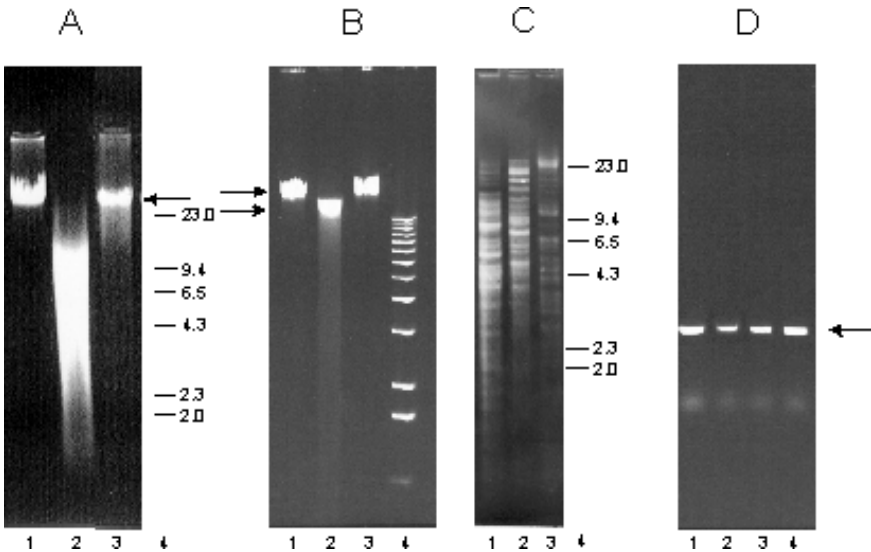


Figure 1. Gel electrophoresis of SRB DNA purified by different methods. DNA fragments were separated by electrophoresis on 0.8 - 1 % agarose gel in standard Tris-borate or Tris-acetate buffers, containing EtBr (Sambrook, et al., 1989). 1 kb DNA ladder (Gibco BRL) and lambda DNA/*Hind*III were used as size markers.

(A). Lane 1, *D. indonensis* DNA purified by GI procedure, lane 2, *D. indonensis* DNA purified by Marmur (1961) method, lane 3, *D. indonensis* DNA purified by standard phenol-chloroform procedure (Ausubel *et al.*, 1995). Lanes 4, lambda DNA/*Hind*III size marker (size given in kb). The arrow indicates the position of chromosomal DNA.

(B). Lane 1, *D. indonensis* DNA purified by GI procedure, lane 2, *D. indonensis* DNA purified by Genomic-tip 20/G (QIAGEN), lane 3, *E. coli* JM 109 DNA, purified by Genomic-tip 20/G (QIAGEN), lane 4, 1 kb DNA ladder (Gibco BRL) size marker. The arrows indicate the position of chromosomal DNA.

(C). Cleavage of *D. indonensis* chromosomal DNA by different restrictases. Lane 1, *Hind*III, lane 2, *Bam*HI, lane 3, *Pst*I, lane 4, lambda DNA/*Hind*III size markers (size given in kb).

(D). PCR amplification of gene fragment of DNA, isolated from different SRB strains. The 658bp DNA fragment of a conserved region of the APS reductase gene of SRB was amplified with forward (dCCAGGGCCTGTCCGCATCAATAC) and reverse (dCCGGGCCGTAACCGTCCTTGAA) primers. The arrow indicates the positions of PCR products. Lane 1, 2R DNA, lane 2, 4R DNA, lane 3, 7R DNA, lane 4, *D. indonensis* DNA.

from *Desulfovibrio desulfuricans*, *Desulfovibrio gigas*, *Desulfovibrio gabonensis* and *Desulfovibrio vulgaris* (data not shown). Thus, a convenient method of recovering intact high molecular weight DNA suitable for molecular biological techniques has been developed. In conclusion, the results have demonstrated that the developed protocol is efficient in isolating high quality and high molecular weight DNA from different SRB. It is expected that this method can also be applied to other Gram-negative microorganisms.

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