

CHAPTER 5

DETECTION OF BACTERIAL DNA BY REAL-TIME POLYMERASE CHAIN REACTION

PCR is a rapid, sensitive and specific method for the detection of bacteria and is increasingly applied to the diagnosis of infectious diseases (White *et al.*, 1992; Tang *et al.*, 1997). It is an improvement on previous methods of bacterial detection and identification including cultivation, immunological and hybridization methods. As reported by Fredricks and Relman (1999), rather than growing an intact microbe one can now amplify a segment of its DNA, replacing culture media with the PCR reaction mix and an incubator with the thermal cycler. A recent advancement in this technology analyses the PCR product formed in real-time rather than the usual end point analysis where PCR components become limited and quantification inaccurate. One example of this technology is the Applied Biosystem ABI-PRISM 7700 (TaqMan) Sequence Detection System (Foster City, CA, USA) which uses the 5' nuclease assay for real-time detection and quantification of PCR products with a dual-labeled fluorogenic probe (Heid *et al.*, 1996).

This chapter begins with a review of PCR technology and its application to bacterial identification and quantitation. Following this is the experimental section which is presented in two parts; the first involves development and testing of the universal amplicon, designed to detect the total bacterial load in a sample. Once achieved, this provided the basis for the second part which examined the issues of extracting DNA from microorganisms while providing protection against the destructive activities of nucleases.

5.1 Review

The commensal flora of the human body has the potential to cause disease when the host defences are impaired. However, in many instances of infection the aetiological agent cannot be identified. It is entirely possible that knowledge of the diversity of human microflora is as inadequate as that in other environments, where less than 0.4% of all bacterial species have

been identified. It, therefore, follows that an inability to cultivate many of the commensal flora may explain the failure in diagnosing and adequately treating related diseases (Relman, 1998).

Results of studies have frequently reported that direct microscopic counts from different environments exceed viable-cell counts by several orders of magnitude. It would appear that the majority of cells seen microscopically are viable but do not form visible colonies on agar. This could be due to the presence of known species unable to be grown under the conditions provided, because they had entered a nonculturable state or unknown species that have not been previously cultivated because of a lack of suitable conditions (Amann *et al.*, 1995). A culture-independent approach for the detection and identification of microbial pathogens is required to overcome these problems and has been achieved by the introduction of molecular techniques using gene sequences to characterise bacteria. This breakthrough was achieved by Carl Woese (1977, 1987) who by comparing ribosomal DNA (rDNA) sequences established a molecular sequence-based phylogenetic tree that could be used to relate all organisms. The construction of a phylogenetic tree involves aligning pairs of rDNA sequences from different organisms and noting the number of nucleotide differences; these differences are determined to be a measure of the "evolutionary distance" between the organisms. The tree can be considered an approximate map of the evolution of the cell lines that constitute modern microorganisms. As molecular identification of organisms is based on gene sequences there is no requirement to capture and grow a functioning cell and therefore unculturable organisms can theoretically be identified and related to known organisms through the phylogenetic tree. As a result of this phylogenetic proximity, predictions regarding the behaviour and properties of the unknown organism can be made with reasonable confidence (Pace, 1997).

Certain features of a genetic sequence are required for it to be useful in identifying uncharacterised microorganisms. Ideally, the sequence must be conserved among a large group of organisms, its rate of change should be constant over long periods, the sequence should not be shared by different organisms through horizontal transmission and, finally, the sequence should be amenable to broad-range detection. The genetic sequences that are most useful as molecular chronometers are those that encode molecules with a highly conserved and essential

biological function. The sequence of the small subunit RNA molecule of the ribosome (16S rRNA) meets these requirements. This molecule has a length of approximately 1,500 nucleotides and folds in a precise way to form ribosomes; highly conserved structures found in all living cells with the function of protein synthesis (Relman, 1993; Relman, 1998). Large databases now exist containing the rDNA sequences for a multitude of organisms.

5.1.1 Development of the polymerase chain reaction

Advances in molecular biology have made it possible to study microbial communities by amplifying gene sequences from different environments followed by purification and sequencing. Initially cloning was used to produce multiple copies of DNA sequences of interest; however, this is a time consuming process and has more recently been replaced by polymerase chain reaction (PCR) technology. This technology, devised by Kary Mullis in 1983 and first published by Saiki *et al.* (1985) described a quick and efficient method for producing many copies of specific regions of DNA; a process that has revolutionised molecular genetics.

PCR exploits certain features of DNA replication. DNA polymerase uses single-stranded DNA as a template for the synthesis of a complementary new strand. The synthesis is directed from a starting point specified by a pair of closely spaced, chemically synthesised oligonucleotide primers that anneal to the template at certain points. The technique of PCR involves the process of repeated cycles to amplify the selected sequence. Each cycle consists of three steps: a DNA denaturation step, in which the double strands of DNA are separated; a primer annealing step, where the primers anneal to their target sequences and an extension step where the DNA polymerase extends the sequences between the two primers. At the completion of each cycle, the quantity of PCR product is theoretically doubled. Therefore, the mechanism of PCR is a primer-directed DNA synthesis, with the advantage that the process is repetitive and the number of copies produced increases exponentially, so that within a few hours (30 to 40 cycles) the original target DNA has been amplified a million-fold or more.

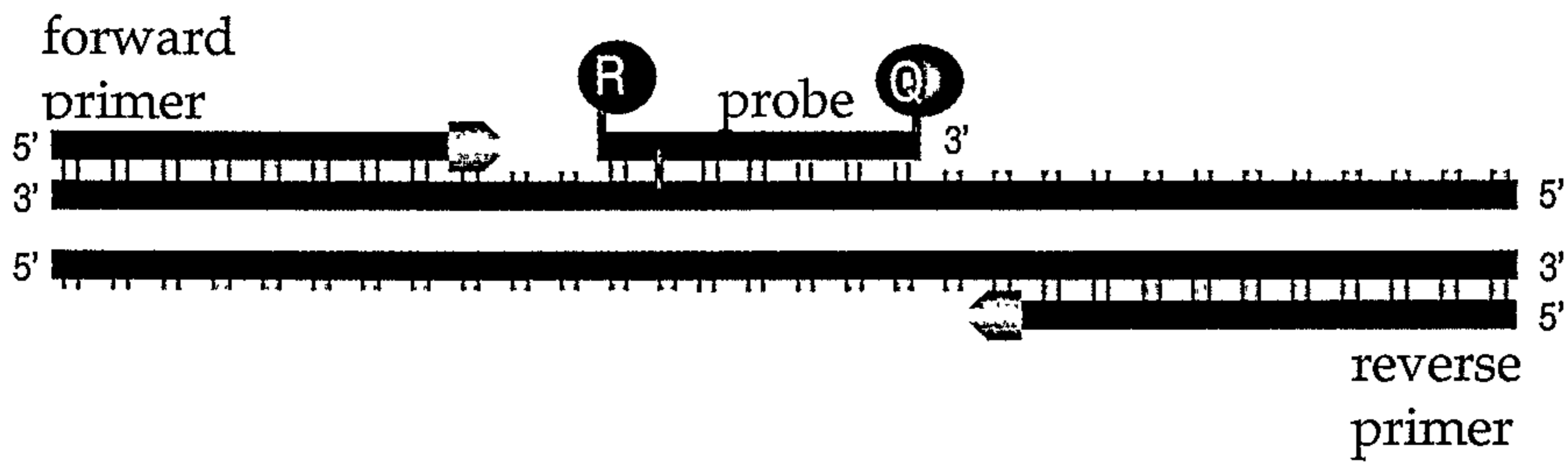
Originally, the Klenow fragment of *E. coli* DNA polymerase I was used to extend the annealed primers, however, this enzyme is heat-sensitive and becomes inactivated at temperatures required to separate double-stranded DNA, thus requiring the addition of fresh

enzyme for each cycle. With the discovery of thermostable DNA polymerases, for example *Taq* (*Thermus aquaticus*) polymerase, the enzyme can be added at the beginning and remains active throughout a complete set of amplification reactions. In addition, these enzymes are active at higher temperatures, thereby increasing the specificity and rate of DNA synthesis. Subsequent automation using dedicated thermal cyclers plus the simplicity of PCR has resulted in its widespread application through many scientific disciplines (O'Leary *et al.*, 1997).

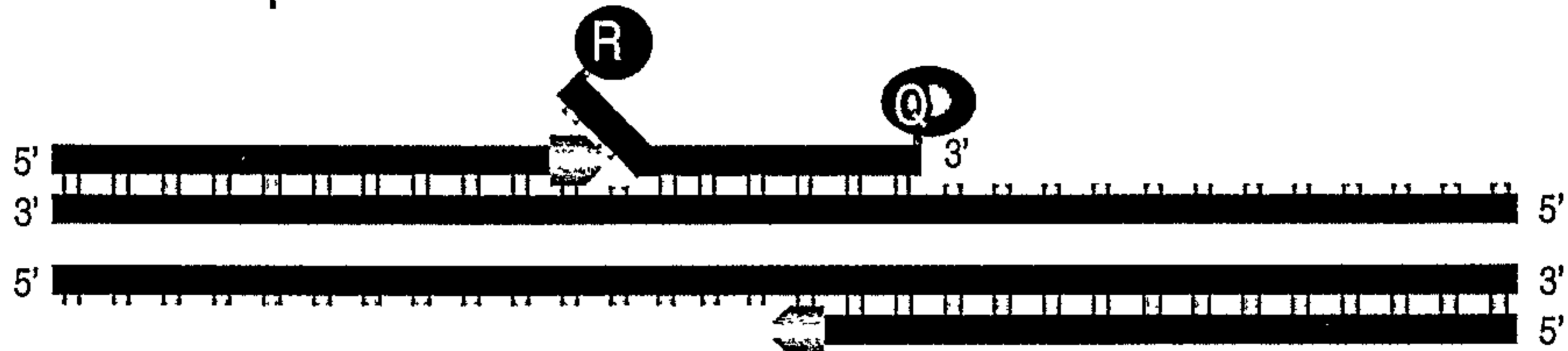
Although a powerful tool, PCR has some disadvantages: the process is labour intensive, post-PCR manipulation of the sample provides the potential for contamination of the amplified products, the quality of the PCR product at the end of the reaction could be affected by depletion of reagents and direct quantitation of the original sample is not possible. Holland *et al.* (1991) devised a method to overcome many of these problems whereby product detection occurs concurrently with target amplification and little or no handling of the post-PCR sample is required. This was achieved by introducing a labelled oligonucleotide probe designed to hybridise with the target DNA. During amplification the 5'→3' exonuclease activity of *Taq* polymerase degrades the probe only when it has been hybridised to the specific target. The amount of probe degradation is therefore proportional to the amount of signal generated, which is proportional to the amount of original sample DNA.

Continued improvements in probe design made use of a probe labelled with two dyes, a fluorescent reporter dye and a fluorescent quencher dye. This TaqMan probe hybridises to an internal region within the amplicon between the primers (Figure 5.1). The reporter dye (6-carboxyfluorescein [FAM], tetrachloro-6-carboxyfluorescein [TET], or 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein [JOE]) is covalently linked to the 5' end and the quencher dye (6-carboxy-tetramethylrhodamine [TAMRA]) is attached at the 3' end. The location of the quencher dye in close proximity to the reporter dye, when the probe is intact, dampens emission of the reporter dye. When hydrolysis of the probe occurs during amplification and the *Taq* polymerase cleaves the probe from its target sequence, separation of the quencher and reporter dyes allows emission of a fluorescence signal. This emission is quantitative for the initial amount of sample DNA (Bassler *et al.*, 1995; Bassam *et al.*, 1996).

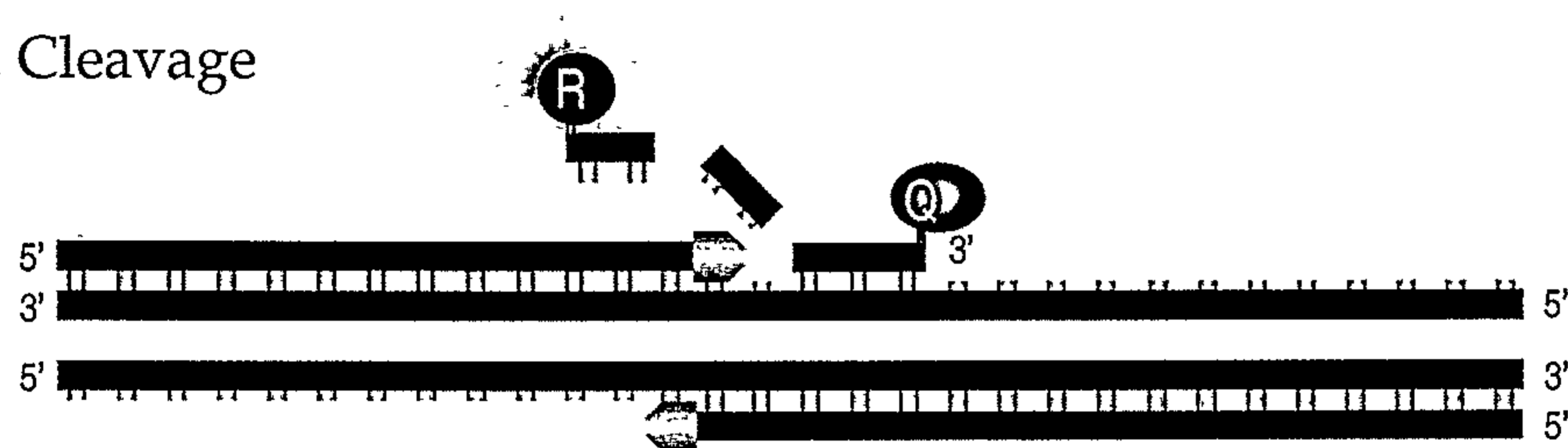
1. Polymerisation



2. Strand displacement



3. Cleavage



4. Polymerisation completed

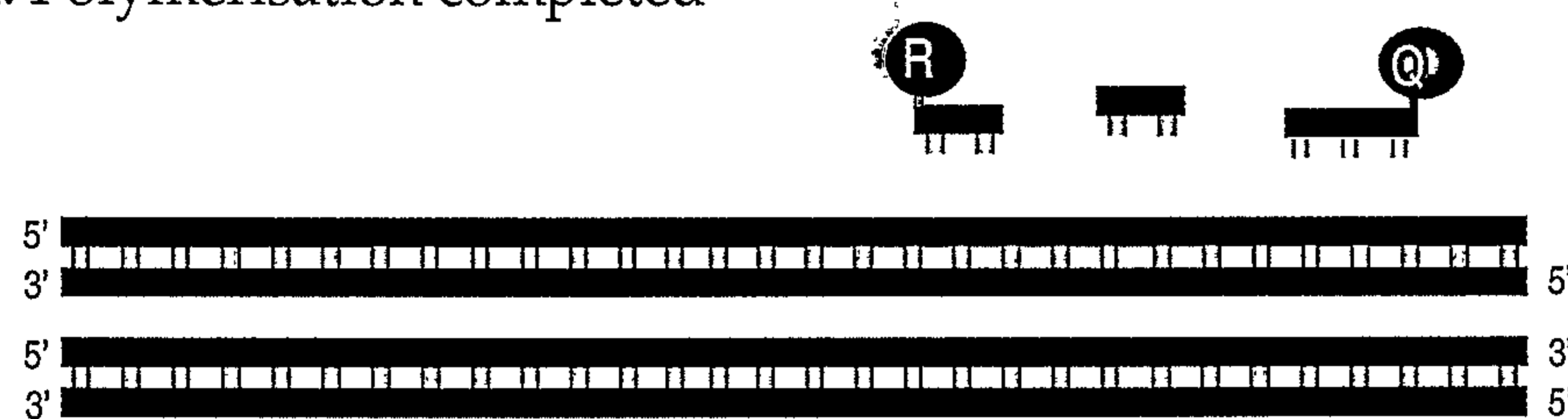


Figure 5.1. Diagram representing stages in the fluorogenic 5' nuclease chemistry. Prior to polymerisation, the primers are annealed to the target DNA and the two fluorescent dyes (R-reporter and Q-quencher) are attached to the probe with the reporter dye emission quenched. During strand displacement the DNA polymerase extends the sequences between the primers with initial displacement of the probe. The extension cycle results in cleavage of the probe with separation of the reporter and quencher dyes resulting in emission of fluorescence from the reporter dye. Polymerisation is complete when new strands of DNA have formed. Diagram provided by Applied Biosystems, Foster City, CA, USA.

Further improvements in instrumentation now allow "real-time" monitoring of the fluorogenic signal as it is generated during amplification. The system integrates a thermal cycler, a laser light source, a fibre-optic network, a cooled CCD camera detector and a computer with dedicated software. Upon collection of the fluorescence signals, the software computes the contribution of each of the dyes to the spectrum and normalises the reporter dye against the standard ROX (6-carboxy-X-rhodamine) passive internal reference dye. Real-time monitoring of the reaction can define the point at which amplification of the PCR product is first detectable. This takes place when fluorescence generated by cleavage of the probe passes a fixed threshold above the baseline. It occurs during the exponential phase of product accumulation, early in the PCR when reagent availability cannot limit the reaction. The higher the original amount of DNA in the sample the fewer the amplification cycles required before a significant increase in fluorescence is recorded. Therefore, measurement of the initial amount of sample DNA is determined from the cycle at which it crosses the baseline (C_T) compared with a standard curve created from known amounts of target DNA (Bassam, *et al.*, 1996; Heid, *et al.*, 1996).

For optimal hybridisation reactions, further development has evolved the use of a "hot start" strategy for PCR. This prevents non-specific primer annealing and extension that might occur when the reaction components are mixed at room temperature. To achieve this, modified forms of the *Taq* polymerase enzyme have been created that are activated only after exposure to an elevated temperature, thus preventing the polymerase from functioning until thermal cycling begins (Birch *et al.*, 1996).

Therefore, current technology using real-time PCR allows amplification of a specific segment of rDNA from a background of mixed DNA in a closed-tube system which requires no post-PCR processing, thereby minimising contamination problems and decreasing processing times. Analysis is performed in the log phase of product accumulation and is consequently not limited by reagent depletion, is claimed to be highly reproducible and allows for a large dynamic assay range.

5.1.2 Detection of oral pathogens using PCR

As previously discussed, conventional diagnostic procedures are based on the ability to culture microorganisms or identify their presence using antibodies. The culture approach is limited as many microbes defy cultivation by standard methods, and of those that can be cultured, many are slow growing with fastidious growth requirements, while antibody testing is relatively insensitive. PCR now provides an effective alternative. Numerous investigators have reported more frequent detection of periodontal pathogens such as *P. gingivalis*, *P. intermedia*, *Bacteroides forsythus*, *Treponema denticola*, *H. actinomycetemcomitans* (Loesche *et al.*, 1992; Ashimoto *et al.*, 1996; van Steenberg *et al.*, 1996; Mättö *et al.*, 1998) and endodontic pathogens, for example, *P. endodontalis* (Dymock *et al.*, 1996) from clinical samples using PCR compared with traditional culture techniques. Other studies have identified unknown species (Harper-Owen *et al.*, 1999) as well as unexpected species in oral environments for example, *T. denticola*, *P. gingivalis*, *Prevotella tanneriae* and *B. forsythus* in endodontic infections (Xia *et al.*, 1999; Siqueira *et al.*, 2000; Siqueira *et al.*, 2001). Within the area of microbiology, PCR has permitted the detection of microorganisms from environments which harbour primarily slow growing obligate anaerobes with fastidious growth requirements and that are often difficult or impossible to culture. Other advantages include the ability to correctly identify bacteria otherwise not adequately identified by screening with phenotypic tests or commercial identification kits (Baumgartner *et al.*, 1999; Haraldsson & Hobrook, 1999) and the opportunity to quantify bacterial load, or particular species, within a mixed environment (Suzuki *et al.*, 2000). In addition, the ability to quantify load allows monitoring of the effectiveness of treatment regimes during therapy (Takamatsu *et al.*, 1999).

5.1.3 Limitations of PCR technology

Despite the obvious advantages already discussed, there are limitations that can interfere with accurate and efficient performance of the PCR, including the possibility of producing misleading or inaccurate data.

5.1.3.1 Design of primers and probes

Primers and probes for PCR are designed from sequences of microorganisms found in databases to target discrete regions of the rDNA gene. The rDNAs of different organisms vary in areas of sequence conservation so that by targeting areas of greater or lesser conservation, primers and probes can be designed to identify individual or groups of microorganisms. It is assumed that the rDNA database sequence entries from which primers and probes are designed are accurate. Unfortunately, there are reports of errors in many of the sequences. Two common problems exist; the first involves contamination by the inclusion of DNA from an unrelated organism following cloning and the second includes entries that contain errors or missing segments (Pennisi, 1999). Another confounding factor, which may compromise the accuracy of primer and probe design, is the presence of many relatively short sequences. Some published sequences are only 500bp long, which represents only one third of the entire 16S rDNA sequence. This may result in insufficient comparative information to allow correct placement in the phylogenetic tree (Hugenholtz *et al.*, 1998).

Certain guidelines must be considered when designing primers and probes to optimise efficient performance for real-time PCR. To assist probe hybridisation, the melting temperature (T_m) of the probe should be between 68-70°C, and 10°C higher than that for the primers (58-60°C). The probe length should ideally be 20-25 nucleotides in length; probes longer than 30 nucleotides have reduced synthesis efficiency and form structures that are likely to interfere with probe hybridisation and cleavage, and DNA amplification. Other design factors, specific for the Applied Biosystems 7700, include a GC content between 30-80%, no runs of greater than three consecutive Gs in either the probe or the primers, the 5' end of the probe should not finish with a G, the last 5 nucleotides at the 3' end of the primers should contain no more than 2 GCs, the probe should be selected from the strand that contains more Cs than Gs and the amplicon length should be between 50-150 base pairs (Bassam, *et al.*, 1996). Other important design considerations include the elimination of secondary structures, duplexes and primer-dimers that might result in the formation of non-specific amplification products. Careful adjustment of temperature, magnesium ion concentration and *Taq* polymerase concentration

within the reaction can eliminate the formation of non-specific PCR products. Software packages are available that provide guidelines and information regarding the design of primers and probes and the avoidance of spurious secondary structures.

Once designed, probe and primer sequences must be checked against database entries to ensure sequence specificity for individual microorganisms and to prevent cross-reactivity with closely related species. Following synthesis of the components and prior to testing with clinical samples, the primers and probe must first be optimised in order to select those concentrations that will provide the most efficient amplification. The primers and probe are then tested against DNA from known microorganisms to verify the specificity of the amplicon.

5.1.3.2 The polymerase chain reaction

The greatest problem affecting PCR usage is that of false positive results which occurs when the majority of molecules detected are from exogenous sources rather than from the test sample. Transfer of even minute quantities of material from a previous PCR, which could contain millions of amplicon copies, is capable of contaminating reagents, buffers, glassware and ventilation systems. To avoid amplicon carry-over, PCR laboratories are required to take special precautions including the use of disposable materials, pre-aliquoted reagents, positive displacement pipettes, premixing of all reagents, avoiding the creation of aerosols and adding DNA as the last step prior to dispensing into the reaction wells. In addition, preparation of reaction mixes should be carried out in a biohazard hood located in a separate area from that of sample preparation and PCR product analysis (Kwok & Higuchi, 1989).

Other techniques to avoid contamination include the use of real-time PCR, where amplification and analysis occurs in a sealed tube, and an amplicon sterilisation step within each PCR run. Amplicon sterilisation involves the substitution of dUTP for TTP in the amplification reaction mixture, resulting in the incorporation of U instead of T in the amplicon and providing a method of discriminating between the original target sequence and the PCR amplicon. The bacterial enzyme uracil-*N*-glycosylase (UNG) is added to the reaction mix and during a brief incubation step the UNG acts by cleaving all uracil residues, thereby

enzymatically degrading any amplicons from previous reactions. Heating the reaction mixture to 94°C then inactivates the UNG (Persing, 1991).

Another source of contamination when using a broad range or universal amplicon to detect total bacterial load comes from bacterial contamination of the *Taq* DNA polymerase. This occurs during manufacture of the polymerase enzyme which is commonly expressed as a recombinant protein in *Escherichia coli* followed by incomplete purification. In the same manner, use of the UNG enzyme will provide additional contamination (Böttger, 1990; Schmidt *et al.*, 1991; Corless *et al.*, 2000).

The use of controls in each PCR experiment is essential to monitor the efficiency of the reaction. False positive reactions are one of the greatest problems with PCR because of the sensitivity of the enzymatic amplification. False positives occur because the PCR amplifies contaminating DNA that may have been present in minute amounts. To detect false positive reactions the PCR should include a negative control or no-template-control. This control consists of all reagents except the DNA sample and will detect contamination in the PCR reagents such as buffers, water and *Taq* polymerase. It may also be necessary to include a no-enzyme-control to check for fluorescence contamination of the sample.

DNA extracted from clinical specimens may contain PCR inhibitors such as potassium ions, blood, urine, vitreous humor, sputum and saliva (Mättö, *et al.*, 1998; Fredricks & Relman, 1999). These inhibitors must be diluted, removed or inactivated to allow DNA amplification to proceed. Fortunately, DNA purification protocols remove most PCR inhibitors. However, unless the inhibitory components are identified and removed, a lack of amplification recorded during the reaction would not necessarily be due to an absence of target DNA, thus creating a false negative result. This can be overcome by monitoring the amplification of a second target nucleic acid during the reaction which serves as an internal control. A limited number of internal control molecules are added to the test sample and co-amplified with the target DNA, therefore, obtaining a positive signal from the internal control indicates successful amplification and can be used to monitor amplification and detection (Rosenstraus *et al.*, 1998). Other reasons for false negative reactions include failure of the DNA extraction procedure to release

DNA, loss of DNA during the purification process, or due to inadequate initial sample volume (Fredricks and Relman, 1999).

Another source of error with PCR is the formation of chimeric gene products. These may occur when genes from mixed bacterial populations are amplified and chimeric products form during the primer extension phase when some templates are incompletely copied. Therefore, during ensuing cycles the partial copies re-anneal to be further extended onto a second template, thus creating chimeric genes of regions copied from different templates. Fortunately, the formation of chimeric products is regarded as a rare event and most likely to occur as a result of low dNTP substrate concentrations in late cycles (Giovannoni, 1991).

5.1.3.3 Preparation of samples for PCR

Sample collection and processing can have a significant impact on the outcome of the PCR assay. Many different types of clinical samples can be used for DNA amplification including tissue, blood, semen, hair, pleural fluid, saliva and archival material, fixed and embedded in paraffin or resin. However, fixation of materials in formaldehyde damages the DNA, often resulting in a smaller sized product available for amplification. Other tissues and fluids require refrigeration and rapid processing or freezing to preserve the DNA and prevent deterioration.

Since quantification of the number of bacteria is entirely dependent on the amount of DNA measured, the extraction and purification protocol becomes a critical step in the procedure. Successful extraction of bacterial DNA from clinical samples often requires the concentration of small amounts of DNA, the inactivation of nucleases and purification to eliminate proteins. Ideally, the method chosen should be sensitive, simple, rapid and reproducible, requiring no specialised equipment or biochemical knowledge. Following extraction, the DNA should be sufficiently pure to allow enzymatic modifications and risk to personnel from any pathogens should be negligible (Boom *et al.*, 1990). Traditionally, the extraction and purification of bacterial DNA has been performed with large numbers of cells using standard procedures to lyse cells with phenol-chloroform followed by extraction with organic solvents to remove residual proteins and cell wall components, followed by precipitation of nucleic acids in ethanol to remove traces of the solvents. However, such techniques incur considerable loss of

DNA and are not applicable to small scale DNA purification. There are reports of various protocols for digesting and purifying clinical samples; some use mechanical, chemical or enzymatic means to break open the bacterial cell walls. Mechanical methods of sample preparation include freeze-thawing, boiling, sonication with or without glass beads, and crushing or homogenising. Other methods use enzymes such as lysozyme, mutanolysin, and proteinase K, chaotropes which include guanidine isothiocyanate, guanidine hydrochloride or hexadecyltrimethyl ammonium bromide (CTAB). In addition detergents, such as sodium dodecyl sulphate (SDS), can be used to disrupt and lyse the bacterial cell wall, digest proteins and inactivate nucleases. Some of these techniques work efficiently for particular microbes, however, there is no single method which optimally digests all bacteria (Fredricks and Relman, 1999).

Numerous commercial kits are also available for the extraction and purification of small quantities of DNA for use with PCR, although published data comparing these kits provides conflicting results. For example, the QIAamp Blood Kit (Qiagen, Clifton Hill, Victoria, Australia) was found by Dixon *et al.* (1998) to be optimal for extracting DNA from serum when comparing 13 different kits, whereas de Kok *et al.* (1998) described the same kit as having very poor isolation efficiency when only a small amount of the target DNA was present.

Correct handling and storage of samples for PCR can impact on the accuracy of bacterial quantitation. Wahlfors *et al.* (1995) found that one additional freeze/thaw cycle of DNA samples weakened the PCR signal obtained. They cautioned that the composition of the sample could be distorted by careless handling, additional freeze/thaw cycles and prolonged storage at inappropriate temperatures, and strongly recommended that analyses be carried out immediately after sample collection. Another study demonstrated that samples stored at 4°C after boiling showed degradation of the DNA (Nakajima *et al.*, 1994). These reports indicate a problem with degradation of sample DNA. Many of the microorganisms found in carious dentine are capable of deoxyribonuclease (DNase) activity. These enzymes have the ability to initially hydrolyse chromosomal DNA into small fractions, followed by total degradation. Although the functions of DNases are unknown, there is speculation regarding roles in

pathogenicity through the facilitation of tissue invasion, provision of nucleic acid precursors for bacterial growth and protection of bacterial integrity from foreign DNA (Rudek & Haque, 1976; Minion *et al.*, 1993; Azcárate Peril *et al.*, 2000). Other authors suggest roles in DNA recombination, replication and repair systems (Yanagida *et al.*, 1982). Nucleases have been found in a number of anaerobic species including fusobacteria, peptostreptococci, propionibacteria, veillonellae, prevotellae (Porschen & Sonntag, 1974) and porphyromonads (Mayrand *et al.*, 1984; Leduc *et al.*, 1995). DNase activity has also been detected in microaerophilic bacteria including lactobacilli (Miller *et al.*, 1971) and oral streptococci (Smith & Bodily, 1967). For most of these microorganisms the DNase is extracellular and does not require lysis of the cell for nuclease activation, however, *Lactobacillus plantarum* produces two nucleases, with one product found in the cytoplasm and membrane fractions of the bacterium (Caso & Suárez, 1997).

Nuclease activity from some bacteria requires the presence of magnesium and/or calcium and incubation at a temperature greater than 16°C. Nuclease activity can usually be controlled by storing bacteria at low temperatures (0-4°C) or exposure to high temperatures (>70°C for 15 minutes). Exceptions are the heat stable DNases of *Yersinia enterocolitica* where boiling is ineffective (Nakajima, *et al.*, 1994). Nuclease activity has also been reported to be inhibited in the presence of actin (Lazarides & Lindberg, 1974), ZnCl₂, ethylenediamine tetra-acetic acid (EDTA) (Leduc, *et al.*, 1995), ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N',-tetra-acetic acid (EGTA) and sodium dodecyl sulphate (SDS) (Bendjennat *et al.*, 1997).

5.1.3.4 Calculation of bacterial numbers following PCR

Quantitation of target DNA is generally accomplished by measuring the intensity of ethidium bromide staining of PCR products in an agarose or polyacrylamide gel which is compared with the band intensity from a known amount of identical bacteria. This procedure has been described as time-consuming and inaccurate (Gibson *et al.*, 1996). The calculation of cell numbers from sample DNA following quantitative PCR uses a standard curve comprising serial dilutions of known amounts of bacterial DNA run in parallel with the sample DNA. The amount of DNA from a reaction can then be determined by interpolation from the standard

curve (Desjardin *et al.*, 1998). The known DNA representing the standard might be made up of the specific bacterial species under study (Lyons *et al.*, 2000), an internal control which uses identical primer binding regions with a unique probe site compared with the target DNA (Rosenstraus, *et al.*, 1998) or an homologous competitor which utilises two primers forming a shorter amplicon than the target (Rupf *et al.*, 1999).

Each of these methods allows for quantitation of the amount of target DNA, although accurate quantitation of bacterial cell numbers requires knowledge of the size and number of the genomes within a cell, in addition to the number of rDNA operons for individual microorganisms. The size of the genome from different bacterial species may range from 600-13,000kb, the number of genomes may also vary while the number of ribosomal operons can range from 1 to 14 and operon copies may be located on two different genomes (Farrelly *et al.*, 1995; Fogel *et al.*, 1999). This information is available for only a limited number of organisms and is unknown for most oral microbes, with the problem further complicated by studying a mixed microbial population, as found in dental caries. In addition, bi-directional replication can further increase the numbers of a given rDNA operon depending on the number of replication forks and the location of the rDNA operon relative to the origin of replication. The number of replication forks is directly related to the generation time (t_d) which in turn depends on the metabolic status of the bacteria at the time of sampling (Neidhardt *et al.*, 1990; Klappenbach *et al.*, 2000). Not knowing the exact number of copies of 16S rDNA operons in any given species at the time of sampling, therefore, represents the main limitation to the absolute determination of bacterial numbers by real-time PCR based on 16S rDNA. However, where a complex multi-species population might be sampled along with impurities, or where bacteria are internalised within a matrix, such as dentine, other methodologies, such as direct microscopic cell counts or fluorescence-based enumeration, are likely to be far less sensitive.

5.1.3.5 Cost

PCR is expensive. The cost of reagents, equipment and the requirement for separate laboratory space is substantial. Real-time PCR requires the use of fluorescent labelled probes and the optimisation of both probes and primers adds considerably to the overall cost.

However, PCR-based tests offer speed and sensitivity that may offset the costs of microbiological tests, treatment and hospitalisation. In addition, miniaturised reactions/arrays and the use of robotics could assist in cost reductions with time (Fredricks and Relman, 1999).

5.2 Experimental sample preparation for real-time PCR

Bacterial analysis of a clinical sample requires not only identification of the microbes present but a determination of the total bacterial load to provide a baseline reference and allow assessment of treatment efficacy in clinical situations. To facilitate this, a universal probe and primers set was designed and used in this study. However, the quantitation of bacteria using nucleic acid-based technology is entirely dependent on the specificity of the designed probes and primers and the amount of DNA measured, therefore the extraction and purification protocol becomes a crucial part of the procedure. Accurate quantification of bacteria also relies on the efficient isolation of DNA from a mixed bacterial population that reflects the true bacterial profile. This is especially important when a low amount of target DNA is present, a limited amount of the clinical sample is available and when PCR inhibitors could be present.

Another factor detrimental to the reliable quantitation of bacteria is the presence of bacterial nucleases. Many of the microbial species found in carious dentine are capable of DNase activity whereby the chromosomal DNA becomes hydrolysed into component nucleotides (Minion, *et al.*, 1993; Leduc, *et al.*, 1995). In this study an internal positive control was used during sample processing and PCR to indicate deterioration of the PCR signal through nuclease activity, although it would not detect DNA hydrolysis following storage.

In the initial stage of the present study a universal amplicon was developed to determine the total bacterial load from samples. In addition, a probe and primers set was designed to enumerate *P. gingivalis*. Following successful testing of these amplicons the second stage of research assessed a series of different DNA extraction procedures for their ability to release DNA from bacterial cells and overcome the presence of nucleases and possible PCR inhibitors. Measurement of DNA was assessed by gel electrophoresis and/or PCR primarily using the universal probe/primers set. The design, optimisation and testing of the universal amplicon represented a conjoint effort involving Dr M Nadkarni and the author, with completion of the

amplicon design by Dr M Nadkarni which is reflected in the authorship of the associated publication (Nadkarni *et al.*, 2002). The experimental data presented in this chapter also represents a convergence of research and where Dr M Nadkarni had the prime role this is indicated in the text. Additionally, where data or gels produced by Dr M Nadkarni have been incorporated in the results, this has been indicated.

5.2.1 Materials and methods

5.2.1.1 Bacterial strains and culture conditions

Escherichia coli strains JM109 (Yanisch-Perron *et al.*, 1985) NM522 (Gough & Murray, 1983) and XL 1 blue (Stratagene, La Jolla, CA, USA) were available from the Institute of Dental Research Culture Collection (Westmead Centre for Oral Health, Westmead, NSW, Australia). *Staphylococcus aureus* strains ATCC 12600, ATCC 9144, ATCC 12598, ATCC BM 10458 and ATCC BM 10143; *Staphylococcus epidermidis* strains ATCC 35983 and ATCC 14990; *Staphylococcus hemolyticus* ATCC 29970 and *S. hemolyticus-infiltrative keratitis isolate*; *Staphylococcus schleferi* ATCC 43808; *Pseudomonas aeruginosa* strains ATCC 19660, ATCC 15442, 6294 and 6206; *Pseudomonas fluorescens-infiltrative keratitis isolate*; *Pseudomonas putida-lens saline isolate*; *Pseudomonas stutzeri-infiltrate isolate*; *Pseudomonas alcaligenes* laboratory-isolate; *Pseudomonas* species and *Serratia marcescens* ATCC 274 were kindly provided by Dr. Mark Willcox, Co-operative Research Centre for Eye Research and Technology, The University of New South Wales, Australia. All *Escherichia*, *Staphylococcus*, *Pseudomonas* and *Serratia* species were grown in Luria Burtani (LB) broth (Miller, 1972) at 37°C in a shaking incubator. *Streptococcus mutans* LT11, *S. gordonii* ATCC 10558 and *S. sanguinis* (formerly *S. sanguis*) ATCC 10556 (obtained from the American Type Culture Collection, Rockville, MD, USA) were grown at 37°C in Brain Heart Infusion broth (Oxoid) under 95% N₂ and 5% CO₂ (v/v); *F. nucleatum* ATCC 25586, *Fusobacterium necrophorum* ATCC 25286, *A. israelii* ATCC 12102 and *A. naeslundii* ATCC 12104 were obtained from the American Type Culture Collection and grown at 37°C in Brain Heart Infusion broth in an anaerobic chamber (85% N₂, 5% CO₂, 10% H₂ [v/v/v]). *Porphyromonas gingivalis* ATCC 33277, *P. melaninogenica* ATCC 25845, *Micromonas micros* (formerly *Peptostreptococcus micros*) ATCC 33270 and *Peptostreptococcus anaerobius* ATCC

27337 were obtained from the American Type Culture Collection and grown at 37°C in an anaerobic chamber in CDC broth [1% (w/v) trypticase peptone and 1% (w/v) trypticase soy broth (Difco, Becton Dickinson, MD, USA), 1% (w/v) yeast extract (Oxoid), 5mg NaCl ml⁻¹, 400µg L-cysteine ml⁻¹ (Sigma Chemical Co., St Louis, MO, USA) containing 5µg haemin ml⁻¹ (Sigma), 2µg menadione ml⁻¹ (Sigma) and 2% (v/v) horse serum (CSL Biosciences, North Ryde, NSW, Australia)}. *Porphyromonas endodontalis* ATCC 35406, obtained from the American Type Culture Collection was also grown in an anaerobic chamber according to the method of Zerr *et al.* (1998). *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus rhamnosus* ATCC 7469 from the Institute of Dental Research Culture Collection (Westmead Centre for Oral Health, Westmead, NSW, Australia) were grown at 37°C in MRS broth (Oxoid) under 95% N₂ and 5% CO₂ (v/v).

5.2.1.2 Sources of other bacterial DNA

DNA from *Legionella pneumophila* serogroup 4 ATCC 33156, serogroup 5 ATCC 33216, serogroup 6 ATCC 33215, serogroup 1 Knoxville-1 ATCC 33153, Philadelphia-1, as well as *Legionella anisa*, *Legionella bozemanii* serogroup-2, *Legionella londiniensis*, *Legionella maceachernii* and *Legionella waltersii* was provided by Mr Rodney Ratcliff, Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, SA, Australia; and from *Mycobacterium tuberculosis* H37RV by Mr Greg James, Microbiology Laboratory, Westmead Hospital, NSW, Australia.

5.2.1.3 Design of primers and probes for real-time PCR

5.2.1.3.1 Universal primers and probe. (Dr M. Nadkarni)

The designed probe and primers set were based on regions of identity within the 16S rDNA following the alignment of sequences from most of the Groups of bacteria outlined in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). The 16S rDNA bacterial sequences with GenBank accession number in parentheses include; *Bacteroides forsythus* (AB035460), *P. gingivalis* (POYRR16SC), *P. melaninogenica* (PVORR16SF), *Cytophaga baltica* (CBA5972), *Campylobacter jejuni* (CAJRRDAD), *Helicobacter pylori* (HPU00679), *Treponema denticola* (AF139203), *Treponema pallidum* (TRPRG16S), *Leptothrix mobilis* (LM16SRR), *Thiomicrospira denitrificans* (TDE243144), *Neisseria meningitidis* (AF059671), *Haemophilus actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) (ACNRRNAJ),

Haemophilus influenzae (HIDNA5483), *E. coli* (ECAT1177T), *Salmonella typhi* (STRNA16), *Vibrio cholerae* (VC16SRRNA), *Coxiella burnetii* (D89791), *L. pneumophila* (LP16SRNA), *P. aeruginosa* (PARN16S), *Caulobacter vibrioides* (CVI009957), *Rhodospirillum rubrum* (RR16S107R), *Nitrobacter winogradskyi* (NIT16SRA), *Wolbachia species* (WSP010275), *Myxococcus xanthus* (MXA233930), *Corynebacterium diphtheriae* (CD16SRDNA), *M. tuberculosis* (MTRRNOP), *Streptomyces coelicolor* (SC16SRNA), *A. odontolyticus* (AO16SRD), *Bacillus subtilis* (AB016721), *Staphylococcus aureus* (SA16SRRN), *Listeria monocytogenes* (S55472), *Enterococcus faecalis* (AB012212), *L. acidophilus* (LBARR16SAZ), *S. mutans* (SM16SRNA), *Clostridium botulinum* (CBA16S), *M. micros* (PEP16SRR8), *Veillonella dispar* (VDRRNA16S), *F. nucleatum* (X55401), *Chlamydia trachomatis* (D89067) and *Mycoplasma pneumoniae* (AF132741) were aligned using the GCG program Pileup (Wisconsin Package Version 8, 1994) accessed through the Australian National Genomic Information Service (ANGIS, <http://www.angis.org.au>).

The Primer Express Software provided by Applied Biosystems was of limited value in determining a universal probe and primers set as the primary selection criterion of the software is the length of the amplicon (50-150bp). Use of this software resulted in a series of best-fit suggestions for the universal primers and probe, leading to unsatisfactory sequence homology for many of the bacterial genera. As a result, the regions of identity within the 16S rDNA were assessed manually, with the Primer Express Software used to check for primer-dimer or internal hairpin configurations, melting temperature (T_m) and percentage GC values within possible primers/probe sets. The designed amplicon (Table 5.1) complied with six of the eight guidelines set by Applied Biosystems for the design of primers and probes. These included a T_m of the DNA between 58–60°C for the primers and 68–70°C for the probe; the G+C content between 30-80%; no runs of more than 3 consecutive Gs in either the primers or the probe; no G on the 5'-end of the probe and the probe selected from the strand with more Cs than Gs. The primers and probe set only deviated from the ideal in that the last 5 nucleotides of the 3'-end of the forward primer contained more than 2 GCs, and that the amplicon of 466bp (based on that generated between residues 331 to 797 on the *E. coli* 16S rDNA gene, GenBank accession no. ECAT1177T) exceeded the 50-150bp recommended. The oligonucleotide probe was labeled

with the fluorescent dyes 5-carboxyfluorescein (FAM) at the 5' end and 6-carboxy-tetramethylrhodamine (TAMRA) at the 3' end.

The universal probe and primers were checked for possible cross hybridization with bacterial genes other than 16S rDNA as well as genes from Eucarya and Archea using the database similarity search program BLAST (Altschul *et al.*, 1990) accessed through ANGIS. The BLAST search results showed only one significant hit; that of a specific breast cancer cell line (BT029) which was detected only by the reverse primer. However, the universal primers did not amplify the human DNA sample supplied by Applied Biosystems in their Beta-actin Detection Kit probe set, thus confirming the specificity of the probe and primers set for the 16S rDNA of the Domain *Bacteria*.

5.2.1.3.2 *P. gingivalis* probe and primers

For the species-specific quantification of *Porphyromonas gingivalis*, a primers-probe set was designed from the variable region of the 16S rDNA sequences reported in Genbank (AB035455, AB035456, AB035457, AB035458, AB035459, AF287987, PGRRNA, POYRR16SC) and accessed through ANGIS. The *P. gingivalis* primers-probe set (Table 5.1) generated a 150bp amplicon spanning nucleotides 590 to 739 (inclusive) in the *P. gingivalis* 16S rDNA sequence (GenBank accession no. L16492) with an internal site for the dual-labelled (FAM and TAMRA) fluorogenic probe. The primers-probe set fulfilled all recommended guidelines set by Applied Biosystems. Once designed, the primers and probes were synthesised by Applied Biosystems.

5.2.1.3.3 Internal positive control. (Dr M. Nadkarni)

A chimeric plasmid was constructed to act as an internal positive control using DNA from the Queensland fruit fly, *Bactrocera tryoni* (M Nadkarni, unpublished data). A primers-probe set was created to enable the detection of the exogenously added internal positive control (IPC-BT) and was designed from the sequence of the *dsX* gene of *B. tryoni* using Primer Express software (Applied Biosystems). The primers-probe set amplified an 89bp region spanning nucleotides 37 to 126 on the *dsX* gene. The probe sequence for the IPC-BT was labelled with the reporter fluorescent dye VIC at the 5' end to differentiate it from the species-specific and

Table 5.1. Primers and probes for detection of the total bacterial load (universal), *P. gingivalis* and *B. tryoni* (internal positive control, IPC-BT).

Primers or probe	Sequence (5'-3')	T _m (°C)
Universal forward primer	TCCTACGGGAGGCAGCAGT	59.4
Universal reverse primer	GGACTACCAGGGTATCTAATCCTGTT	58.1
Universal probe	[6-FAM]CGTATTACCGCGGCTGCTGGCAC [TAMRA]	69.9
<i>P.gingivalis</i> forward primer	TCGGTAAGTCAGCGGTGAAAC	58.8
<i>P.gingivalis</i> reverse primer	GCAAGCTGCCTTCGCAAT	58.7
<i>P.gingivalis</i> probe	[6-FAM] CTCAACGTTTCAGCCTGCCGTTGAAA [TAMRA]	68.8
<i>B. tryoni</i> forward primer	GGAAGGTAAGTTGCATTTTCAGCA	59.3
<i>B. tryoni</i> reverse primer	GCGTACTTATCATGGTAAATTAAGTCAATT	58.6
<i>B. tryoni</i> probe	[VIC] TCCCGTTACAAAATCGTGTTTACATCGTATACTCG [TAMRA]	69.1

universal probes which were labelled at the 5' end with the fluorescent reporter dye FAM (Table 5.1). Due to software limitations, the VIC probe cannot be used to quantify amounts of DNA, therefore results are expressed in terms of C_T values only.

5.2.1.4 Conditions for real-time PCR

Precautions were taken at all stages to avoid contamination and the possibility of false positive PCR results. These included the use of disposable materials where possible with UV sterilisation prior to use, pre-aliquoted reagents, preparation of all reaction mixes for PCR in a biohazard hood located in an area remote from DNA preparation and the addition of DNA as the last component to the reaction mix. Amplification and detection of DNA by real-time PCR used the ABI PRISM 7700 Sequence Detection System with a 96-well plate format. PCR was carried out in duplicate or triplicate reactions, in a 25 μ l reaction volume containing 300nM of each of the universal primers, and 100nM of the universal probe and 100nM each of the primers and probe for the Internal Positive Control (IPC-BT) and *P. gingivalis* set using the TaqMan Universal PCR Master Mix or the TaqMan PCR Core Reagents Kit (Applied Biosystems). The reaction conditions for amplification of DNA were 95°C for 10min and 40cycles of 95°C for 15s and 60°C for 1min. Data were analysed using the Sequence Detection Software Version 1.6.3 supplied by Applied Biosystems.

5.2.1.4.1 DNA standards used for determining bacterial number by real-time PCR

Most experiments used *E. coli* DNA to generate a standard graph, within the range 238fg-2.38ng, for the determination of bacterial numbers by real-time PCR. *Porphyromonas gingivalis* was also used to generate a standard graph using DNA within the range 360fg-3.6ng. Standard graphs were always prepared from data accumulated at the same time as the test samples in order to act as internal controls.

5.2.1.4.2 Bacterial cultures used in isolation experiments

No attempts were made to standardise optical densities of the different bacterial cultures as comparison of DNA recovery from the various bacterial species with each other was not required within the experiments. Therefore, only relative assessments have been made regarding the DNA measured from each of the methods tested. It should be noted that it was not

possible to purify all the DNA isolated or quantify all the results by PCR because of the costs involved.

Unless stated otherwise all procedures reported for the isolation and quantitation of bacterial cultures were repeated at least once to confirm reproducibility of the results.

5.2.1.5 Extraction and protection of bacterial DNA

As a survey of the literature revealed no clear methodology for the extraction of bacterial DNA combined with appropriate protection against the action of nucleases, an incremental experimental approach was used to determine the most efficient procedure to address both aims. Initially different mechanical methods were used to facilitate the release of bacterial DNA from a Gram-positive bacterium *S. mutans* and a Gram-negative bacterium, *P. gingivalis*. These bacteria were selected to provide representative examples of microbes found in dental caries that provided difficulties in DNA extraction and nuclease control, respectively.

Following successful testing and optimisation of both the universal and *P. gingivalis* probe/primers sets, the universal amplicon was used to quantitate DNA from a number of experiments involving the extraction and protection of DNA to provide comparative data, while the *P. gingivalis* amplicon was used in an experiment assessing nuclease activity.

5.2.1.5.1 Mechanical extraction procedures

5.2.1.5.1.1 Freeze-boil method

P. gingivalis and *S. mutans* cells ($\sim 10^9$ cells) were harvested by centrifugation (14,000 g, 2min, 18–20°C) (Biofuge Pico, Heraeus Instruments, Sydney, Australia) and resuspended in 200µl of buffer containing 10mM sodium phosphate pH 6.7 and frozen at –20°C (2–16h) before being boiled for 10min. After cooling to room temperature (18–20°C) the samples were incubated at 37°C for 10min following the addition of 1mg DNase-free RNase ml⁻¹ (Sigma). The unpurified samples were diluted 100-fold and a 2.5µl aliquot quantified using the ABI PRISM 7700 Sequence Detection System with the universal primers-probe set (Table 5.1) and the TaqMan Universal PCR Master Mix. Triplicate measurements were taken based on a standard graph generated by known amounts of *E. coli* DNA.

5.2.1.5.1.2 Freeze-thaw method

P. gingivalis and *S. mutans* ($\sim 10^9$ cells) were harvested as described and resuspended in 200 μ l of buffer containing 10mM sodium phosphate pH 6.7 and frozen at -20°C . After thawing and RNase treatment, the samples were diluted 100-fold for PCR quantitation as described above.

5.2.1.5.1.3 Sonication

P. gingivalis and *S. mutans* ($\sim 10^9$ cells) were harvested and resuspended in 200 μ l of buffer containing 10mM sodium phosphate pH 6.7 prior to continuous sonication for 3, 6 or 9min at 75W using a Branson Sonifier (Model 250; Branson Ultrasonics Corporation, Danbury, CT, USA). Aliquots were collected at each time interval and following RNase treatment, samples were diluted 100-fold for PCR quantitation as described.

5.2.1.5.2 Nuclease detection

Different experiments were designed to assess whether the loss of *P. gingivalis* DNA noted following mechanical treatments was the result of shearing or DNase activity.

5.2.1.5.2.1 Isolation of *P. gingivalis* DNA in the presence of the internal positive control (IPC-BT) (Dr M. Nadkarni)

P. gingivalis cells ($\sim 2.5 \times 10^8$) were harvested by centrifugation (14,000g, 2min, 18 – 20 $^\circ\text{C}$) and the pellet resuspended in 100 μ l of buffer containing 10mM sodium phosphate pH 6.7 and 1 μ l (620pg/ μ l) IPC-BT DNA. Following the freeze/thaw and freeze/boil procedures, described previously, the samples were diluted 100-fold with 2.5 μ l aliquots used for real-time PCR. Quantitation of DNA used the *P. gingivalis* and IPC-BT primers-probe sets with the TaqMan PCR Core Reagents Kit. Duplicate measurements were taken from a standard curve generated from known amounts of *P. gingivalis* DNA.

5.6.1.5.2.2 Detection of nuclease activity in *P. gingivalis*

Exogenous *P. gingivalis* DNA (300–400ng), prepared using the ATL method (5.2.1.5.3.10) and purified using the QIAamp DNA Mini Kit (Qiagen) was added to samples containing approximately 300–400ng DNA prepared using the freeze/thaw, freeze/boil, 3 and 6 min sonication procedures, prior to incubation at 50 $^\circ\text{C}$ for 30min. The presence of DNA was

determined visually following electrophoresis of samples in 1% (w/v) agarose gels in TAE and stained with ethidium bromide.

5.2.1.5.3 Chemical DNA isolation procedures

Further experiments were conducted to establish a protocol for DNA extraction and isolation designed to release the maximum amount of polymeric DNA and inhibit nuclease activity from an expanded group of representative Gram-positive and Gram-negative bacteria using a variety of enzymes and chemicals.

5.2.1.5.3.1 Enzymatic, ZnCl₂ and EDTA methods

Actinomyces israelii, *F. nucleatum*, *L. acidophilus*, *P. gingivalis*, *P. melaninogenica* and *S. mutans* cells ($\sim 2.5 \times 10^8$ of each bacterial species) were harvested by centrifugation (14,000g, 2min, 18–20°C) and resuspended in 100µl of 10mM sodium phosphate buffer pH 6.7 containing 1mg lysozyme ml⁻¹ and 2mg mutanolysin ml⁻¹ (lysis buffer), with and without the addition of 5mM ZnCl₂ or 100mM EDTA. After incubation at 60°C for 30min, the bacteria were lysed in the presence of 1%SDS and incubated at 37°C for 10min following the addition of 1mg RNase ml⁻¹. Quantitation of unpurified DNA using real-time PCR used 2.5µl aliquots of 100-fold diluted samples with the universal primers-probe set and the TaqMan Universal PCR Master Mix. Triplicate measurements were taken from a standard graph generated from known amounts of *E. coli* DNA.

5.2.1.5.3.2 Enzymatic, EDTA, EGTA, DEPC and combination methods

Actinomyces israelii, *F. nucleatum*, *L. acidophilus*, *P. gingivalis*, *P. melaninogenica* and *S. mutans* cells ($\sim 5 \times 10^8$ of each bacterial species) were harvested by centrifugation (14,000g, 4min, 18–20°C) and resuspended in 200µl of 10mM sodium phosphate buffer pH 6.7 containing 1mg lysozyme ml⁻¹ and 2mg mutanolysin ml⁻¹, with and without 100mM EDTA, 100mM EGTA, 20mM diethylpyrocarbonate (DEPC), 100mM EDTA plus 20mM DEPC and 100mM EGTA plus 20mM DEPC. After incubation at 60°C for 30 min, the bacteria were lysed in 1% SDS, incubated at 56°C for 10min with 2mg proteinase K ml⁻¹ (Qiagen) and finally incubated at 37°C for 10min following the addition of 1mg RNase ml⁻¹. The samples were purified using the QIAamp DNA Mini Kit and eluted into AE buffer (proprietary buffer,

Qiagen). DNA was quantified using 2.5µl aliquots of a 1:100 dilution of purified DNA, in triplicate, with the universal primers-probe set as described.

5.2.1.5.3.3 Extended incubation method

Attempts were made to improve the recovery of DNA from the Gram-positive bacteria *A. israelii*, *L. acidophilus* and *S. mutans* while controlling the nuclease activity of *P. gingivalis* by incorporating proteinase K into the buffer and extending the incubation period. Cells of *A. israelii*, *F. nucleatum*, *L. acidophilus*, *P. gingivalis*, *P. melaninogenica* and *S. mutans* (~5 × 10⁸ of each) were harvested by centrifugation (14,000g, 4min, 18–20°C) and resuspended in 200µl of 10mM sodium phosphate buffer pH 6.7 containing 2mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹, 2mg proteinase K ml⁻¹ and 20mM DEPC, with and without the addition of 100mM EDTA and 10mM MgCl₂. After a prolonged incubation of 150min at 56°C the bacteria were lysed in 1% SDS. The presence of DNA was determined visually following electrophoresis of samples in agarose gels.

5.2.1.5.3.4 MES buffer method

Further attempts to improve the recovery of DNA from the Gram-positive microorganisms were based on the methods described by Pollock *et al.* (1987). Cells of *A. israelii*, *F. nucleatum*, *L. acidophilus*, *P. gingivalis*, *P. melaninogenica* and *S. mutans* (~5 × 10⁸ of each), and mixtures of (~2.5 × 10⁸) of *L. acidophilus* plus *S. mutans*, and *P. gingivalis* plus *S. mutans* were harvested by centrifugation (14,000g, 4min, 18–20°C) and resuspended in 200µl of 25mM MES (2-[N-morpholino] ethane sulphonic acid) buffer pH 5.2 containing 2mg lysozyme ml⁻¹ and 2mg proteinase K ml⁻¹, with and without 20mM DEPC, 100mM EDTA and 100mM DEPC plus 100mM EDTA. Following incubation for 120min at 37°C, 10mM NaHCO₃ and 100mM NaCl were added and incubated for a further 120min at 37°C. Aliquots were taken both before and after the addition of 1% SDS and the presence of DNA assessed by agarose gel electrophoresis.

5.2.1.5.3.5 Comparison of buffers

This experiment compared the sodium phosphate and MES buffers with a shorter incubation time. Cells of *A. israelii*, *F. nucleatum*, *L. acidophilus*, *P. gingivalis*, *P. melaninogenica* and *S. mutans* (~5 × 10⁸ of each) and mixtures of (~2.5 × 10⁸) of *L. acidophilus* plus *S. mutans*,

and *P. gingivalis* plus *S. mutans* were harvested by centrifugation (14,000g, 4min, 18–20°C) and resuspended in 200µl of buffer containing either 10mM MES pH 5.2 or 10mM sodium phosphate pH 6.7 plus 2mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹, 2mg proteinase K ml⁻¹ and 20mM DEPC. Following incubation for 15min at 60°C, 10mM NaHCO₃ and 100mM NaCl were added and the cells lysed with 1% SDS. The presence of DNA was determined visually following gel electrophoresis.

5.2.1.5.3.6 Phosphate lysis buffer with additional DNase inhibitors

Cells of *A. israelii*, *F. nucleatum*, *L. acidophilus*, *P. endodontalis*, *P. gingivalis*, *P. melaninogenica*, *M. micros* and *S. mutans* (~5 × 10⁸ of each) were harvested by centrifugation (14,000g, 4min, 18–20°C) and resuspended in 200µl buffer containing 10 mM sodium phosphate pH 6.7, 2mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹ and 2mg proteinase K ml⁻¹ with one of the following; 10mM ascorbic acid (reducing agent), 0.1mM aurin tricarboxylic acid (topoisomerase inhibitor), 50mM trisodium citrate (chelator), or a combination of 10mM ascorbic acid plus 50mM trisodium citrate, with and without 20mM DEPC. Following 20min storage on ice or 6min pulse sonication, the samples were incubated for 30min at 56°C, lysed in 1% SDS or purified using the QIAamp DNA Mini Kit. Aliquots were loaded into agarose gels to visualise the DNA recovered.

5.2.1.5.3.7 Varying the lysozyme concentration

Bacterial cultures (~5 × 10⁸) of *A. israelii*, *F. nucleatum*, *L. acidophilus*, *P. endodontalis*, *P. gingivalis*, *P. melaninogenica*, *M. micros* and *S. mutans* were harvested by centrifugation (14,000g, 4min, 18–20°C) and resuspended in 144µl buffer (10mM sodium phosphate pH 6.7) containing 20mM DEPC. Cell suspensions were incubated on ice for 20min or sonicated in pulse mode for 6min at 75W (Branson Sonifier), followed by the addition of 56µl of 1mg or 5mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹ and 2mg proteinase K ml⁻¹. Another group was resuspended in the same buffer containing the lysis enzymes and 20mM DEPC, and stored on ice for 20min. The cell suspensions were then incubated at 56°C for 40min with intermittent vortexing for 10sec after every 10min. Cells were lysed with 1% SDS and 1mg RNase ml⁻¹ was

added, followed by a further incubation at 37°C for 10min. The unpurified DNA was visualised following gel electrophoresis.

5.2.1.5.3.8 Isolation of DNA in DEPC

Bacterial cultures ($\sim 5 \times 10^8$) of *A. israelii*, *F. nucleatum*, *L. acidophilus*, *P. endodontalis*, *P. gingivalis*, *P. melaninogenica*, *M. micros* and *S. mutans* were harvested by centrifugation (14,000g, 4min, 18–20°C) and resuspended in 200µl of buffer containing 10mM sodium phosphate pH 6.7, 5mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹ and 2mg proteinase K ml⁻¹, with and without the addition of 20mM DEPC. The cell suspensions were incubated at 56°C for 40min with intermittent vortexing for 10sec after every 10min. Cells were lysed with 1% SDS, 1mg RNase ml⁻¹ was added, and the sample incubated for a further 10min at 37°C. The DNA was purified using the QIAamp DNA Mini Kit. The presence and quality of the DNA was determined visually following electrophoresis of samples in agarose gels.

5.2.1.5.3.9 Isolation of DNA using a modified DEPC method

Bacterial cultures ($\sim 5 \times 10^8$) of *A. israelii*, *F. nucleatum*, *L. acidophilus*, *P. endodontalis*, *P. gingivalis*, *P. melaninogenica*, *M. micros* and *S. mutans* were harvested by centrifugation (14,000g, 4min, 18–20°C) and resuspended in 144µl buffer (10mM sodium phosphate pH 6.7) containing 20mM DEPC. Cell suspensions were incubated on ice for 10min or sonicated in pulse or continuous mode for 6min followed by the addition of 56µl of 5mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹ and 2mg proteinase K ml⁻¹. The cell suspensions were incubated at 56°C for 40min with intermittent vortexing for 10sec after every 10min. Cells were lysed with 1% SDS, 1mg RNase ml⁻¹ was added, followed by further incubation at 37°C for 10min. DNA was purified using a QIAamp DNA Mini Kit. Quantitation of DNA using the universal primers-probe set and the TaqMan PCR Core Reagents Kit was carried out in triplicate using 2.5µl aliquots of a 1:100 dilution of purified DNA, based on a standard graph generated from known amounts of *E. coli* DNA.

5.2.1.5.3.10 Isolation of DNA in ATL buffer from the QIAamp DNA Mini Kit

Bacterial cultures ($\sim 5 \times 10^8$) of *A. israelii*, *F. nucleatum*, *L. acidophilus*, *P. endodontalis*, *P. gingivalis*, *P. melaninogenica*, *M. micros* and *S. mutans* were pelleted by centrifugation (14,000g,

4min, 18–20°C). Cell pellets were resuspended in 180µl ATL buffer (Qiagen) and 400µg proteinase K (Qiagen). The cell suspensions were incubated at 56°C for 40min with intermittent vortexing for 10sec every 10min. Ribonuclease (1mg ml⁻¹) was added, followed by a further incubation at 37°C for 10min. DNA was purified using the QIAamp DNA Mini Kit and quantitation of DNA using the universal primers-probe set was carried out as described previously. The presence and quality of the DNA was determined visually following electrophoresis of samples in agarose gels.

5.2.1.5.4 *The effect of ZnCl₂ as a PCR inhibitor (Dr M Nadkarni)*

In order to determine whether ZnCl₂ acted as an inhibitor of real-time PCR, *P. gingivalis* cells (~2.5 × 10⁸) were harvested by centrifugation (14,000g, 2min, 18–20°C) and resuspended in 100µl of 10mM sodium phosphate buffer pH 6.7 containing 1mg lysozyme ml⁻¹ and 2mg mutanolysin ml⁻¹ with the addition of 5mM, 50mM and 500mM concentrations of ZnCl₂. After incubation at 60°C for 30min, the samples were either lysed in 1% SDS, or were purified using the QIAamp DNA Mini Kit following the manufacturer's instructions before being diluted, with 2.5µl aliquots used for real-time PCR. DNA preparations with and without purification were diluted to theoretically contain equivalent amounts of DNA prior to PCR. Quantitation of DNA, in triplicate, used the universal primers-probe set and a standard curve generated from known amounts of *E. coli* DNA, as described previously.

5.2.1.5.5 *Additional extraction methods*

Other extraction methods not described in detail in the preceding methods included: combinations of different chemicals with boiled or autoclaved suspensions; ZnCl₂/DEPC combination in phosphate buffer; the use of Triton X-100 to remove SDS which was found to interfere with the action of mutanolysin; the use of chloroform in the initial suspension; TRIS as an alternate buffer and the use of 10 and 100mM dipyriddy, dimethylsulphoxide (DMSO), HgCl₂, histidine, mannitol and actin in the initial suspension as nuclease inhibitors (Gottesfeld *et al.*, 1971; Pirt, 1975; Dhermy *et al.*, 1977).

5.2.1.6 Statistical analyses

Basic descriptive statistics were applied to the PCR results following DNA quantitation producing mean, standard deviation and standard error information.

5.2.2 Results

5.2.2.1 Sensitivity of the universal probe and primers in detecting *E. coli* rDNA

TaqMan technology determines the PCR cycle at which the increase in fluorescence of the reporter dye reaches a threshold cycle (C_T) (Figure 5.1a). The C_T is proportional to the log of the amount of target DNA and hence the log of the number of bacteria in the sample, provided there is only one copy of the reported sequence within the genome. The standard graph based on *E. coli* rDNA (Figure 5.1b) theoretically equates one *E. coli* cell to the detection of 4.96fg DNA, provided the seven copies of rDNA in each copy of the chromosome (Farrelly, *et al.*, 1995) are not taken into consideration. Using *E. coli* as a standard, between 238fg of *E. coli* DNA (corresponding to 48 *E. coli* cells) and 2.38ng of *E. coli* DNA (corresponding to 4.8×10^5 *E. coli* cells) was consistently detected. It should be noted that at extreme high and low C_T values, a two-fold error in the estimation of the relative amount of DNA can occur. C_T values below 0.1pg and above 1000pg DNA should therefore be avoided in calculating the amount of DNA in a sample provided alternative dilutions are practical (Fig. 5.1b).

Detection in the apparent range of 4.8 to 48 cells was limited by contamination from bacterial DNA in the commercially supplied reagents. The degree of contamination varied with different kits of the TaqMan Universal PCR Master Mix and TaqMan PCR Core Reagents Kit. To minimise this problem different lot numbers of TaqMan Universal PCR Master Mix and TaqMan PCR Core Reagents Kit supplied by Applied Biosystems were tested, and only those with minimum contaminating DNA were used. Although 40 cycles were theoretically available for the reaction, contamination of reagents manifest in the no-template-control restricted the sensitivity of the reaction to C_T values below 33–38 cycles depending on the batches of reagents used. In Figure 5.1a, the no-template-control is seen at C_T of 37.7. This contamination is not detected when using the species-specific probes and primers.

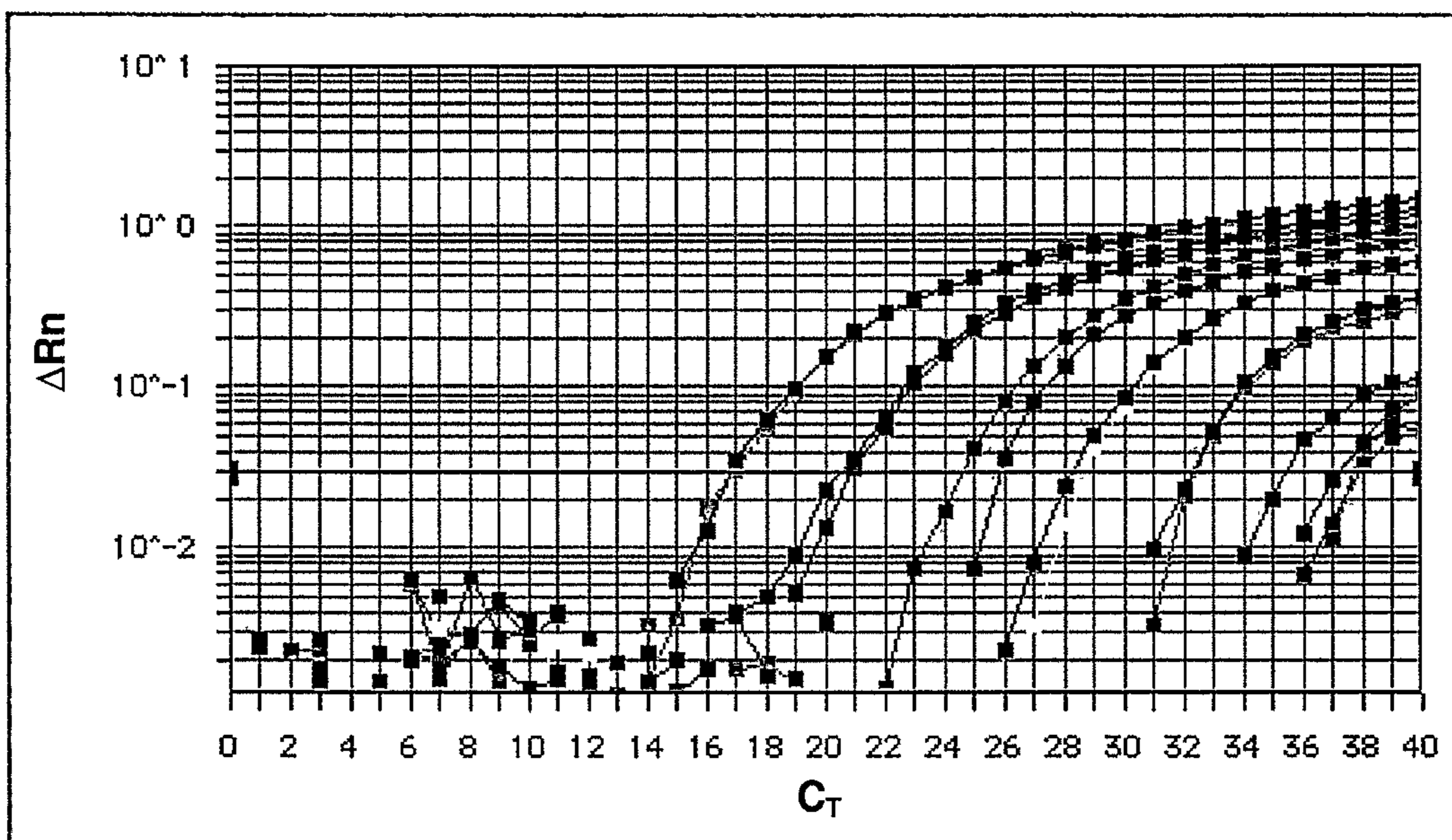


Figure 5.1a. Sensitivity of the universal probe and primers set in detecting *Escherichia coli* rDNA. Purified *E. coli* DNA was used as the template in quantities of 2380pg, 238pg, 23.8pg, 2.38pg, 238fg and 23.8fg, representing C_T values in the range 16.9-35.3 where the intercept of the magnitude of the fluorescence signal (ΔR_n) with the horizontal threshold line in bold represents the C_T value for a given sample. The fluorescence signal at C_T 37.7 corresponds to the no-template control and represents bacterial DNA contamination in the commercially supplied reagents.

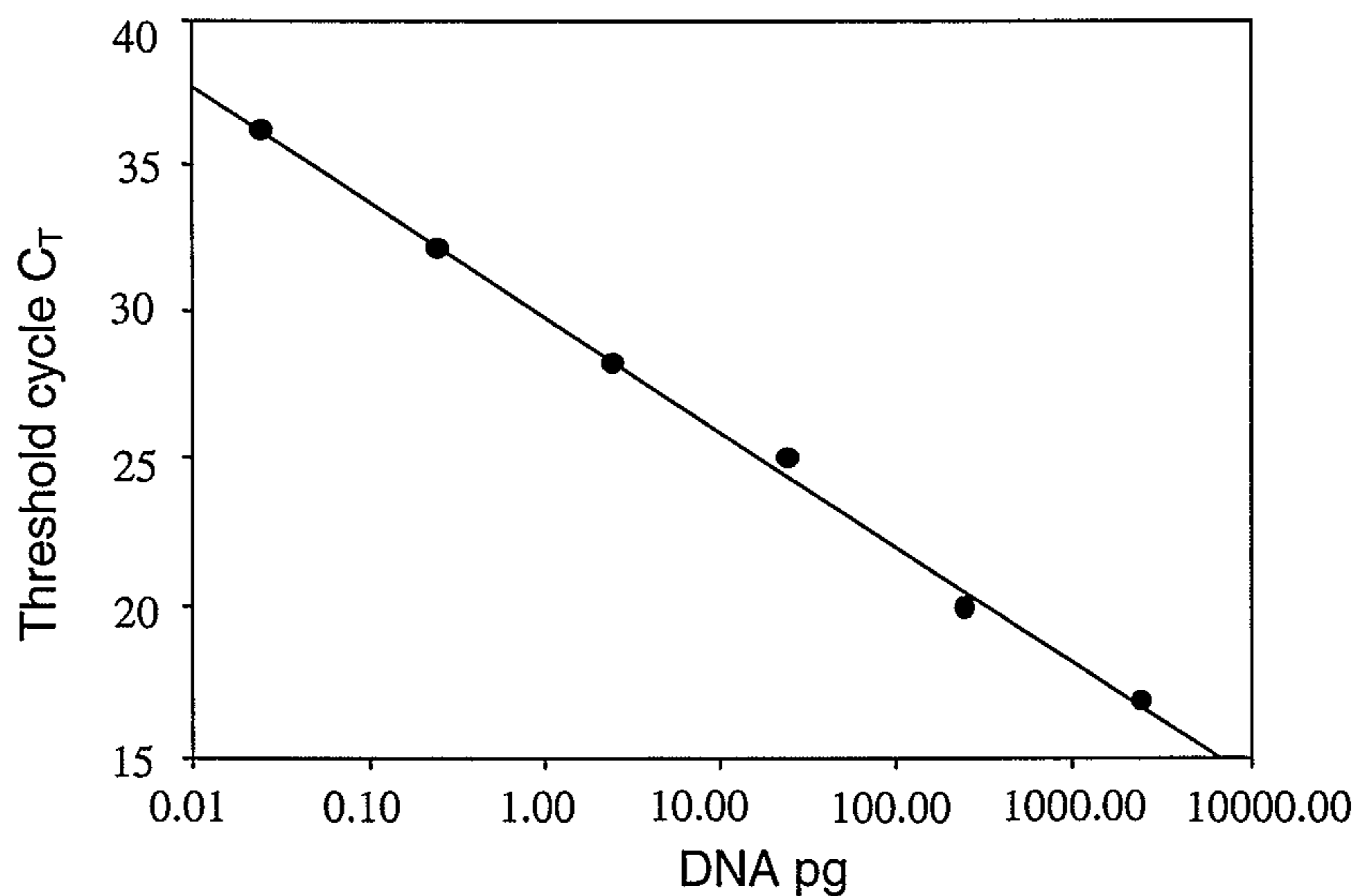


Figure 5.1b. Relation between the threshold cycle and the amount of *E. coli* DNA using TaqMan real-time PCR. Each point represents an amount of *E. coli* DNA corresponding to the C_T value using the universal probe and primers set. The correlation coefficient of the straight line, R^2 , was 0.994.

5.2.2.2 Broad range detection of bacterial species by the universal probe and primers set

In order to determine the ability of the universal probe and primers set to detect a broad range of bacteria, samples of DNA extracted from 49 strains representing 34 different species from the major Groups of bacteria listed in Bergey's Manual of Determinative Bacteriology (Holt, *et al.*, 1994) were subjected to real-time PCR using the probe/primers set. All the selected species were detected within a C_T range of 17.05–34.00 (Table 5.2). For each species there was little variance in the value of 2.00×10^2 (range 1.98 – 2.06×10^2) *E. coli*-equivalent bacteria per pg DNA when *E. coli* DNA was used as a standard, indicating that the source of DNA was not influencing the level of detection and that the probe and primers set was equally efficient in detecting the DNA irrespective of the species from which it was extracted. Only in the case of *M. micros* was there a mismatch in identity between the probe and primers set and the 16S rDNA. This constituted a single nucleotide deletion in the 16S rDNA compared with the 5'-end of the forward primer. This sequence discrepancy was clearly tolerated during real-time PCR detection of *M. micros* DNA (Table 5.2).

5.2.2.3 Detection using the *P. gingivalis* and internal positive control probe and primers

Specificity of the *P. gingivalis* probe and primers set was tested against *S. mutans* LT11 (Tao *et al.*, 1993), *F. nucleatum* ATCC 25586, *F. necrophorum* ATCC 25286, *A. israelii* ATCC 12102, *A. naeslundii* ATCC 12104, *P. gingivalis* ATCC 33277, *P. melaninogenica* ATCC 25845, *M. micros* ATCC 33270 and *P. anaerobius* ATCC 27337, *P. endodontalis* ATCC 35406, *L. acidophilus* ATCC 4356 and human Beta-actin DNA (Applied Biosystems), and found to detect only *P. gingivalis* DNA.

The addition of a chimeric plasmid containing unique non-bacterial DNA to mixed bacterial samples allowed both a determination of the efficiency of DNA recovery following sample preparation and the detection of potential PCR inhibitors in the reaction mix during real-time PCR. The IPC-BT produced a fluorescence signal with real-time PCR, measured by the C_T reading, confirming an internal location for the probe within the amplicon (Fig. 5.2).

Table 5.2. Representative bacterial species detected by real-time PCR using the universal probe and primers set.

Bacterial Species*	C _T [†]
Gram negative aerobic bacteria[†]	
<i>Pseudomonas aeruginosa</i> ATCC 19660	18.14
<i>Pseudomonas aeruginosa</i> ATCC 15442	18.46
<i>Pseudomonas aeruginosa</i> 6294	18.26
<i>Pseudomonas aeruginosa</i> 6206	19.67
<i>Pseudomonas fluorescens</i>	19.19
<i>Pseudomonas putida</i>	22.35
<i>Pseudomonas stutzeri</i>	18.87
<i>Pseudomonas alcaligenes</i>	19.33
<i>Pseudomonas species</i>	19.52
<i>Legionella pneumophila</i> knoxville-1 ATCC 33153	21.93
<i>Legionella pneumophila</i> serogroup 4 ATCC 33156 21	34.00
<i>Legionella pneumophila</i> serogroup 5 ATCC 33216	<u>20.08</u>
<i>Legionella pneumophila</i> serogroup 6 ATCC 33215	21.19
<i>Legionella pneumophila</i> philadelphia-1 ATCC 33152	25.18
<i>Legionella anisa</i>	24.02
<i>Legionella bozemanii</i> serogroup 2	21.46
<i>Legionella londiniensis</i>	20.50
<i>Legionella macearchernii</i>	22.97
<i>Legionella waltersii</i>	21.96
Gram negative facultative anaerobic bacteria	
<i>Escherichia coli</i> JM109	19.86
<i>Escherichia coli</i> NM522	28.22
<i>Escherichia coli</i> XL 1 blue	26.95
<i>Serratia marsescens</i> ATCC 274	20.96
Gram negative anaerobic bacteria	
<i>Porphyromonas gingivalis</i> ATCC 33277	23.50
<i>Porphyromonas endodontalis</i> ATCC 35406	22.05
<i>Prevotella melaninogenica</i> ATCC 25845	20.48
<i>Fusobacterium necrophorum</i> ATCC 252	23.15
<i>Fusobacterium nucleatum</i> ATCC 25586	21.05
Gram positive bacteria	
<i>Staphylococcus aureus</i> ATCC 12600	16.15
<i>Staphylococcus aureus</i> ATCC 9144	29.57
<i>Staphylococcus aureus</i> ATCC 12598	27.41
<i>Staphylococcus aureus</i> ATCC BM 10458	<u>26.32</u>
<i>Staphylococcus aureus</i> ATCC BM 10143	27.20
<i>Staphylococcus epidermidis</i> ATCC 35983	17.88
<i>Staphylococcus epidermidis</i> ATCC 14990	22.27
<i>Staphylococcus hemolyticus</i> ATCC 29970	21.14
<i>Staphylococcus hemolyticus</i>	<u>22.28</u>
<i>Staphylococcus schleferi</i> ATCC 43808	22.29
<i>Streptococcus sanguis</i> ATCC 10556	17.05
<i>Streptococcus salivarius</i> ATCC 25975	20.27
<i>Streptococcus gordonii</i> ATCC 10558	20.03
<i>Streptococcus mutans</i> LT11	18.78
<i>Peptostreptococcus anaerobius</i> ATCC 27337	22.36
<i>Peptostreptococcus micros</i> ATCC 33270	22.83
Gram positive asporogenous bacteria	
<i>Lactobacillus acidophilus</i> ATCC 4356	20.73
<i>Lactobacillus rhamnosus</i> ATCC 7469	24.53
Actinomycetes	
<i>Actinomyces naeslundii</i> ATCC 12104	24.32
<i>Actinomyces israelii</i> ATCC 12102	26.38
<i>Mycobacterium tuberculosis</i> H37RV	26.00

* Groups of bacteria based on Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

† DNA was either extracted from equivalent volumes of cultured bacteria or obtained from independent sources and diluted to be within the range of the threshold cycle (C_T) of the standard graph C_T vs *Escherichia coli* DNA. The data are the means of duplicate determinations. Variation in the duplicates was $\leq 3.7\%$, except where underlined the duplicates varied between 6.0 and 11.8%.

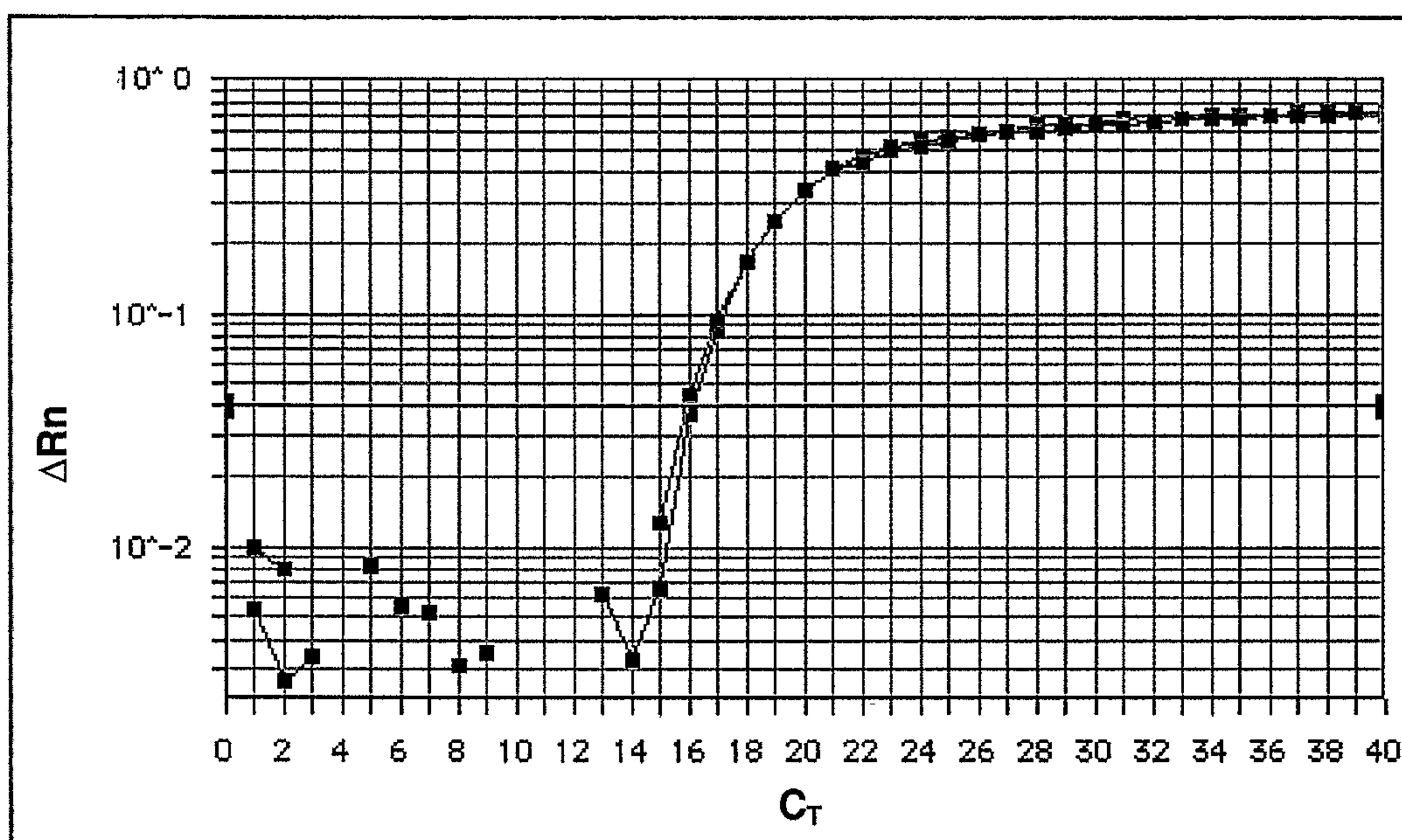


Figure 5.2. Real-time PCR amplification curve produced by the fluorescence signal of IPC-BT DNA using the IPC-BT primers and probe and showing an internal site for the probe within the amplicon.

5.2.2.4 Mechanical DNA isolation procedures.

These experiments were repeated three times due to variations in the amount of *P. gingivalis* isolated from different cultures. Overall results showed the largest amount of DNA measured for *P. gingivalis* following the freeze/boil procedure, with decreasing amounts of DNA measured following the freeze/thaw and sonication methods. In contrast, *S. mutans* showed more effective release of DNA following sonication, with less DNA quantified after the freeze/thaw and freeze/boil processes (Figure 5.3).

5.2.2.5 Nuclease detection

5.2.2.5.1 Isolation of *P. gingivalis* DNA in the presence of the internal positive control

When *P. gingivalis* DNA was processed together with the internal positive control (IPC-BT), the amount of DNA recovered after the freeze/thaw process was substantially reduced, as recorded by the C_T value for both the *P. gingivalis* and the IPC-BT probe and primers when compared with DNA recovered following the freeze/boil method (Table 5.3). Deterioration of both the *P. gingivalis* and the IPC-BT signal indicated the action of a DNA degrading agent which was inactivated by boiling the sample.

5.2.2.5.2 Detection of nuclease activity in *P. gingivalis*

The presence of nuclease activity was confirmed by agarose gel electrophoresis where purified exogenous *P. gingivalis* DNA was degraded by *P. gingivalis* DNA prepared by the freeze/thaw process and following 3min and 6min sonication times. A band of *P. gingivalis* DNA with smearing was noted following incubation with the freeze/boil prepared *P. gingivalis* culture (Figure 5.4), whereas no DNA was recovered following the other procedures.

5.2.2.6 Chemical DNA isolation procedures.

5.2.2.6.1 Enzymatic, $ZnCl_2$ and EDTA methods

The enzymes lysozyme and mutanolysin were added to the phosphate buffer to encourage cell wall lysis, particularly for the Gram-positive bacteria. Additionally $ZnCl_2$ and EDTA were incorporated as potential nuclease inhibitors. Following incubation, PCR results showed that similar amounts of *A. israelii* and *F. nucleatum* DNA were extracted by each procedure, whereas

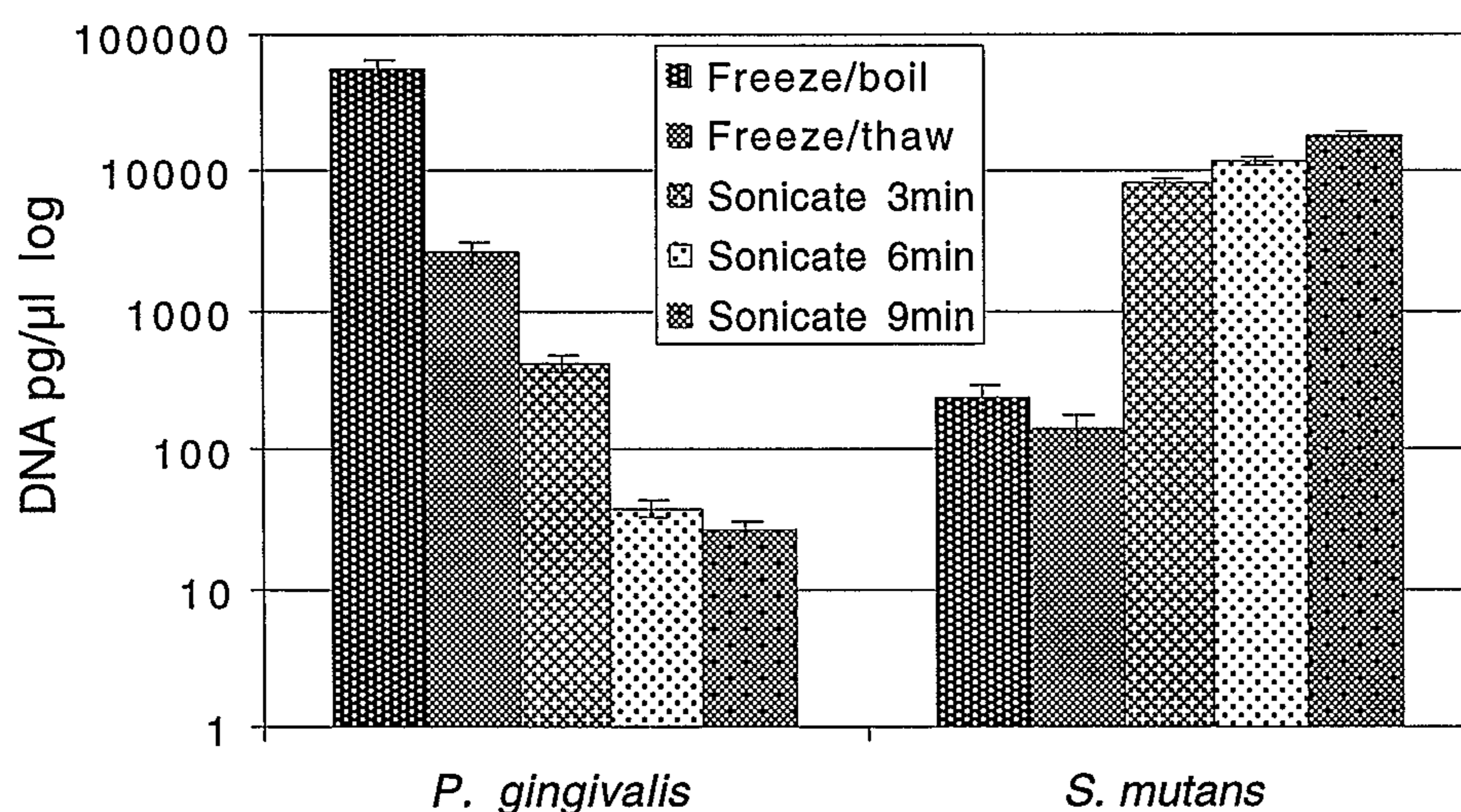


Figure 5.3. PCR quantitation of DNA from cultures of *P. gingivalis* and *S. mutans* isolated in 10mM sodium phosphate buffer and subjected to mechanical treatments including: freeze/boil, freeze/thaw and continuous sonication for 3, 6 and 9 min. Data shows one representative experiment of three, expressed as mean values from triplicate readings \pm SEM.

Table 5.3. Isolation of *P. gingivalis* DNA in the presence of the internal positive control (IPC-BT). Quantitation used both the *P. gingivalis* and IPC-BT probe-primer sets utilising different fluorescent reporter dyes FAM and VIC, respectively. Data provided by Dr M Nadkarni.

DNA isolation method	<i>P. gingivalis</i> C _T (FAM) (St Dev)	IPC-BT C _T (VIC) (St Dev)
Freeze/boil	16.69 (0.06)	16.65 (0.30)
Freeze/thaw	22.40 (0.06)	24.40 (0.16)

Note: The higher the original amount of sample DNA, the lower the C_T value.

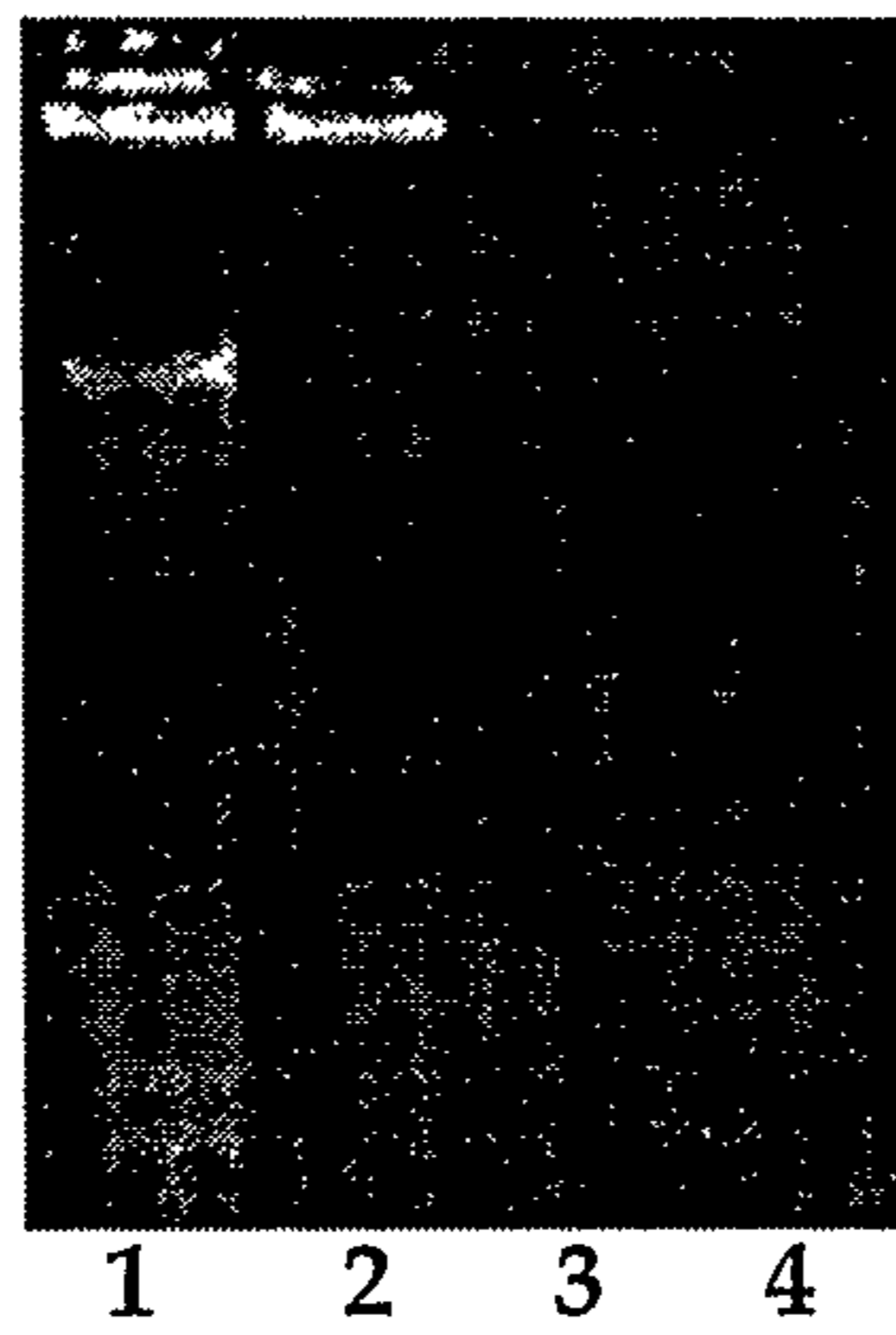


Figure 5.4 Agarose gel electrophoresis of DNA from *P. gingivalis*. Lane 1: purified exogenous *P. gingivalis* DNA incubated with freeze/boil prepared *P. gingivalis* DNA.; Lane 2: purified exogenous *P. gingivalis* DNA incubated with freeze/thaw prepared *P. gingivalis* DNA; Lane 3: purified exogenous *P. gingivalis* DNA incubated with 3 min sonicated *P. gingivalis* DNA; Lane 4: purified exogenous *P. gingivalis* DNA incubated with 6 min sonicated *P. gingivalis* DNA.

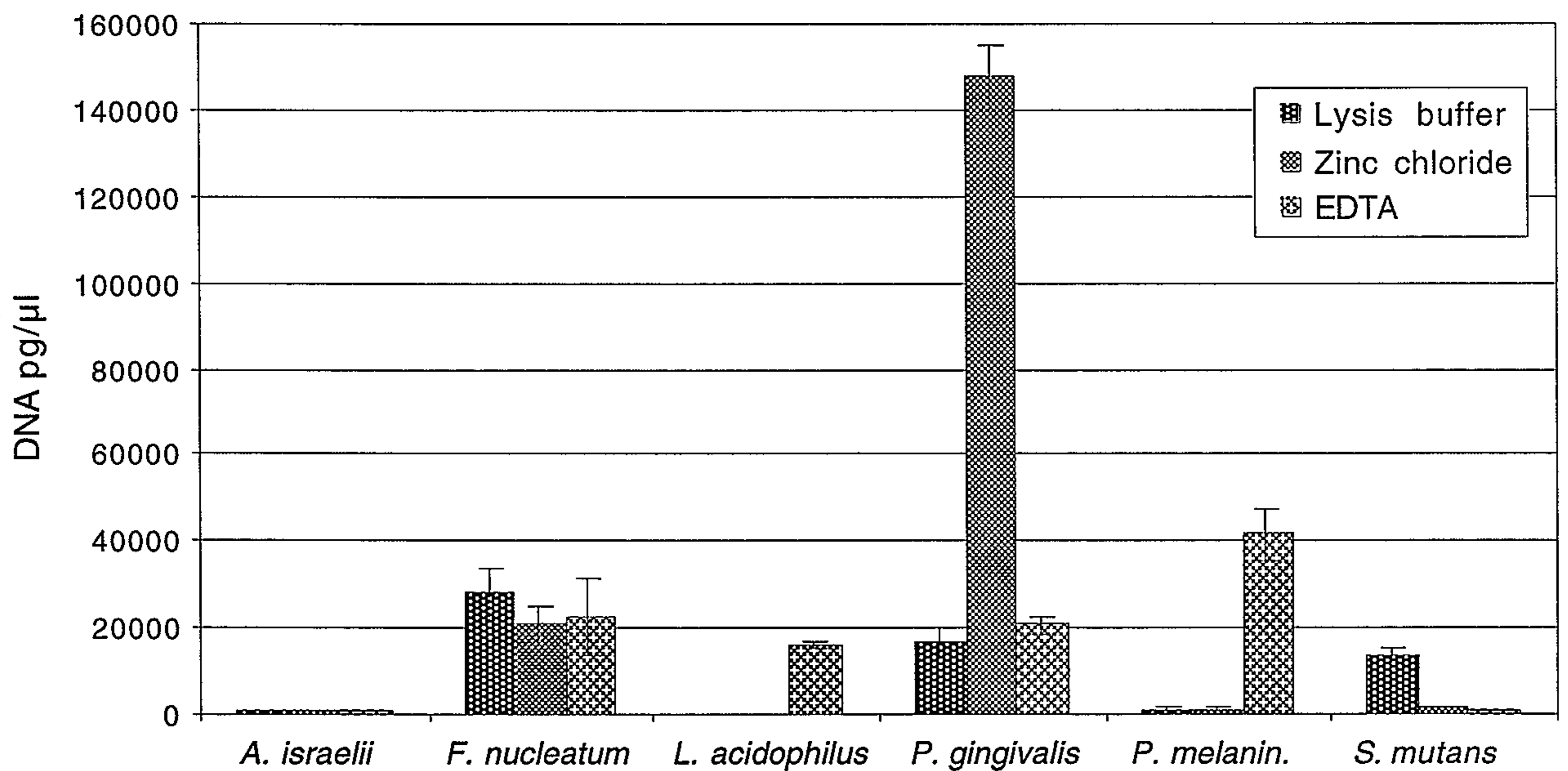


Figure 5.5. PCR quantitation of DNA from cultures of *A. israelii*, *F. nucleatum*, *L. acidophilus*, *P. gingivalis*, *P. melaninogenica* and *S. mutans* isolated in lysis buffer containing 10mM sodium phosphate, 1mg lysozyme ml⁻¹ and 2mg mutanolysin ml⁻¹, with and without 5mM zinc chloride and 100mM EDTA. Data shows one representative experiment of two, expressed as mean values from triplicate readings \pm SEM.

more DNA was recovered from *P. gingivalis* suspended in $ZnCl_2$, from *P. melaninogenica* and *L. acidophilus* in EDTA and from *S. mutans* in the lysis buffer (Figure 5.5).

5.2.2.6.2 Enzymatic, EDTA, EGTA, DEPC and combination methods

Following PCR most DNA was quantified from *F. nucleatum* suspended in the lysis buffer alone, and lysis buffer containing DEPC and EGTA/DEPC; for *L. acidophilus* the EDTA containing buffer; for *P. gingivalis* the DEPC containing buffer; for *P. melaninogenica* the EDTA/DEPC combination and for *A. israelii* and *S. mutans*, the lysis buffer (Figure 5.6).

Experiments from 5.2.2.6.1 and 5.2.2.6.2 were repeated three times as the DNA recovery from cultures of *A. israelii*, *P. gingivalis* and *L. acidophilus* varied depending on the culture batch used.

5.2.2.6.3 Extended incubation method

In an attempt to improve DNA recovery from the Gram-positive bacteria, while controlling nuclease activity, proteinase K was included in the lysis buffer and incubated for a prolonged period, with and without the addition of EDTA and $MgCl_2$. The gel image shows minimal DNA bands for *A. israelii* and obvious bands for *F. nucleatum*, *P. gingivalis* and *P. melaninogenica* for both procedures. However, when EDTA and $MgCl_2$ were absent a distinct band was seen for *S. mutans* but not for *L. acidophilus* (Figure 5.7a), whereas the reverse was seen when EDTA and $MgCl_2$ were present (Figure 5.7b).

5.2.2.6.4 MES buffer method

An acidic buffer (MES) with lysozyme and proteinase K was used in combination with $NaHCO_3$ and NaCl and an extended incubation to assist cell lysis of the Gram-positive microbes. The agarose gel images of the cultures prior to SDS treatment (Figure 5.8a) showed good recovery of DNA from only *S. mutans*, with less recovered from the mixture of *S. mutans* and *L. acidophilus*, and none recovered from *L. acidophilus*. However, following SDS treatment only minimal bands of DNA were noted for the Gram-negative bacterial cultures. The combination of *S. mutans* and *P. gingivalis* prior to SDS treatment produced a smeared image indicating a lack of *P. gingivalis* nuclease inhibition. Adding DEPC (Figure 5.8b) improved the recovery of *F. nucleatum*, *P. gingivalis* and the *S. mutans/P. gingivalis* combination prior to SDS

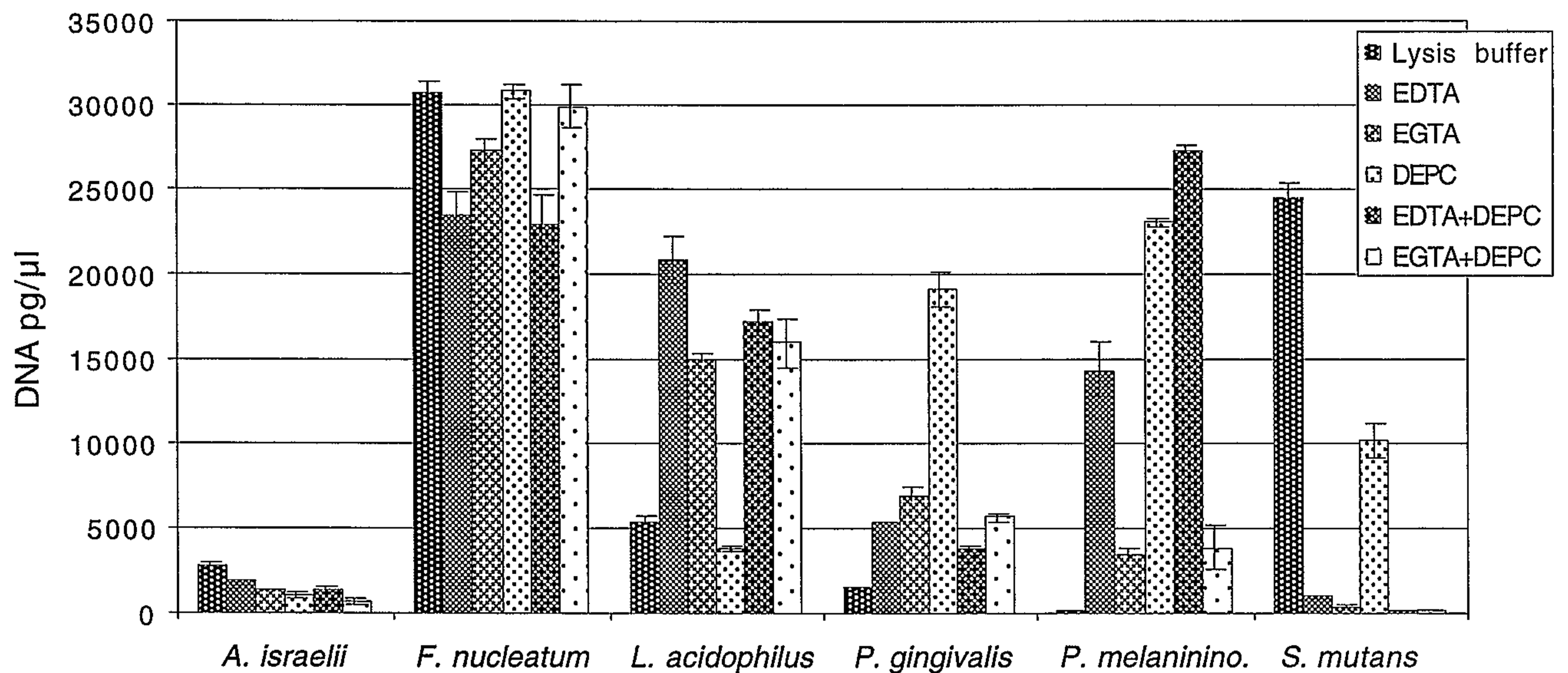


Figure 5.6. PCR quantitation of DNA isolated from cultures of *A. israelii*, *F. nucleatum*, *L. acidophilus*, *P. gingivalis*, *P. melaninogenica* and *S. mutans* following incubation in lysis buffer containing 10mM sodium phosphate, 1mg lysozyme ml⁻¹ and 2mg mutanolysin ml⁻¹ with or without 100mM EDTA, 100mM EGTA, 20mM DEPC, 100mM EDTA/20mM DEPC or 100mM EGTA/20mM DEPC. Representative experiment of three, expressed as mean values from triplicate readings \pm SEM.



Figure 5.7. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate, 2mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹ and 2mg proteinase K ml⁻¹ (Figure 5.7a) and with the addition of 100mM EDTA and 50mM MgCl₂ (Figure 5.7b).

Lane 1: *A. israelii*; Lane 2: *F. nucleatum*; Lane 3: *L. acidophilus*; Lane 4: *P. gingivalis*; Lane 5: *P. melaninogenica*; Lane 6: *S. mutans*.

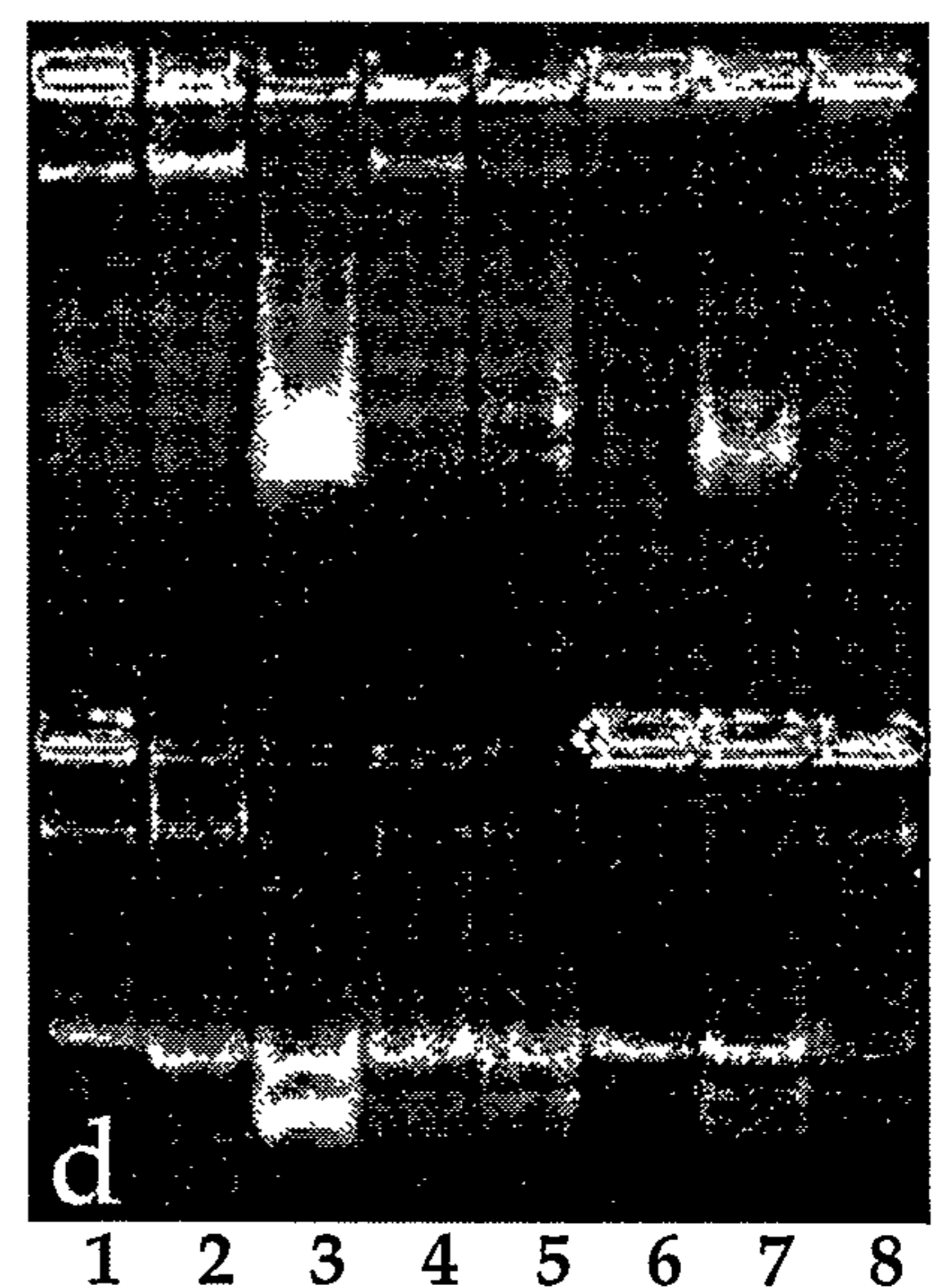
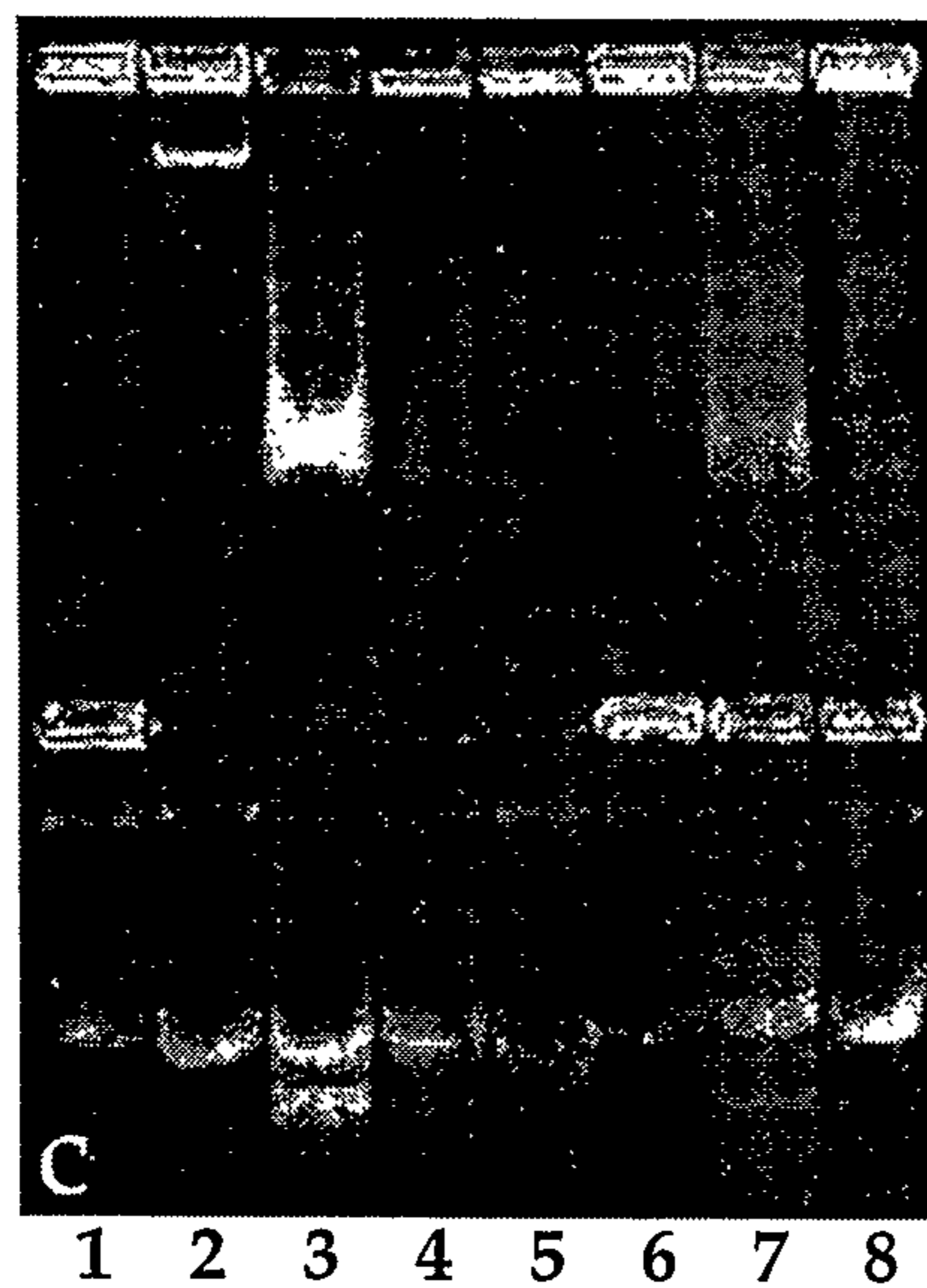
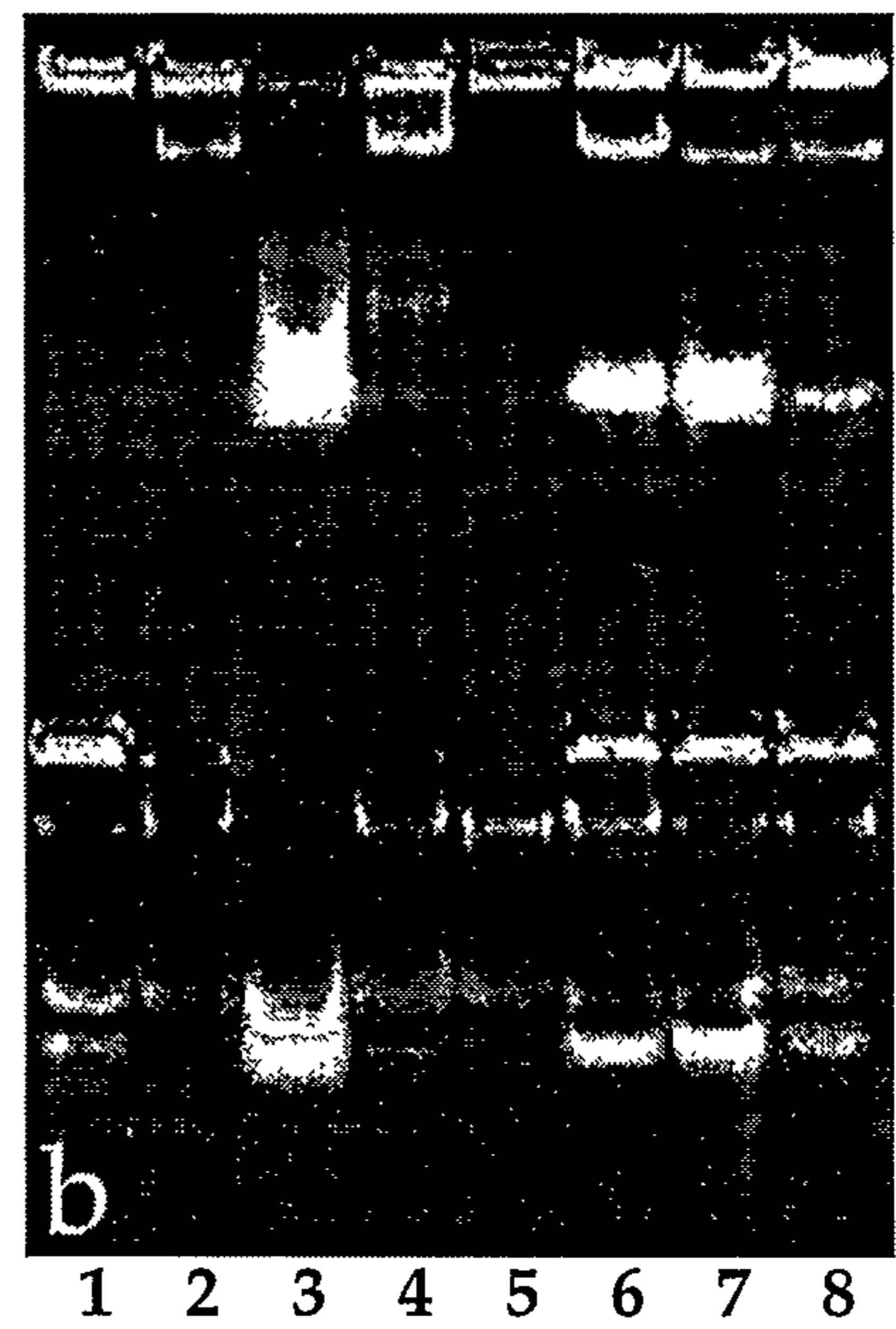
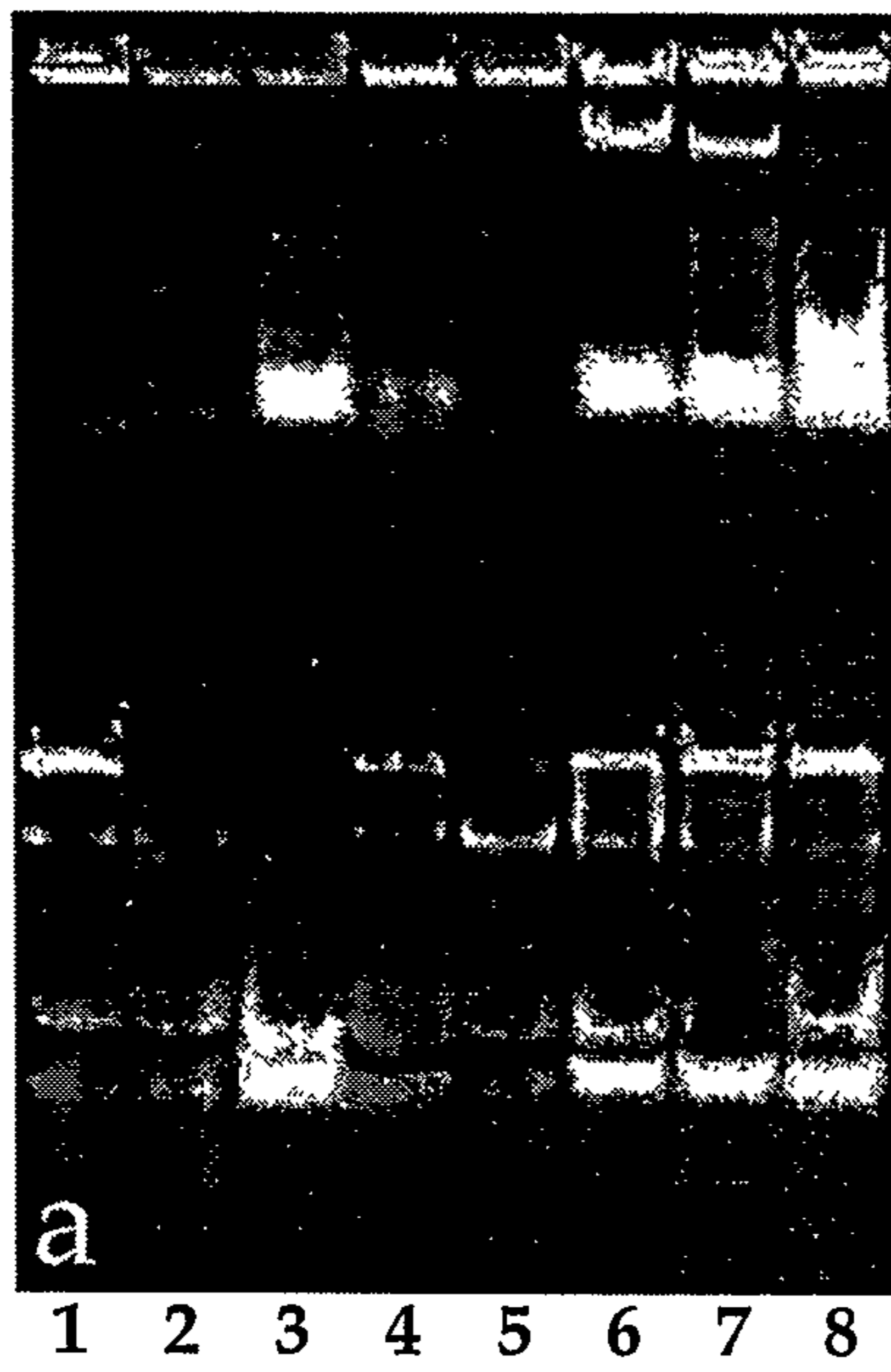


Figure 5.8. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 25mM MES buffer containing 2mg lysozyme ml⁻¹ and 2mg proteinase K ml⁻¹ (Figure 5.8a), with the addition of 20mM DEPC (Figure 5.8b), 100mM EDTA (Figure 5.8c), or 20mM DEPC and 100mM EDTA (Figure 5.8d). The upper rows show DNA isolation prior to 1% SDS treatment, the lower rows show DNA isolation after SDS treatment..

Lane 1: *A. israelii*; Lane 2: *F. nucleatum*; Lane 3: *L. acidophilus*; Lane 4: *P. gingivalis*; Lane 5: *P. melaninogenica*; Lane 6: *S. mutans*; Lane 7: *L. acidophilus* and *S. mutans*; Lane 8: *P. gingivalis* and *S. mutans*. Figures 5.8c and 5.8d were provided by Dr M Nadkarni.

treatment. The addition of EDTA (Figure 5.8c) recovered only *F. nucleatum* and the addition of DEPC/EDTA (Figure 5.8d) assisted the DNA extraction of *A. israelii*, *F. nucleatum* and *P. gingivalis*. Minimal DNA was recovered from the remaining Gram-negative bacteria after SDS treatment and marked smearing of the DNA was noted with *L. acidophilus* for each method. This experiment was repeated three times as the results for *P. gingivalis* and *L. acidophilus* isolation varied with different culture batches.

5.2.2.6.5 Comparison of buffers

Using a modified form of the previous experiment including mutanolysin and DEPC, the action of MES was compared with phosphate buffer with an incubation period of 15 min. Following incubation in phosphate buffer (Figure 5.9a), the agarose gel showed bands for all cultures except *A. israelii*; those bands for *L. acidophilus* and *S. mutans*, individually or combined, were less intensely stained than the bands of the Gram-negative bacteria. Following incubation in MES buffer the Gram-negative bacteria showed bands with intense staining, while those for the Gram-positive bacteria were quite faint (Figure 5.9b).

5.2.2.6.6 Lysis buffer with additional DNase inhibitors

DNA bands were seen in the agarose gel images for all cultures with each of the combinations (10mM ascorbic acid, 0.1mM aurin tricarboxylic acid and 50mM trisodium citrate), however, considerable smearing was noted for all except *A. israelii* and *M. micros* (Figures 5.10a-c). With the addition of DEPC (Figures 5.10d-g), the combinations showed increased staining intensity for all cultures except *A. israelii*. Following pulse sonication (Figures 5.10h-k), deterioration of the band intensities was noted for all combinations except ascorbic acid/trisodium citrate/DEPC (Figure 5.10k). Of the various combinations, ascorbic acid/DEPC and ascorbic acid/citric acid/DEPC/6 min pulse sonication showed the most intensely stained bands from each of the cultures. However, following purification of the samples, DNA from the Gram-negative bacteria appeared in the gels with marked smearing and from the Gram-positive bacteria as faint bands (Figures 5.10l, m).

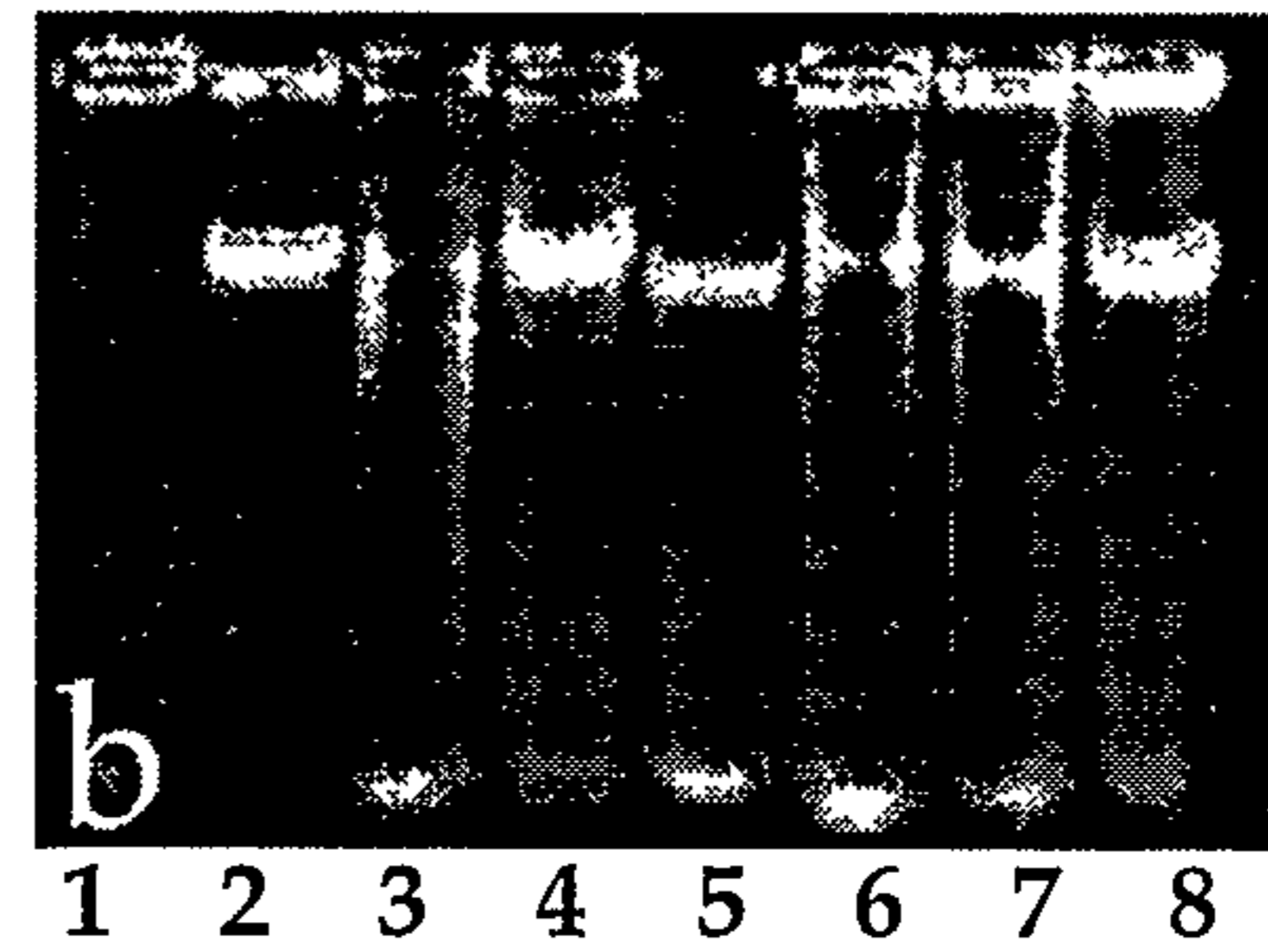
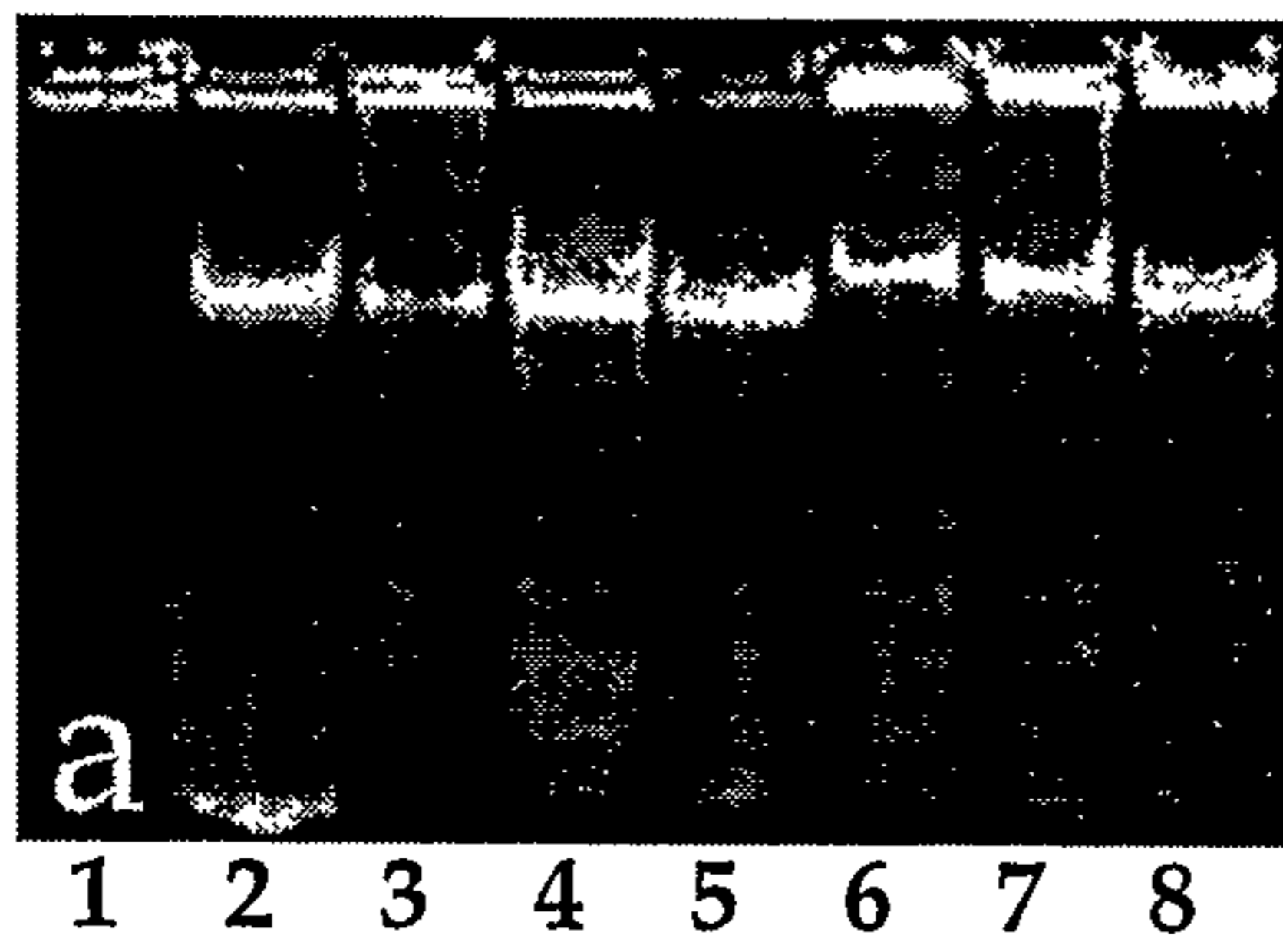


Figure 5.9. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer (Figure 5.9a) and MES buffer (Figure 5.9b) containing 2mg lysozyme ml^{-1} , 2mg mutanolysin ml^{-1} , 2mg proteinase K ml^{-1} and 20mM DEPC.

Lane 1: *A. israelii*; Lane 2: *F. nucleatum*; Lane 3: *L. acidophilus*; Lane 4: *P. gingivalis*; Lane 5: *P. melaninogenica*; Lane 6: *S. mutans*; Lane 7: *L. acidophilus* and *S. mutans*; Lane 8: *P. gingivalis* and *S. mutans*.

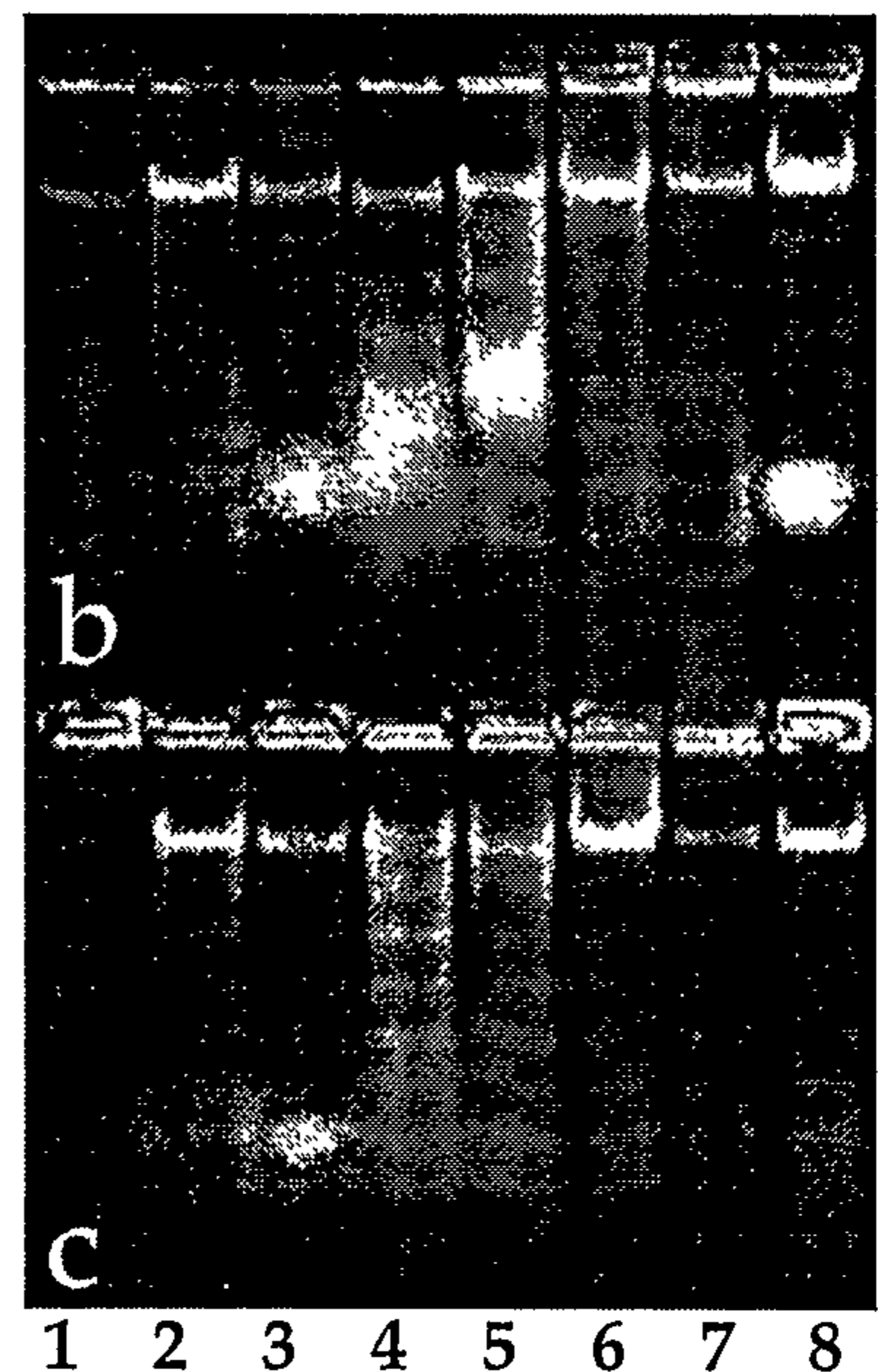
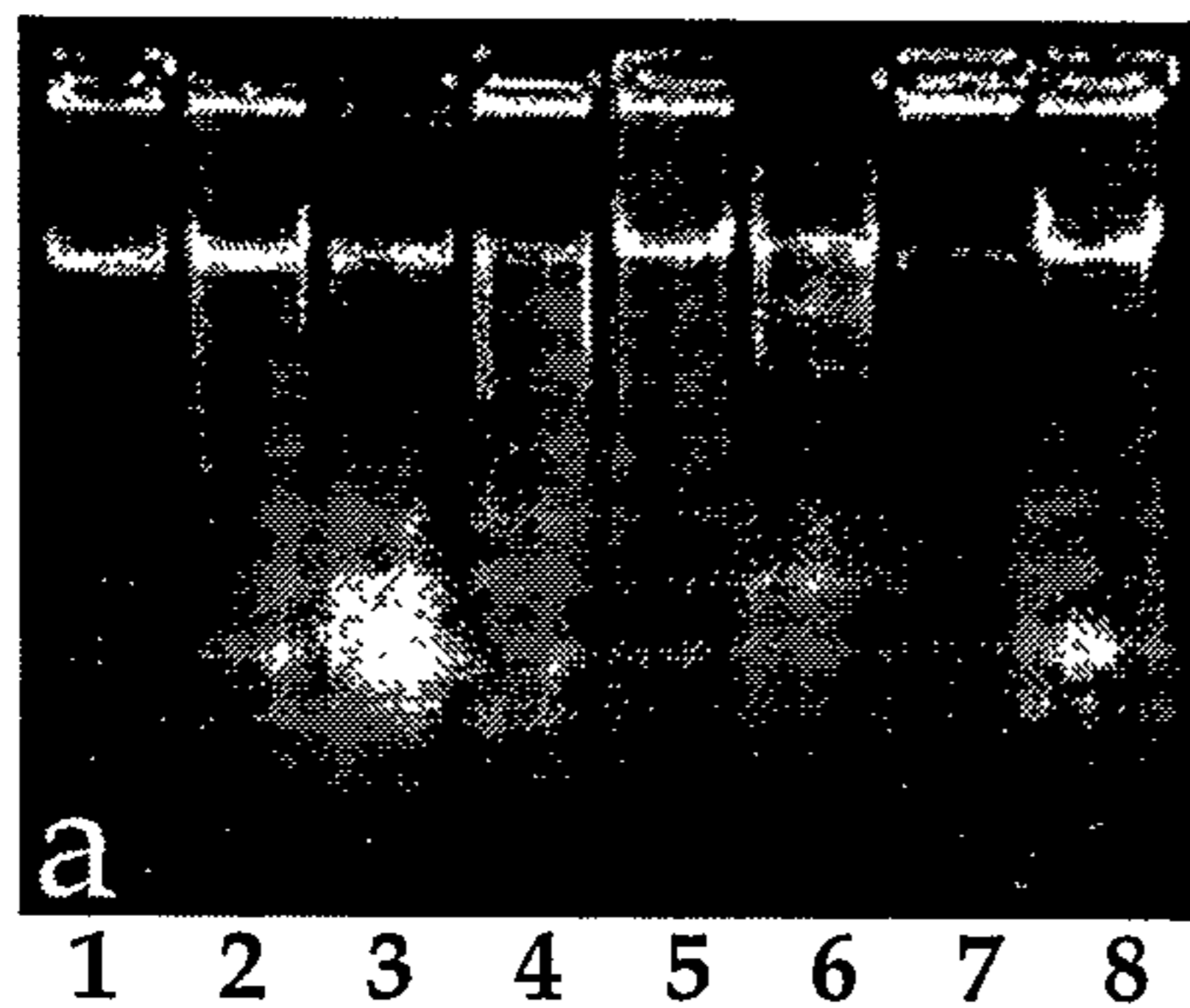


Figure 5.10a-c. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing 2mg lysozyme ml^{-1} , 2mg mutanolysin ml^{-1} and 2mg proteinase K ml^{-1} with the addition of 10mM ascorbic acid (Figure 5.10a), 0.1mM aurin tricarboxylic acid (Figure 5.10b) or 50mM trisodium citrate (Figure 5.10c).

Lane 1: *A. israelii*; Lane 2: *F. nucleatum*; Lane 3: *L. acidophilus*; Lane 4: *P. endodontalis*; Lane 5: *P. gingivalis*; Lane 6: *P. melaninogenica*; Lane 7: *M. micros*; Lane 8: *S. mutans*.

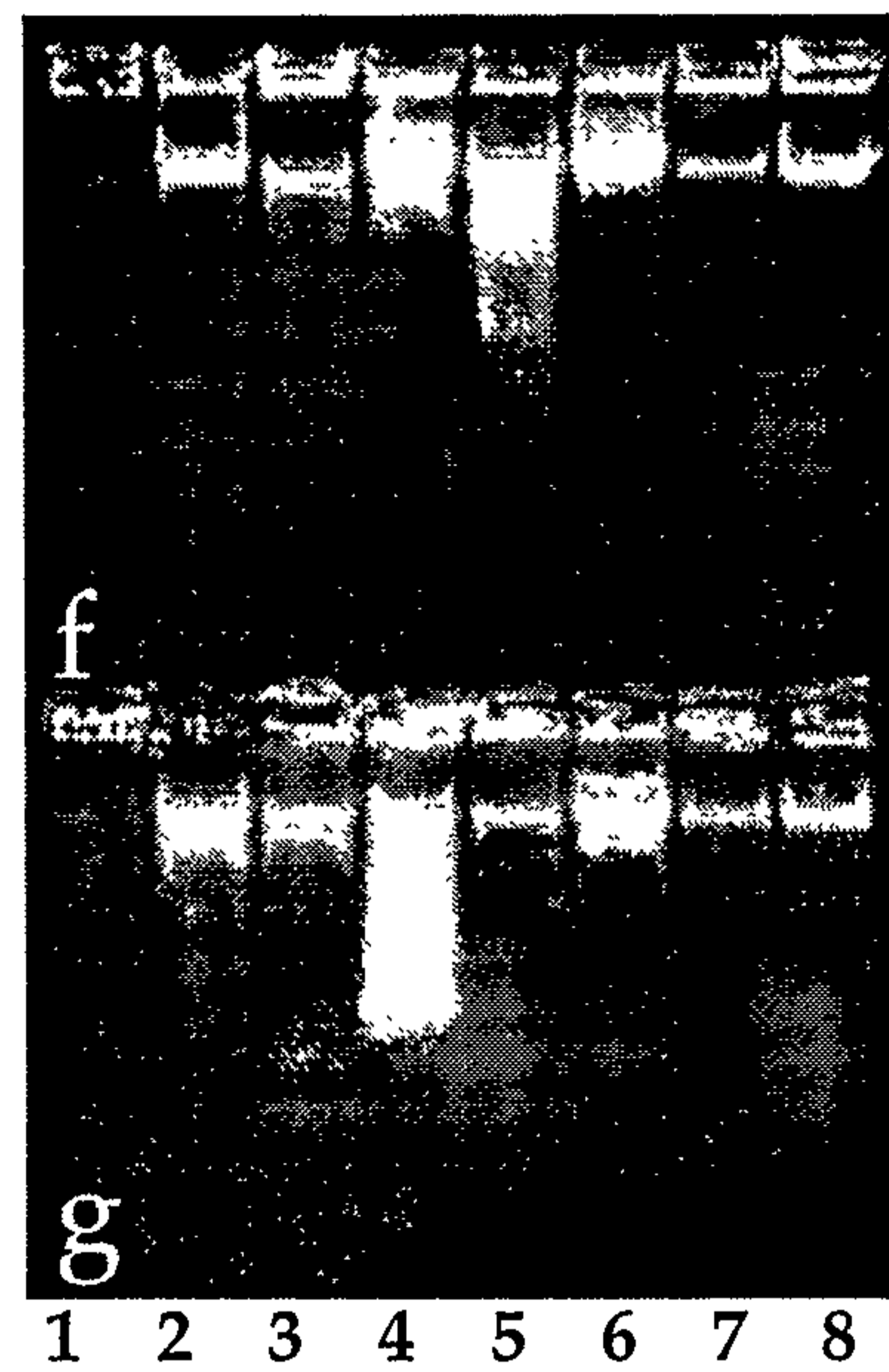
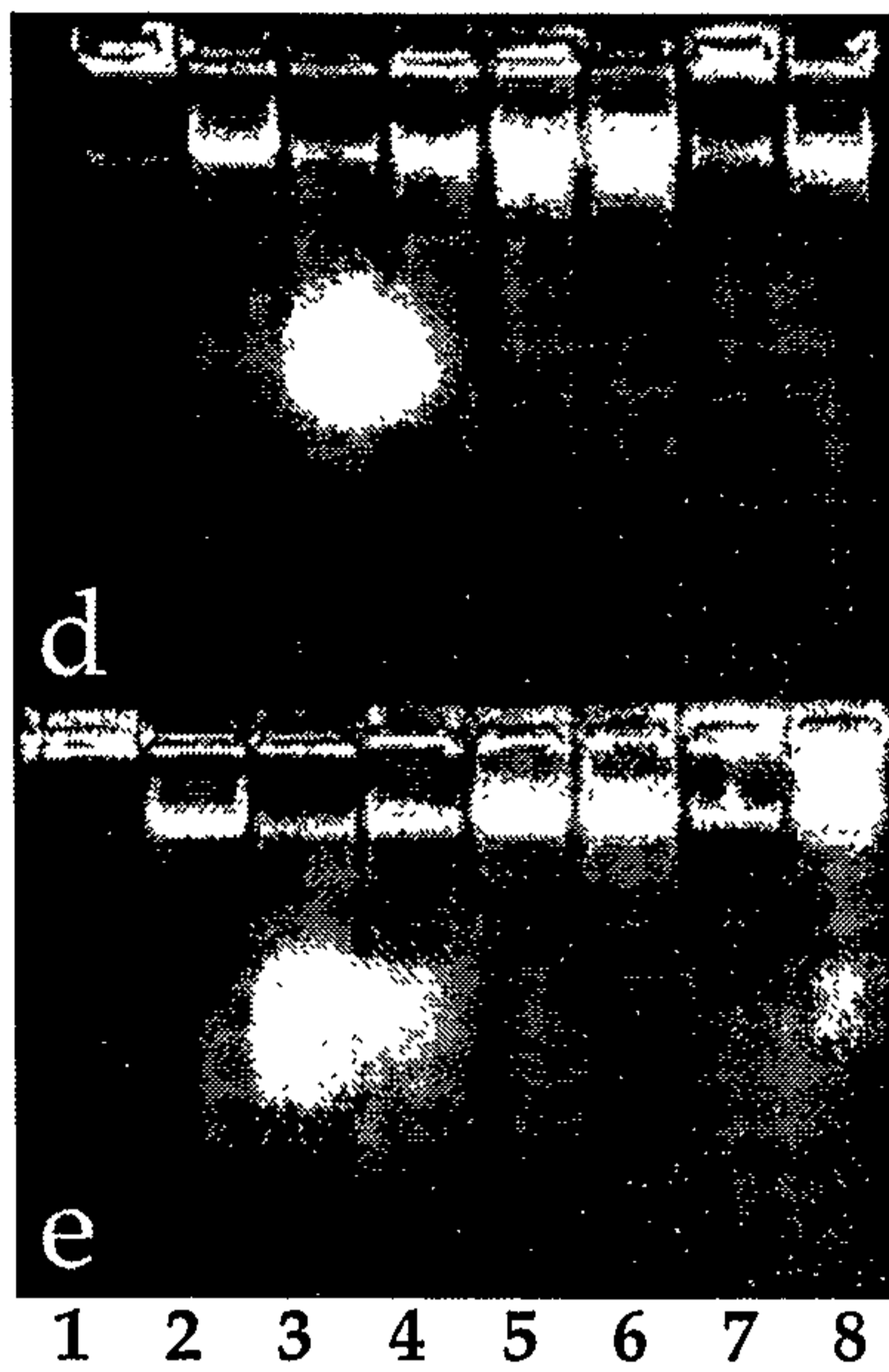


Figure 5.10d-g. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing 2mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹, 2mg proteinase K ml⁻¹ and 20mM DEPC with 10mM ascorbic acid (Figure 5.10d), 0.1mM aurin tricarboxylic acid (Figure 5.10e), 50mM trisodium citrate (Figure 5.10f) or 10mM ascorbic acid plus 50mM trisodium citrate (Figure 5.10g).

Lane 1: *A. israelii*; Lane 2: *F. nucleatum*; Lane 3: *L. acidophilus*; Lane 4: *P. endodontalis*; Lane 5: *P. gingivalis*; Lane 6: *P. melaninogenica*; Lane 7: *M. micros*; Lane 8: *S. mutans*.

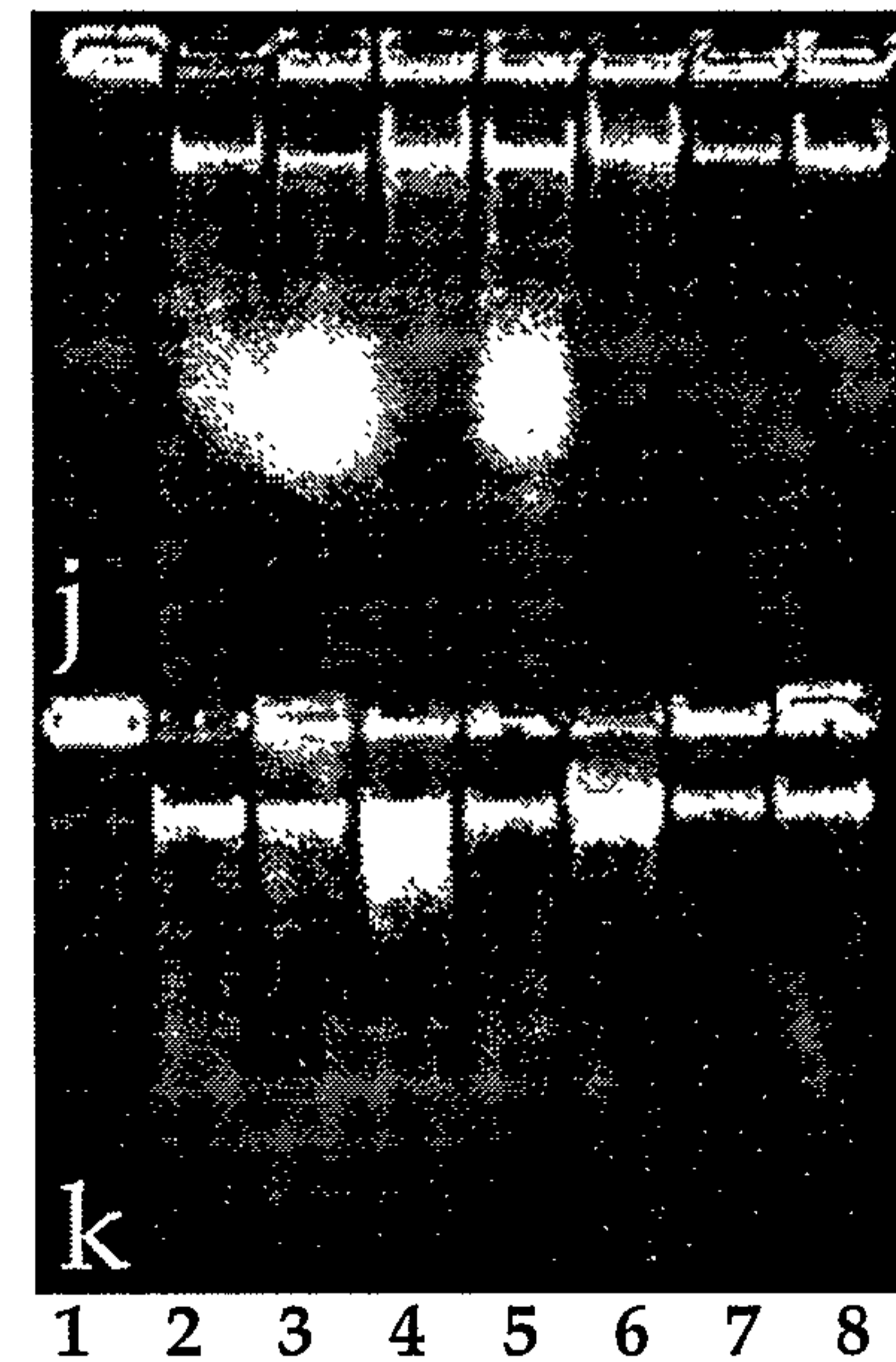
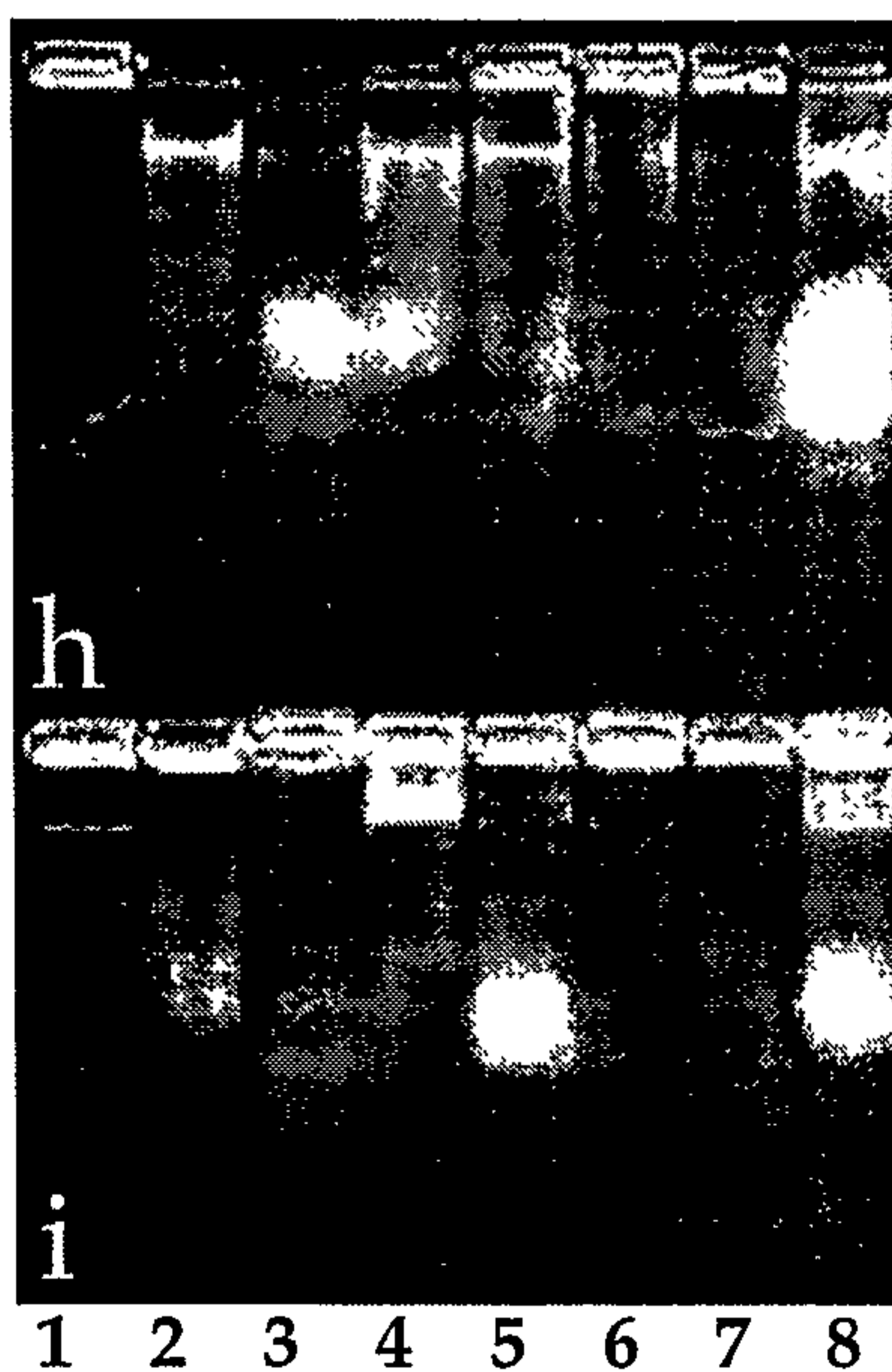


Figure 5.10h-k. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing 2mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹, 2mg proteinase

K ml⁻¹ and 20mM DEPC with 10mM ascorbic acid (Figure 5.10h), 0.1mM aurin tricarboxylic acid (Figure 5.10i), 50mM trisodium citrate (Figure 5.10j) for 10mM ascorbic acid plus 50mM trisodium citrate (Figure 5.10k) with 6min pulse sonication.

Lane 1: *A. israelii*; Lane 2: *F. nucleatum*; Lane 3: *L. acidophilus*; Lane 4: *P. endodontalis*; Lane 5: *P. gingivalis*; Lane 6: *P. melaninogenica*; Lane 7: *M. micros*; Lane 8: *S. mutans*.

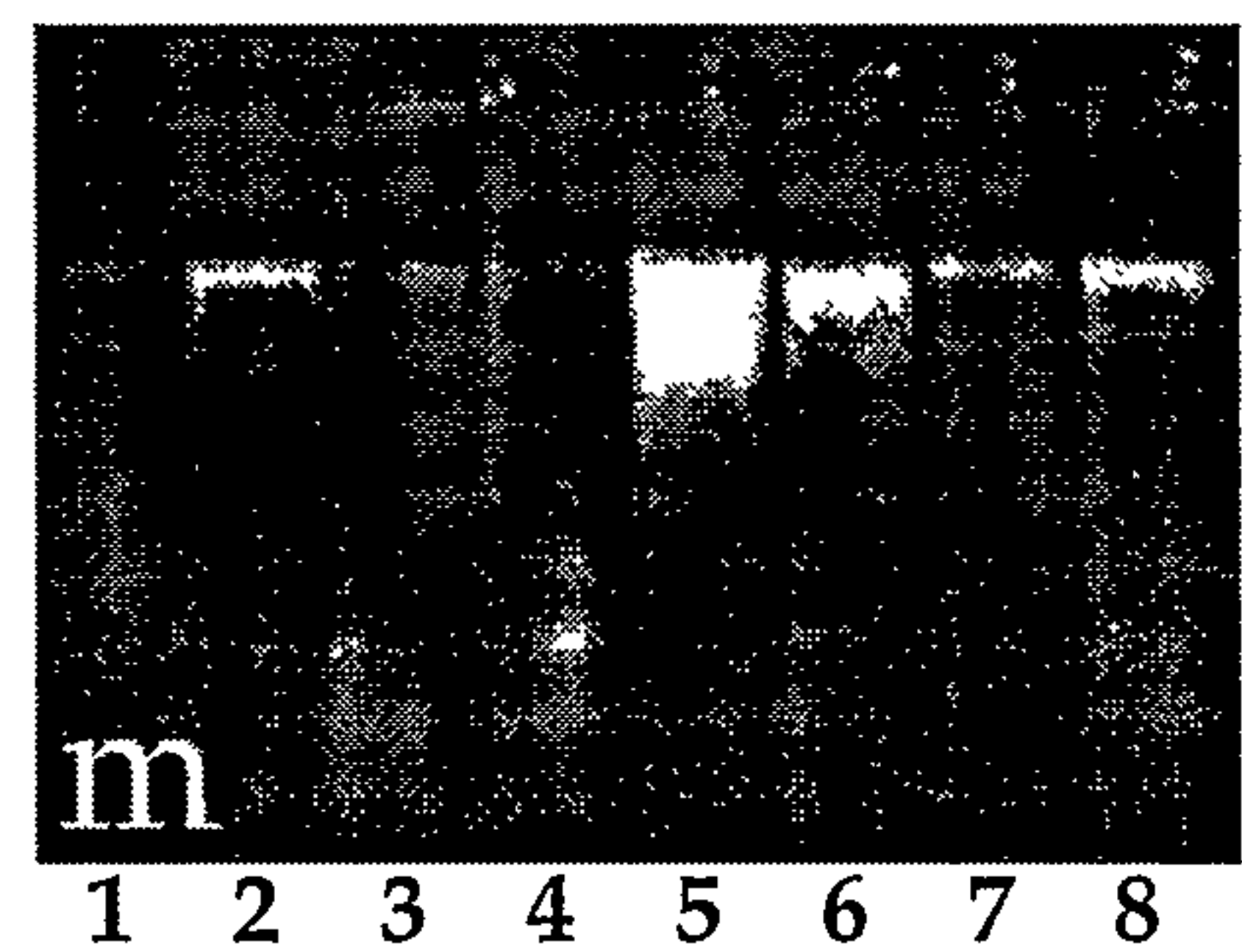
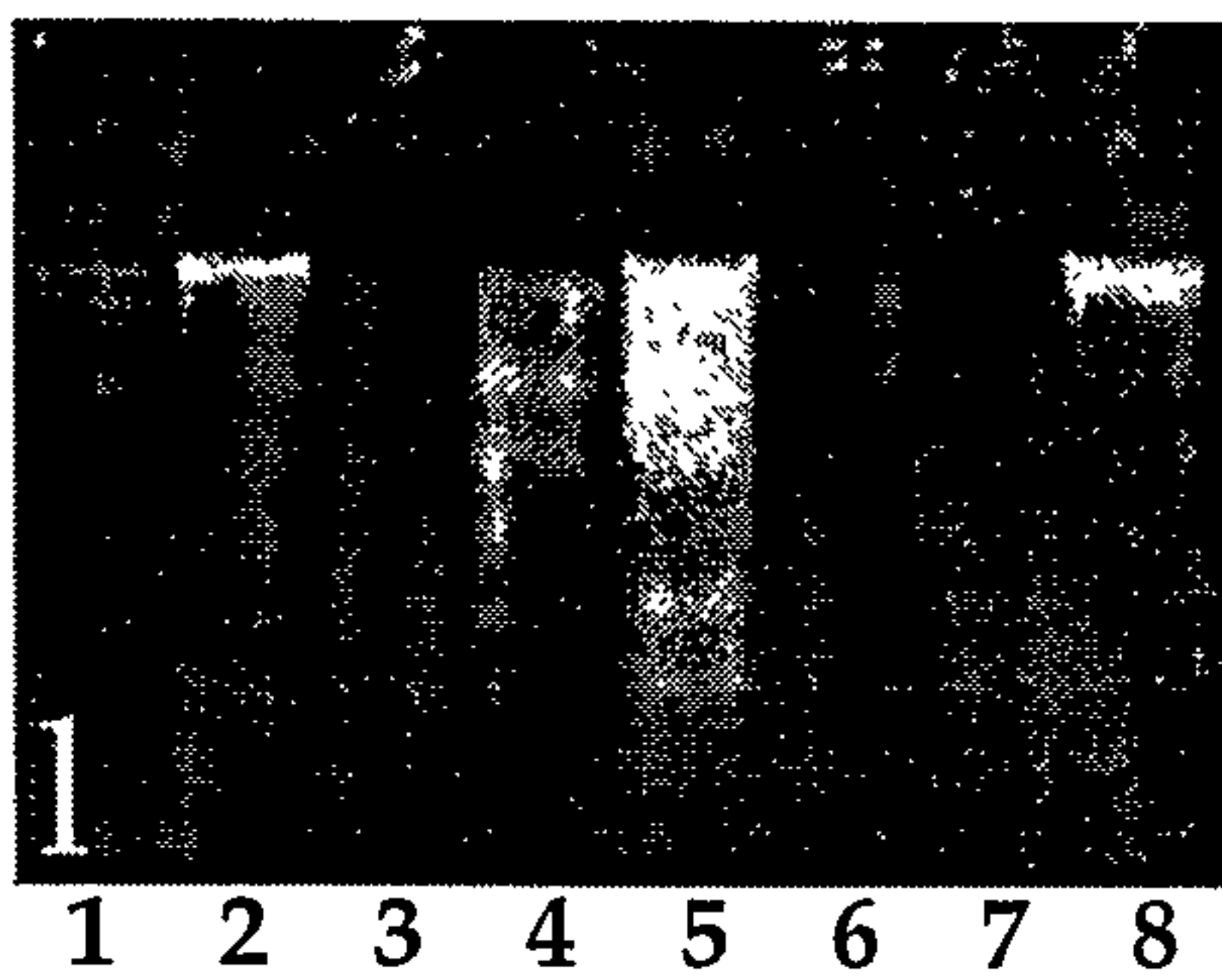


Figure 5.10l-m. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing 2mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹, 2mg proteinase K ml⁻¹ and 20mM DEPC with 10mM ascorbic acid (Figure 5.10l) or 10mM ascorbic acid plus 50mM trisodium citrate with 6min pulse sonication (Figure 5.10m) following purification.

Lane 1: *A. israelii*; Lane 2: *F. nucleatum*; Lane 3: *L. acidophilus*; Lane 4: *P. endodontalis*; Lane 5: *P. gingivalis*; Lane 6: *P. melaninogenica*; Lane 7: *M. micros*; Lane 8: *S. mutans*.

5.2.2.6.7 Varying the lysozyme concentration

In this experiment the concentration of lysozyme was varied and the procedure was separated into two parts; initially the culture pellets were suspended in buffer and DEPC, then following incubation or sonication the enzymes were added. To provide a comparison, another group of cultures was suspended in buffer with DEPC plus enzymes for 20min storage on ice. Results from the agarose gels showed an increased staining intensity of the DNA bands for those cultures isolated in buffer containing 5mg lysozyme ml⁻¹, particularly for *A. israelii*, *P. endodontalis* and *P. melaninogenica* compared with 1mg lysozyme ml⁻¹ (Figures 5.11a-d). The results following storage of the cultures on ice with the later addition of the enzymes, showed an increase in the recovery for *A. israelii* and *M. micros* for both methods, and in 1mg lysozyme ml⁻¹ an increased detection for *P. melaninogenica*, although with reduced detection of *P. endodontalis* (Figures 5.11a-d). Following sonication prior to the addition of enzymes (Figures 5.11e-h) the gel images showed a loss of intensity for *M. micros* following both pulse and continuous sonication, while for *F. nucleatum* (5mg lysozyme ml⁻¹) and *P. gingivalis* (1mg and 5mg lysozyme ml⁻¹) loss of staining intensity resulted from continuous sonication. The remaining sonicated cultures produced bands that appeared similar to those stored on ice for 20 min in the two-stage procedure.

5.2.2.6.8 Isolation of DNA in DEPC

Purified DNA preparations extracted in the absence of DEPC (Figure 5.12a) were visualised in an agarose gel and showed bands for *F. nucleatum*, *P. endodontalis*, and *S. mutans*, and faint bands were seen for *P. melaninogenica*, *M. micros* and *L. acidophilus*. DNA extracted in the presence of 20mM DEPC (Figure 5.12b) showed DNA bands evident for *F. nucleatum*, *P. endodontalis*, *P. gingivalis*, *P. melaninogenica* and *S. mutans*. Without DEPC in the suspension, *P. gingivalis* could not be recovered, however, the inclusion of DEPC reduced the recovery of *F. nucleatum*, *M. micros* and *S. mutans* (Figures 5.12a and b).

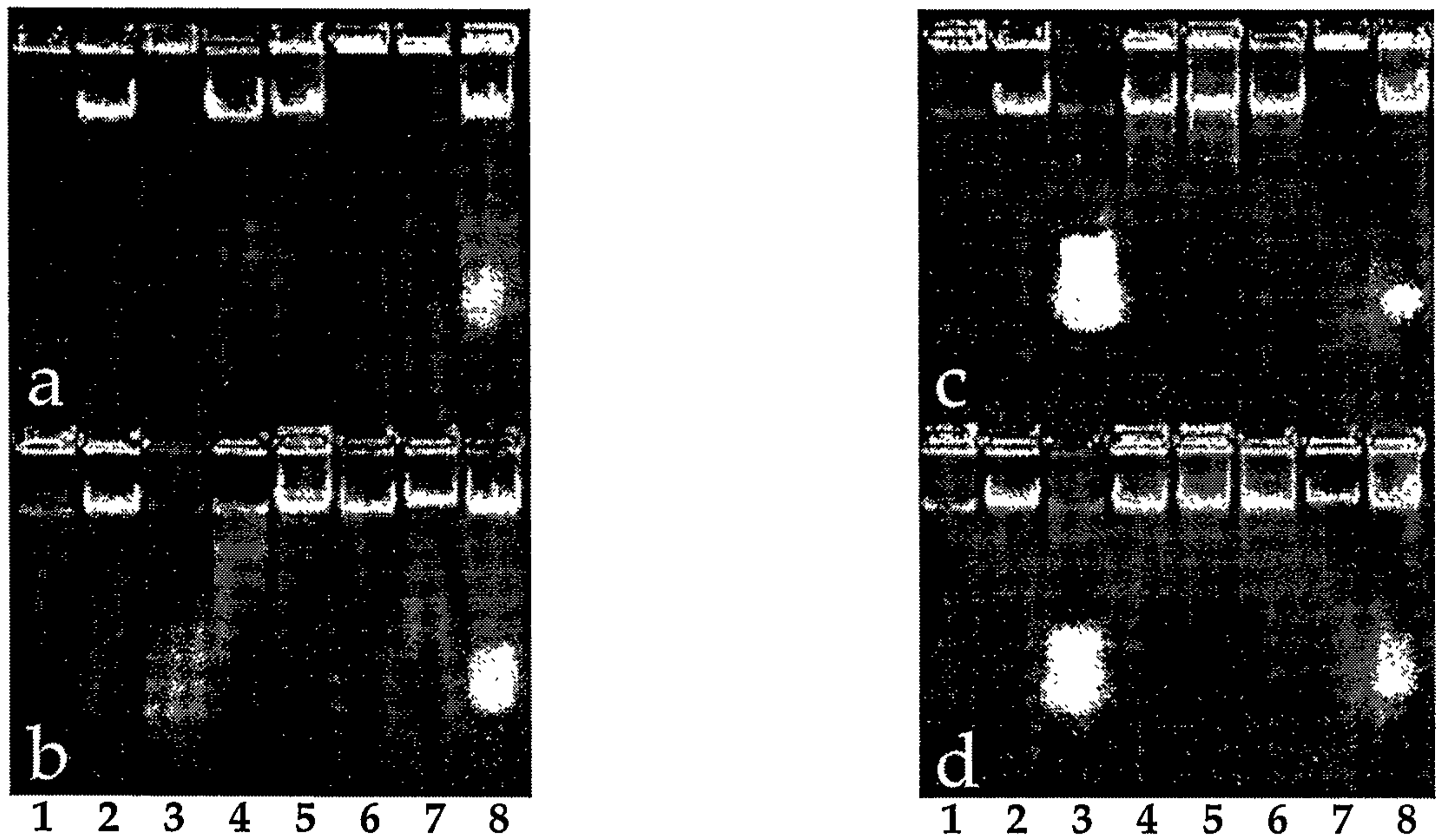


Figure 5.11a-d. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing, 2mg mutanolysin ml⁻¹, 2mg proteinase K ml⁻¹, 20mM DEPC with 1mg lysozyme ml⁻¹ (Figure 5.11a, b) or 5mg lysozyme ml⁻¹ (Figure 5.11c, d). The cultures from Figure 5.11a and Figure 5.11c were suspended in the lysis buffer and stored for 20min on ice, those cultures from Figure 5.11b and Figure 5.11d were suspended in 10mM sodium phosphate and 20mM DEPC and stored on ice for 20min prior to addition of the enzymes. Lane 1: *A. israelii*; Lane 2: *F. nucleatum*; Lane 3: *L. acidophilus*; Lane 4: *P. endodontalis*; Lane 5: *P. gingivalis*; Lane 6: *P. melaninogenica*; Lane 7: *M. micros*; Lane 8: *S. mutans*.

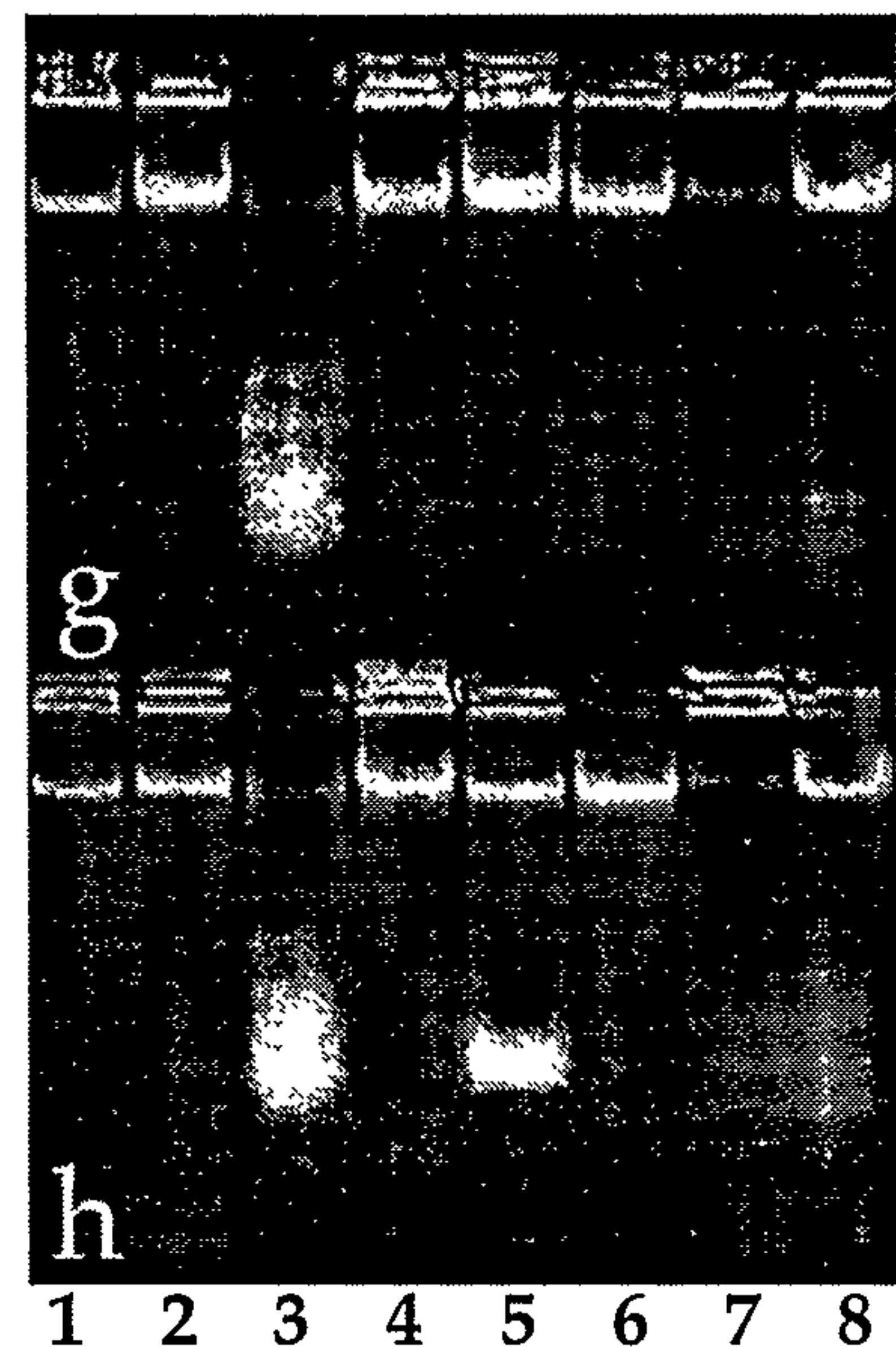
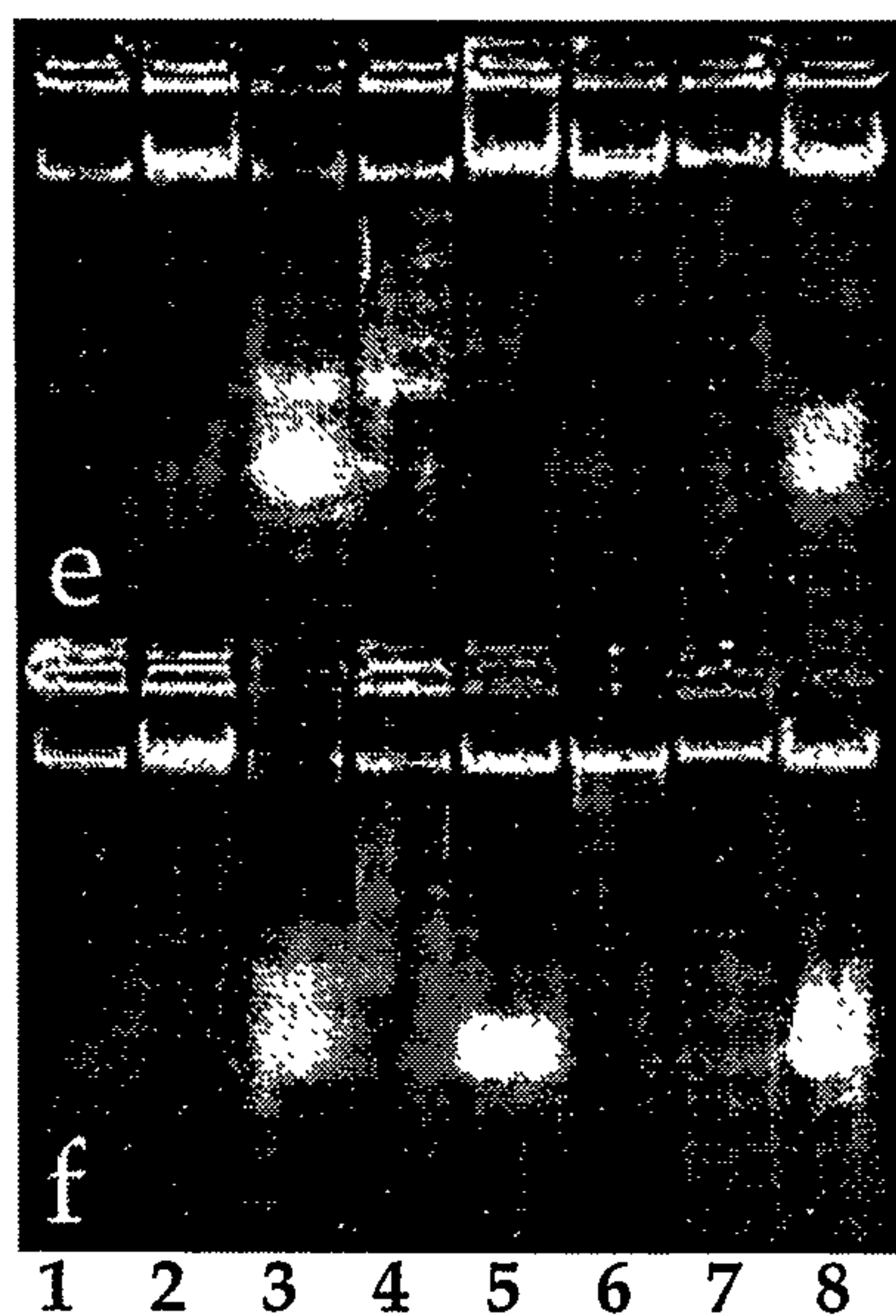


Figure 5.11e-h. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing, 2mg mutanolysin ml⁻¹, 2mg proteinase K ml⁻¹, 20mM DEPC with 1mg lysozyme ml⁻¹ (Figure 5.11e,f) or 5mg lysozyme ml⁻¹ (Figure 5.11g,h). The cultures from Figure 5.11e and Figure 5.11g were suspended in 10mM sodium phosphate and 20mM DEPC and sonicated in pulse mode for 6min prior to the addition of the enzymes, those cultures from Figure 5.11f and Figure 5.11h were suspended in 10mM sodium phosphate and 20mM DEPC and sonicated in continuous mode for 6min prior to addition of the enzymes.

Lane 1: *A. israelii*; Lane 2: *F. nucleatum*; Lane 3: *L. acidophilus*; Lane 4: *P. endodontalis*; Lane 5: *P. gingivalis*; Lane 6: *P. melaninogenica*; Lane 7: *M. micros*; Lane 8: *S. mutans*.

Figures 5.11f and 5.11h were provided by Dr M Nadkarni.

5.2.2.6.9 Isolation of DNA using a modified DEPC method

Quantitation of DNA using the DEPC method containing lysis buffer comparing 6 min pulse or continuous sonication with storage on ice showed similar DNA recovery following all procedures, except for *P. melaninogenica* where 6min pulse sonication provided higher DNA quantitation (Figure 5.13). The recovery of *A. israelii*, although improved following sonication, was still low compared with the other cultures. This experiment was repeated three times, and in separate instances, improved DNA recovery from *A. israelii* and *L. acidophilus* was achieved, but this data was not reproducible and varied with the culture batch used.

5.2.2.6.10 Isolation of DNA using ATL buffer from the QIAamp DNA Mini Kit

PCR quantitation of cultures incubated in ATL buffer prior to purification showed improved DNA recovery for all Gram-negative bacteria when compared with data from the same cultures following isolation in DEPC with different procedures (Figure 5.13). However, the amount of DNA retrieved from the Gram-positive cultures was almost negligible. Agarose gel images from DEPC 6min pulse prepared cultures showed an intensely stained band representing *S. mutans* with other preparations producing smearing, except for *A. israelii* and *L. acidophilus* which produced faint bands (Figure 5.14a). The ATL-treated cultures exhibited distinct DNA bands for the Gram-negative bacteria and a faint band for *M. micros* (Figure 5.14b).

5.2.2.6.11 Effect of $ZnCl_2$ as an inhibitor of PCR.

Cultures of *P. gingivalis* isolated in the presence of 5mM $ZnCl_2$ and diluted 100-fold (final concentration 0.05mM) prior to PCR showed no inhibition of the PCR reaction. However, with approximately the same number of cells in a final concentration of 0.5mM $ZnCl_2$, DNA quantitation was reduced by approximately 100-fold and in a concentration of 5mM $ZnCl_2$, DNA recovery was completely inhibited (Figure 5.15). Following purification with the QIAamp DNA Mini Kit, the inhibitory action of 0.5mM $ZnCl_2$ was controlled, however, the 5mM concentration still incurred some inhibition despite purification.



Figure 5.12. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing 5mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹ and 2mg proteinase K ml⁻¹ (Figure 5.12a) and with the addition of 20mM DEPC (Figure 5.12b).

Lane 1: *F. nucleatum*; Lane 2: *P. endodontalis*; Lane 3: *P. gingivalis*; Lane 4: *P. melaninogenica*; Lane 5: *M. micros*; Lane 6: *S. mutans*; Lane 7: *L. acidophilus*; Lane 8: *A. israelii*. Gel images provided by Dr M Nadkarni.

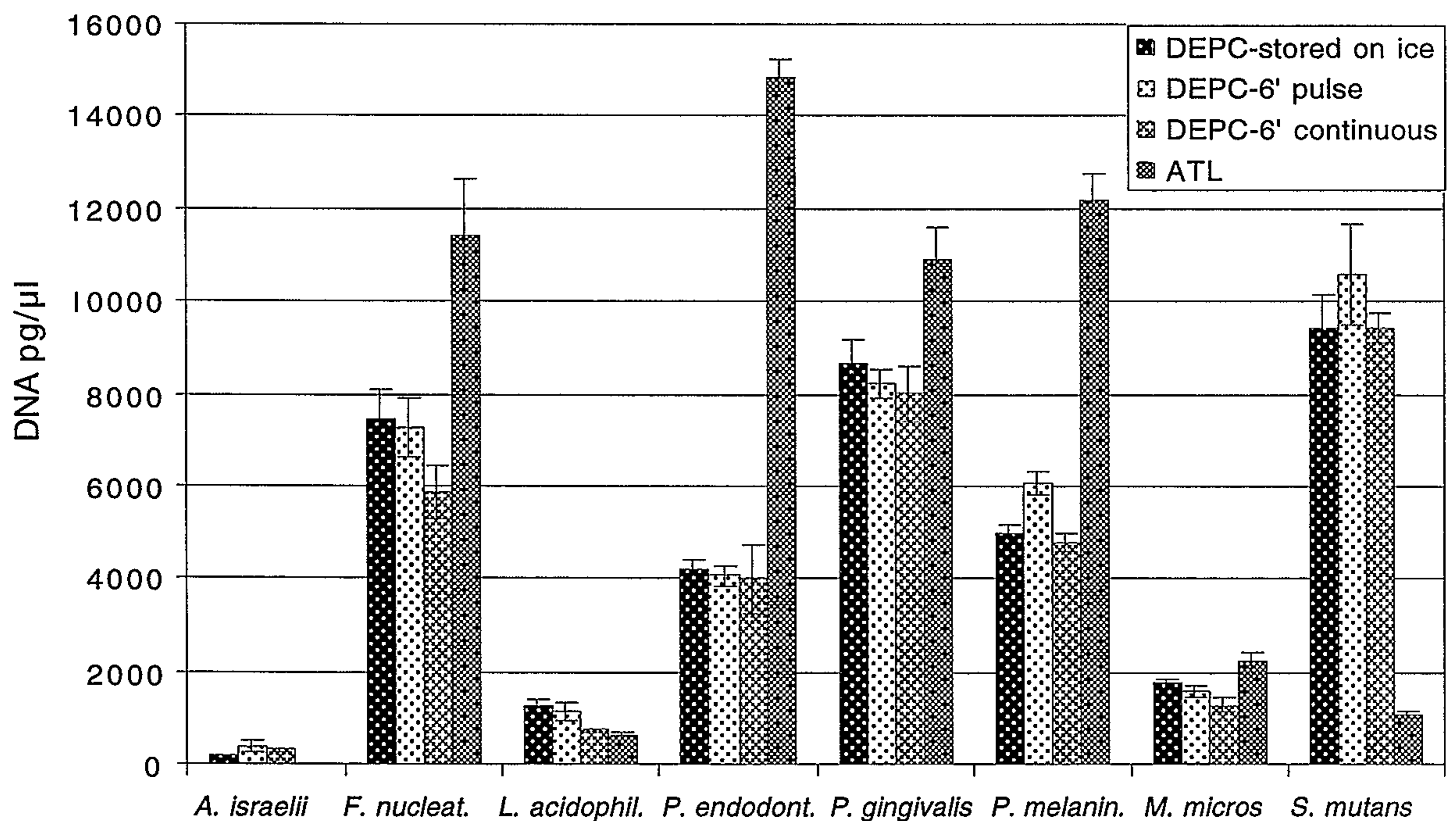


Figure 5.13. PCR quantitation of DNA isolated from cultures of *A. israelii*, *F. nucleatum*, *L. acidophilus*, *P. endodontalis*, *P. gingivalis*, *P. melaninogenica*, *M. micros*, and *S. mutans* suspended in 5mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹, 2mg proteinase K ml⁻¹ and 20mM DEPC, comparing storage of the culture on ice with 6min pulse and 6min continuous sonication, and isolation in ATL. Data shows one representative experiment of three, expressed as mean values from triplicate readings \pm SEM.

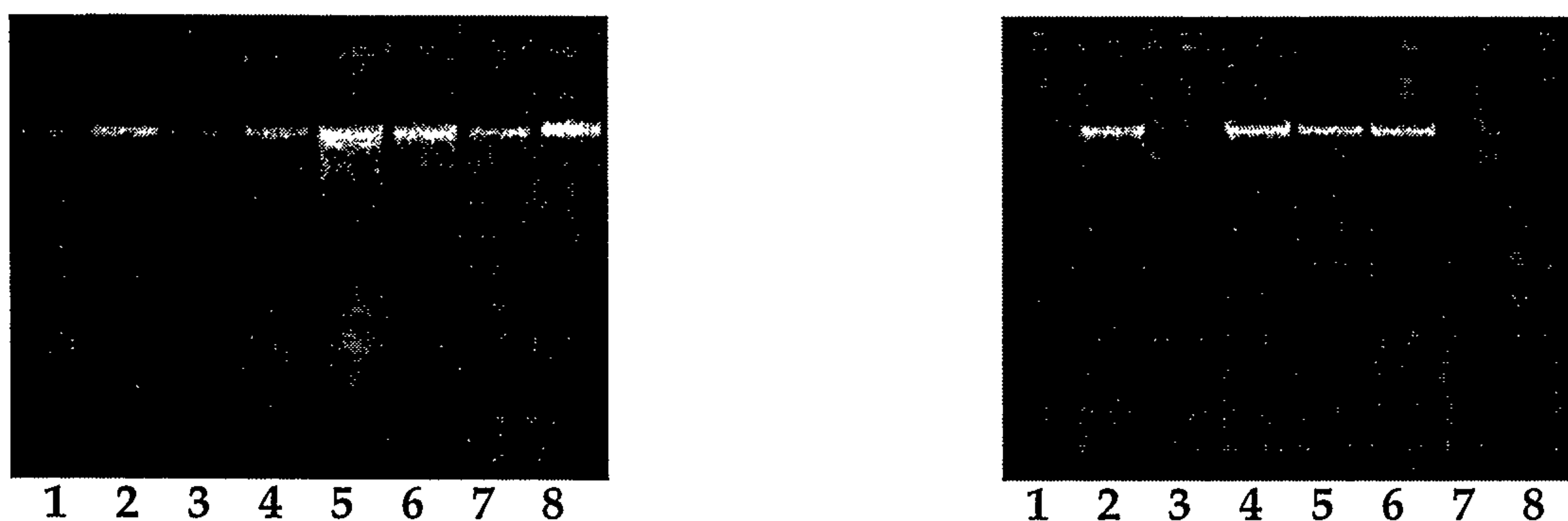


Figure 5.14. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing 5mg lysozyme ml⁻¹, 2mg mutanolysin ml⁻¹, 2mg proteinase K ml⁻¹ and 20mM DEPC with 6min pulse sonication (Figure 5.14a) and using the ATL method (Figure 5.14b). Lane 1: *A. israelii*; Lane 2: *F. nucleatum*; Lane 3: *L. acidophilus*; Lane 4: *P. endodontalis*; Lane 5: *P. gingivalis*; Lane 6: *P. melaninogenica*; Lane 7: *M. micros*; Lane 8: *S. mutans*.

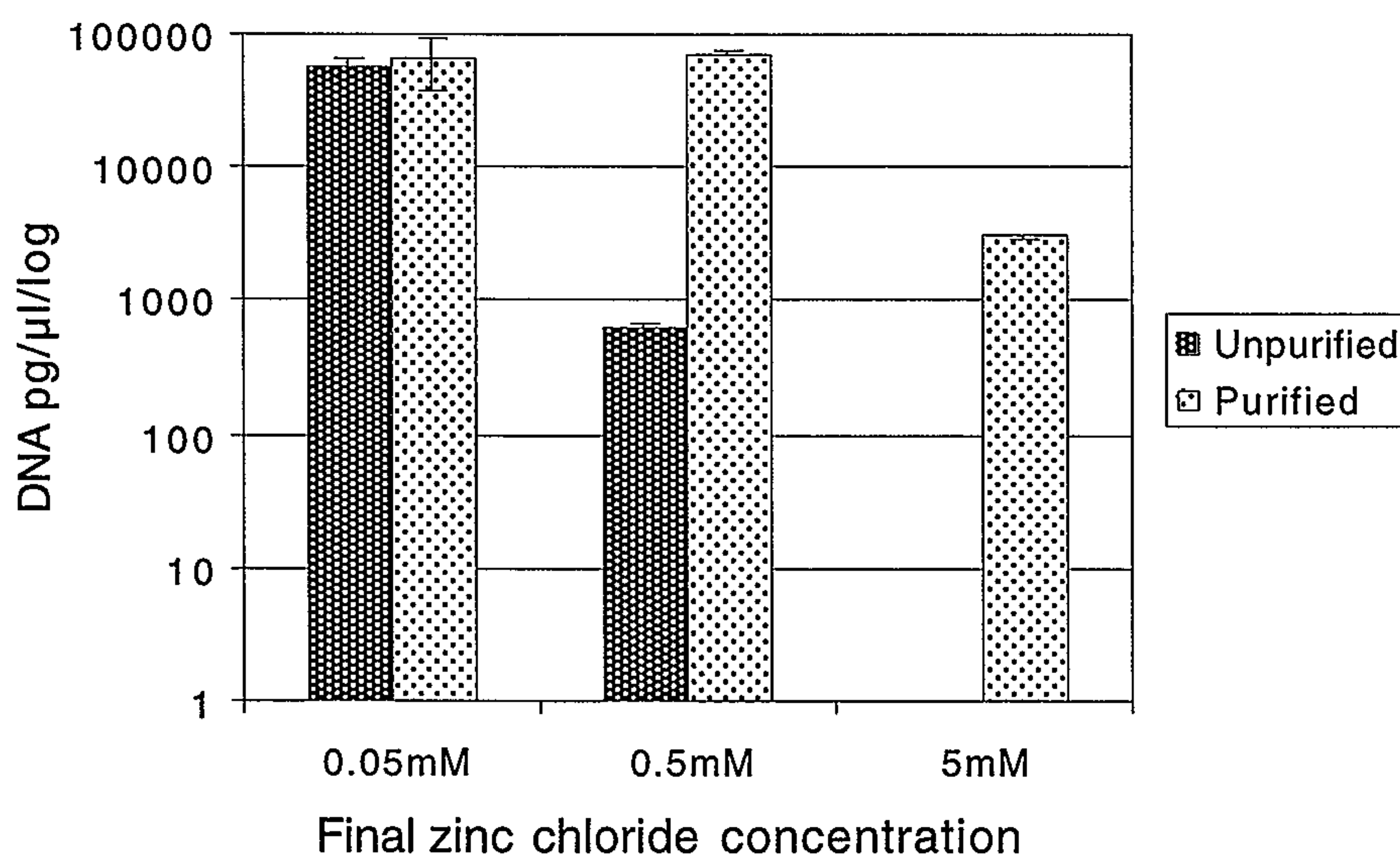


Figure 5.15. PCR quantitation of DNA isolated from cultures of *P. gingivalis* in lysis buffer containing 10mM sodium phosphate, 1mg lysozyme ml⁻¹ and 2mg mutanolysin ml⁻¹ with final concentrations of 0.05mM, 0.5mM and 5mM zinc chloride. Data are expressed as mean values from triplicate readings \pm SEM. Data provided by Dr M Nadkarni.

5.2.3 Discussion

Approximately 500 bacterial species are estimated to inhabit the oral cavity. The composition of the oral microflora is extremely complex and diverse comprising aerobic and anaerobic Gram-positive and Gram-negative bacteria (Paster *et al.*, 2001). In recent years considerable changes have occurred in the taxonomy of anaerobic bacteria, primarily based on DNA sequence information, which has led to the introduction of many new species or renaming of old species (Jousimies-Somer & Summanen, 1999). Traditional methods of microbial detection are often unable to detect uncultured or fastidious microbes (Amann, *et al.*, 1995; Wade *et al.*, 1997; Hugenholtz, *et al.*, 1998; Kroes *et al.*, 1999; Spratt *et al.*, 1999; Paster, *et al.*, 2001) and as the cultivation of microbes often takes days to weeks for initial growth, phenotypic testing and identification, molecular techniques offer a considerable advance. The advent of sequence-based identification has not only overcome the laborious methods of identification by traditional culture but has also indicated that less than 1% of the microorganisms present in nature have been cultivated (Amann, *et al.*, 1995; Hugenholtz, *et al.*, 1998). In contrast, PCR-based methods allow both accurate identification and quantification of a small number of organisms in a substantially shorter time (White, *et al.*, 1992; Chen *et al.*, 1997; Wahlfors, *et al.*, 1995). The development of a rapid molecular testing method should allow the investigation of a mixed microflora associated with disease in its entirety without the inherent bias of culture (Amann, *et al.*, 1995; Parrish & Greenberg, 1995; Dymock, *et al.*, 1996; Relman, 1998; Conrads *et al.*, 1999; Harper-Owen, *et al.*, 1999; Kroes, *et al.*, 1999; Paster, *et al.*, 2001). These advances should improve the knowledge of disease diagnosis and treatment monitoring.

Primers with broad inter-species specificity have been designed to amplify 16S rDNA by PCR and have been used to determine bacterial numbers in complex communities (Wilson *et al.*, 1990; Greisen *et al.*, 1994; Hykin *et al.*, 1994; Marchesi *et al.*, 1998; Klausegger *et al.*, 1999; Suzuki, *et al.*, 2000;). A majority of these studies, however, report the use of more than a single set of primers to detect the bacteria of interest. In addition, some techniques, such as competitive PCR (Blok *et al.*, 1997; Rupf, *et al.*, 1999) are labour intensive and require the

analysis of results from multiple reactions for each test sample. In contrast, the universal primers and probe set used in this study (Nadkarni, *et al.*, 2002) provides an efficient method for determining the total bacterial load in a sample. Although larger than the 150bp limit set in the Applied Biosystems protocol, the 466bp amplicon was found to be uniformly successful in detecting a wide range of bacteria. Takai and Horikoshi (2000) reported an amplicon from the same region of the genome which effectively retrieved phylogenetically diverse prokaryotic rDNA, although designed with variable bases. Corless *et al.* (2000) have reported a universal primers-probe set as a tool for the rapid detection of bacteria by real-time PCR. However, analysis of their forward and reverse primers and probe showed multiple mismatches with most of the dental pathogens including *B. forsythus*, *P. gingivalis*, *P. melaninogenica*, and one or more mismatches with many other bacteria at the 5'- as well as the 3'-ends. Similarly, the universal primers-probe set described by Lyons *et al.* (2000) for the detection of total bacteria in dental plaque has one or two mismatches within the probe for the 16S rDNA of *S. aureus*, *C. jejuni*, *H. pylori*, *Wolbachia* species, *M. micros*, *F. nucleatum*, *M. pneumoniae*, *L. mobilis* and *T. denitrificans*, and their reverse primer has no corresponding 16S rDNA sequences in the databases for numerous Gram-positive and Gram-negative bacteria. Furthermore, their primers-probe set exceeds a number of the guidelines set by Applied Biosystems for TaqMan technology. Their probe was selected from the strand with more Gs than Cs, which could affect the amount of fluorescence measured. The T_m of 48.4°C and 35.4°C for their forward and reverse primers, respectively (calculated from their reported sequences using the Primer Express Software) contrasts with the 58°C–60°C set by Applied Biosystems and necessitated the authors using additional annealing steps at 52°C for 1min and 72°C for 2min to allow for the elongation of their longer amplicon (727bp). This extended the total reaction time by at least 70min when using the ABI-PRISM 7700 Sequence Detection System for real-time PCR. The total time taken for quantification was further increased by the requirement for an initial PCR reaction.

One limitation of any broad-range or universal probe is the sensitivity limitation created by contamination. This contaminating DNA is thought to be present in either the enzyme

preparations (*Taq* polymerase or UNG) from organisms used to prepare the enzyme (*Thermus aquaticus* or *E. coli*) or from bacterial or fungal growth in cultures, chromatography columns or buffers used during enzyme purification (Böttger, 1990; Rand & Houck, 1990; Schmidt, *et al.*, 1991; Maiwald *et al.*, 1994; Corless, *et al.*, 2000; Lyons, *et al.*, 2000); an observation verified in the current study by the presence of rDNA in reagent mixes and negative controls containing no added *E. coli* DNA (Fig. 5.1a). Corless *et al.* (2000) reported contamination from the no-template control appearing in the 24th cycle, severely limiting the sensitivity of their reaction. Contamination in this study was restricted to 33-38th cycles by careful choice of the reagents. Other authors have attempted to remove contamination from the reagents using various methods including DNase I treatment (Lyons, *et al.*, 2000), restriction enzymes followed by DNase I treatment (Corless, *et al.*, 2000), UV irradiation (Hitti *et al.*, 1997; Schmidt, *et al.*, 1991), UV treatment and pre-PCR uracil DNA glycosylase digestion (Niederhauser *et al.*, 1994) or UV irradiation in the presence of 5-methoxypsoralen (Klausegger, *et al.*, 1999). However, Corless *et al.* (2000) reported that it was not possible to eliminate endogenous contamination from the *Taq* polymerase without severely compromising the efficiency of the PCR with a resultant reduction in sensitivity.

Detection and enumeration of *P. gingivalis* in previous studies has involved the use of amplicons ranging in length between 197bp to 527bp, with most requiring post-PCR processing (Wahlfors, *et al.*, 1995; Ashimoto, *et al.*, 1996; García *et al.*, 1998; Rupf, *et al.*, 1999; Tran & Rudney, 1999). The designed *P. gingivalis* primers-probe set used in this study was shown to be not only specific for *P. gingivalis*, but its design fell within the guidelines suggested by Applied Biosystems to optimise bacterial quantification in generating a 150bp amplicon. This is considerably shorter than the 900bp *P. gingivalis* primers-probe set designed by Lyons *et al.* (2000) for real-time PCR which incorporates part of the interspacer region as well as the 16S gene.

Since quantitation of the number of bacteria is entirely dependent on the amount of DNA measured, the DNA extraction and purification protocol becomes a critical step in the procedure. At this stage there is no universal method to optimise the digestion of all microbes

in all tissues (Fredricks and Relman, 1999). There are reports of some DNA purification protocols working efficiently with samples designed to amplify one or a limited number of microbes (Desjardin, *et al.*, 1998; Higgins *et al.*, 1998; Sen, 2000). Complex microbial communities, such as carious dentine, harbour a wide variety of both Gram-negative and Gram-positive species and difficulties arise when applying strategies that will efficiently lyse Gram-positive bacteria without shearing the DNA of more fragile organisms. A further complication of DNA extraction concerns the presence of nucleases. Results from this study indicated a deteriorating PCR signal for *P. gingivalis* DNA suggestive of DNA degradation. Nuclease activity has been reported for many bacteria (Park *et al.*, 1980; Flint & Thomson, 1990; Gibson & McKee, 1993; Nakajima, *et al.*, 1994; Ruiz *et al.*, 2000), including those found in the oral cavity such as fusobacteria, porphyromonads, prevotellae, bacteroides, peptostreptococci (Porschen and Sonntag, 1974; Rudek and Haque, 1976), lactobacilli (Smith and Bodily, 1967) and streptococci (Miller, *et al.*, 1971). The presence of nuclease activity in *P. gingivalis* was demonstrated by its ability to degrade *P. gingivalis* DNA as well as the internal positive control (IPC-BT) DNA. Therefore, all DNA extraction procedures tested in this study required consideration of not only the type of cell wall treatment, but also the possibility of nuclease activity of the organisms.

Despite the investigation of different techniques using an extensive series of experiments, it was not possible to efficiently extract DNA from both Gram-negative and Gram-positive bacteria, while also preventing nuclease degradation. Problems were noted with a number of the techniques which prevented efficient DNA extraction. In summary, DEPC, EDTA, EGTA and SDS showed some incompatibility with mutanolysin, thereby preventing lysis of Gram-positive bacteria. However, the Gram-negative microbes required DEPC, EGTA and/or EDTA for nuclease control and SDS for cell lysis, and attempts to remove SDS, by washing or Triton X-100 treatment, to facilitate the action of mutanolysin were ineffective.

Boiling the sample was shown to release and protect the DNA of *P. gingivalis* to a limited extent and has been used in a number of studies as the DNA extraction method (Söder *et al.*, 1993; Slots *et al.*, 1995; Ashimoto, *et al.*, 1996; García, *et al.*, 1998; Conrads, *et al.*, 1999;

Corless, *et al.*, 2000; Siqueira, *et al.*, 2001). However, Bickler *et al.* (1992) reported that nuclease activity could be restored following heat treatment or by changes in Mg^{2+} concentration and cautioned that heat treatment alone was inadequate for DNase inactivation.

Other published extraction methods use a lysis buffer (usually 10mM tris hydrochloride or 10mM sodium phosphate) generally containing 1-10mg ml⁻¹ lysozyme (Parrish and Greenberg, 1995; Dymock, *et al.*, 1996) and/or 0.1-1mg ml⁻¹ proteinase K (Smith *et al.*, 1989; Conrads *et al.*, 1997). Other additions include 1% SDS, 1-20mM EDTA or 10mM EGTA (Conrads, *et al.*, 1997; Jung *et al.*, 2000; Odell *et al.*, 1999). Lysozyme is used to weaken the cell wall of Gram-negative organisms which subsequently lyse on the addition of a detergent (Chassy & Giuffrida, 1980). Proteinase K is also used for cell lysis and has been demonstrated to be effective in lysing some strains of *P. intermedia* and Gram-positive species such as *Actinomyces odontolyticus*, *A. naeslundii* and *Eubacteria saburreum* (Grenier, 1994). Other Gram-negative species lysed to a lesser extent by proteinase K included *Capnocytophaga ochracea*, *F. nucleatum*, *Treponema denticola* and *H. actinomycetemcomitans*. Overall the Gram-positive species are resistant to the action of lysozyme and proteinase K, with the exception of those previously mentioned, and all strains of *S. mutans* have been found to be quite resistant (Grenier, 1994). Digestion of the Gram-positive cell wall is an essential pre-requisite prior to lysis with a detergent or the DNA yield from a sample will be substantially reduced (Dymock, *et al.*, 1996; Harper-Owen, *et al.*, 1999). Therefore, other methods were tested for their ability to lyse these bacteria including lysozyme at higher concentrations and for longer incubation periods (Roussel *et al.*, 1993), lysozyme incubation with a protease in an acidic buffer followed by incubation with bicarbonate anions at physiologic concentrations (Pollock *et al.*, 1987) and the use of 2mg ml⁻¹ mutanolysin, which hydrolyses the peptidoglycan of the Gram-positive cell wall (Simpson *et al.*, 1993). In the present study 2mg ml⁻¹ mutanolysin was used in a phosphate buffer in place of a Tris-EDTA buffer for all experiments to optimize the activity of the mutanolysin (Simpson, *et al.*, 1993). Those experiments that incorporated bicarbonate in the presence of lysozyme and proteinase K with an extended incubation period showed good recovery of DNA from only *S.*

mutans. The gel images of DNA from *L. acidophilus* appeared smeared and only minimal amounts of DNA were recovered for the remaining organisms.

Some bacterial nucleases were inactivated and DNA degradation inhibited by boiling, or the addition of 5mM ZnCl₂, 100mM EDTA, 100mM EGTA or 20mM DEPC during DNA isolation. This is supported by the findings of Leduc *et al.* (1994) who demonstrated nuclease inhibition of *P. gingivalis* by heating above 70°C or treatment with 5mM ZnCl₂ or 100mM EDTA, and Bendjennat *et al.* (1997) who showed the effectiveness of 5mM EDTA, 5mM EGTA and 0.1%DEPC on *Mycoplasma pneumoniae* nuclease inhibition. In the present study 5mM ZnCl₂ was unable to prevent the degradation of *L. acidophilus* and although 100mM EDTA was useful in minimising *L. acidophilus* degradation it was observed to inhibit the action of mutanolysin on *S. mutans*. The presence of 20mM DEPC was an effective nuclease inhibitor for the DNA isolation of *F. nucleatum*, *P. melaninogenica* and *P. gingivalis*, moderately effective against *A. israelii* and *S. mutans*, and least effective with *L. acidophilus*. Other chemicals assessed as possible nuclease inhibitors included aurin tricaboxylic acid, a topoisomerase inhibitor (Hallick *et al.*, 1977), trisodium citrate (Ruiz, *et al.*, 2000) and ascorbic acid. The hydroxyl radical scavengers were only minimally effective in inhibiting nuclease activity as evidenced by smearing of the DNA bands in the agarose gel images. Additional experiments were then conducted to explore the effect of DEPC under different conditions in an attempt to find a general nuclease inhibitor.

Successful extraction of DNA from bacterial cultures in the presence of DEPC with an increased lysozyme concentration was noted in agarose gel images for all the Gram-negative bacteria and *S. mutans*. To increase the recovery of DNA from the remaining Gram-positive microbes and to dissociate aggregations or "corn-cob" arrangements of bacteria, additional mechanical treatments were included. However, only *A. israelii*, *P. melaninogenica* and *S. mutans* showed increased DNA recovery following sonication.

As DNA could only be reliably extracted from the Gram-negative bacteria, a comparison was made between DNA recovery following the DEPC procedures and the ATL buffer from the QIAamp DNA Mini Kit. Data from this experiment demonstrated that for each of the

Gram-negative cultures the DNA recovered using ATL was significantly greater than that following the DEPC procedures, with less deterioration of the DNA as shown by the clear bands in the ATL gel images. Although the ATL buffer was ineffective in lysing Gram-positive bacteria, *M. micros* showed increased DNA release following isolation in ATL compared with DEPC. This behaviour might be explained by disruption of the cell wall integrity that has been reported when anaerobic bacteria are exposed to oxygen (Johnson *et al.*, 1995a).

Clinical samples are known to contain PCR inhibitors such as potassium ions, blood, urine, vitreous humor, sputum, saliva (Mättö, *et al.*, 1998; Fredricks and Relman, 1999). Inhibitors from other sources include sodium phosphate from transport media (Johnson *et al.*, 1995b), calcium alginate and aluminium swab shafts (Wadowsky *et al.*, 1994). These inhibitors must be diluted, removed or inactivated to allow DNA amplification to proceed. The inhibitory effect of ZnCl₂ has been demonstrated and adequate dilution and/or purification allowed the sample to be quantified. Therefore, purification was considered to be an essential component of DNA preparation of the clinical samples prior to PCR. Of those studies which incorporated DNA purification in their extraction process, the QIAamp Tissue Kit Protocol (Mini Kit) has been most commonly reported (Oberst *et al.*, 1998; Conrads, *et al.*, 1999; Odell, *et al.*, 1999; Tran and Rudney, 1999). However, studies directly comparing the efficiency of purification kits provide conflicting reports (de Kok *et al.*, 1998; Dixon *et al.*, 1998). To ensure that PCR had successfully been carried out and that inhibition had not created false negative results, monitoring the amplification of a second target nucleic acid, the IPC-BT, during the reaction served as a useful internal control.

In summary, some success was found in extracting DNA from the anaerobic Gram-negative bacteria and the Gram-positive *M. micros* in ATL, and *S. mutans* in DEPC. However, reasonable amounts of DNA could not be reliably recovered by any technique from either *A. israelii* or *L. acidophilus*, the latter almost always seen on gel images with marked smearing indicating considerable deterioration of the DNA product. As the culture data of Gram-positive microaerophilic bacteria from the dentine samples (Chapter 4) provided information comparable with the findings of other authors, PCR quantitation of these microbes from the

carious samples was not attempted. However, as a result of limitations regarding the efficient culture of anaerobic microorganisms, PCR quantitation of the anaerobic microbes from the clinical samples was carried out using the proprietary buffer ATL, followed by purification with the QIAamp DNA Mini Kit. The results of this study are reported in the next chapter.