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The Sub1 nuclear protein protects DNA from oxidative damage

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Abstract Reactive oxygen species are a by-product of aerobic metabolism that can damage lipid, proteins, and nucleic acids. Oxidative damage to DNA is especially critical, because it can lead to cell death or mutagenesis. Previously we reported that the yeast *sub1* deletion mutant is sensitive to hydrogen peroxide treatment and that the human *SUB1* can complement the sensitivity of the yeast *sub1* mutant. In this study, we find that Sub1 protects DNA from oxidative damage in vivo and in vitro. We demonstrate that transcription of *SUB1* mRNA is induced by oxidative stress and that the *sub1Δ* mutant has an increased number of chromosomal DNA strand breaks after peroxide treatment. We further demonstrate that purified Sub1 protein can protect DNA from oxidative damage in vitro, using the metal ion catalyzed oxidation assay.

Keywords Reactive oxygen species · Oxidative damage · Oxidative stress · Oxidation resistance · Sub1 protein

Introduction

All aerobic organisms from bacteria to humans reduce molecular oxygen to produce energy generating reactive oxygen species (ROS) as a by-product [1]. ROS can damage cellular components including lipids, proteins, RNA and DNA, triggering a variety of diseases including cancers, neurodegenerative diseases and aging [2–4]. To combat ROS, cells contain numerous antioxidant defenses that prevent or repair cellular damage [5].

The cell nucleus is a more reducing environment than the cytoplasm [5]; however, oxidative DNA damage still occurs and numerous mechanisms function to repair oxidative DNA damage [6]. To date, no antioxidant proteins have been reported to function exclusively in the nucleus, or to specifically protect DNA in eukaryotes, although some isoforms of cytoplasmic antioxidant proteins have been found in the nucleus [5, 7].

The human Sub1 protein, also known as PC4, is an abundant nuclear protein with only 127 amino acids that were originally isolated from HeLa cell nuclear extracts and shown to enhance transcription in vitro, a function requiring the serine- and lysine-rich N terminal domain (amino acids 1–40) [8]. It is also a DNA-binding protein and Sub1 binds to both double-strand DNA (dsDNA) and single-strand DNA (ssDNA), without apparent sequence specificity but with higher affinity to partially unpaired dsDNA and ssDNA [9, 10]. This DNA-binding function requires the carboxyl-terminal ssDNA-binding activity (amino acids 63–91) [8, 11]. Sub1 is highly conserved in all eukaryotic cells. The yeast Sub1 has 47 % identity with the human homolog in the conserved DNA binding and dimerization domains. It has been shown that Sub1 interacts with the transcription factor TFIIB and stimulates the GCN4 and HAP4 promoters in yeast [12, 13]. Both the yeast and human Sub1 bind nonspecifically to ssDNA and dsDNA with higher affinity to ssDNA [12].

Previously, we reported that human Sub1 prevents oxidative mutagenesis when expressed in bacteria and that this function requires its DNA-binding domain. In yeast,

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SUB1 is required for peroxide resistance and the human Sub1 complements the yeast *sub1Δ* mutant [11]. However, many questions remain to be answered, for example, whether *SUB1,* like many other oxidative stress resistance proteins, is induced in response to oxidative stress [14, 15]. Because Sub1 is a transcription cofactor, it remains to be tested if Sub1 has oxidation resistance functions that are independent of its role in transcription regulation. Here we report that the *SUB1* gene is induced by oxidative stress that *sub1Δ* mutants suffer from increased single- and double-strand DNA breaks after peroxide treatment, and that purified Sub1 protein can directly protect DNA from oxidative damage in vitro.

Results

SUB1 encodes a nuclear protein that is inducible by oxidative stress

The nuclear localization of Sub1 is inferred from its role in transcription regulation, but it has not been directly tested. We fused the Green fluorescence protein (GFP) gene to the yeast *SUB1* gene to test its cellular localization. Sub1-GFP appears to distribute evenly and exclusively in the nucleus in yeast, whether its transcription is driven from its own promoter on the chromosome (Fig. 1a), or from an inducible methionine promoter (Fig. 1b).

To test if Sub1 is inducible by oxidative stress, we treated yeast cells with 1 and 5 mM peroxide for 30 min and examined *SUB1* mRNA expression by Northern blot analysis. As shown in Fig. 2a, b *SUB1* mRNA levels are substantially increased after peroxide treatment, especially with the 5 mM H_2O_2 challenge, demonstrating that it is an

(a)

(b)
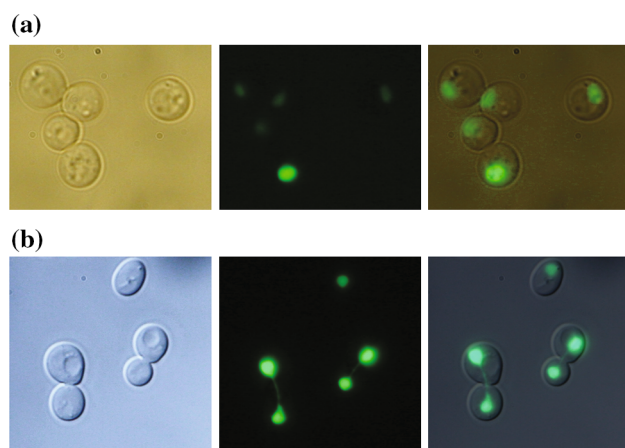
Fig. 1 Nuclear localization of Sub1 shown by GFP fusions under fluorescence microscope. *Left panels* Phase contrast; *middle* GFP; *right* overlaps. **a** The GFP gene is fused to the chromosomal SUB1 gene. **b** The GFP-SUB1 fusion gene is placed after the methionine promoter in plasmid pMV1327, cells are cultured in methionine drop out media

oxidative stress inducible gene. By contrast the constitutively expressed *PDR5* gene shows a dose-dependent decline in mRNA levels, presumably due to the accumulation of oxidative DNA damage in the template DNA. We confirmed the induction of *SUB1* by quantitative reverse transcription quantitative PCR (qRT-PCR). After normalizing to the internal control the ribosomal RNA *RND18*, *SUB1* mRNA is significantly induced after H_2O_2 treatment ($p = <0.01$, ANOVA test).

Elevated levels of oxidative DNA damage in the *sub1Δ* mutant after peroxide treatment

Single-strand DNA breaks (SSB) are a common DNA lesion caused by oxidative damage [16] and can be visualized by the alkaline comet assay [17]. Figure 3a shows that yeast chromosomal DNA migrates out of the nucleus in an electric field in the comet assay, forming a comet-like shape with a bright nuclear center. After treating the cells with peroxide, more DNA migrates out of the nucleus diminishing its intensity and the DNA becomes more diffuse as a result of SSBs produced by peroxide treatment (Fig. 3a). Comets of the *sub1Δ* mutant are essentially devoid of their nuclei and are more diffuse than those of wild-type after peroxide treatment, indicating peroxide produces more oxidative DNA damage in the *sub1Δ* mutant. Quantification of 30 comets showed the *sub1Δ* mutant has significantly more diffused comets than wild-type after peroxide treated ($p < 0.001$, Fig. 3b).

Double-strand DNA breaks (DSBs) are a more lethal form of oxidative DNA damage, which can be produced directly by ROS, or converted from SSBs during DNA replication. We used pulse field gel electrophoresis (PFGE) to test if peroxide produces more DSBs in the *sub1Δ* mutant. In this assay, a single DSB in a chromosome reduces the number of chromosomes found in a full-length chromosomal band. Figure 3c demonstrates that the chromosomes of the *sub1Δ* mutant decline in intensity more rapidly than in wild-type. This is most clearly seen in the larger chromosomes because they are larger targets and therefore more likely to acquire a DSB. We measured the peak vs background intensities in each lane. The ratios of the peak to background intensity declined more rapidly in the *sub1Δ* mutant than in the wild-type (Fig. 3d). These results demonstrate that the increased peroxide sensitivity of the *sub1Δ* mutant is accompanied by the increased accumulation of DSBs when compared with wild-type after treatment with hydrogen peroxide.

Purified Sub1 protects DNA in vitro

We previously reported that the expression of the human SUB1 gene complements the peroxide sensitivity of the
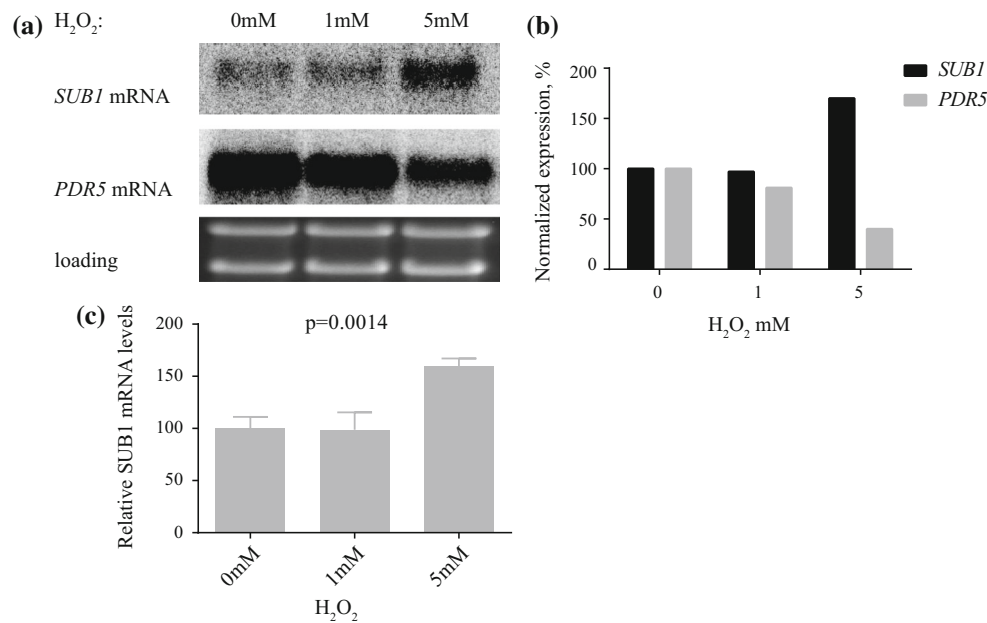
Fig. 2 Expression of *SUB1* after peroxide treatment. **a** Northern blot analysis of yeast mRNA extracted from wild-type cells treated with peroxide at the indicated concentrations for 30 min. The *PDR5* mRNA is a random control. The rRNA (*lower panel*) is shown as the loading control. **b** Quantitative representation of the blots. Gene expressions were first normalized to the ribosome bands then to the levels at 0 mM treatment. Photoshop CS6 was used to quantify the blots in a. **c** RT-PCR analysis of *SUB1* mRNA from wild-type cells treated with peroxide at the indicated concentrations for 30 min. The relative levels of *SUB1* mRNA are normalized to the ribosome RNA *RND18*. Results of three independent experiments were shown. One-way ANOVA test was used to compute the *p* value

yeast *sub1Δ* mutant, suggesting a conserved oxidation-protection function in this family of proteins. Therefore, to test if Sub1 can function directly to reduce oxidative damage to DNA, we purified the human Sub1 protein to homogeneity according to the previously published methods [18] (Fig. 4b) and tested its ability to directly protect DNA from oxidative damage in vitro. The metal ion catalyzed oxidation (MCO) assay is an in vitro assay that uses the mixture of Fe^{3+} and DTT in the presence of oxygen to produce ROS, particularly H_2O_2 and hydroxyl radicals, which in turn produce single-strand DNA breaks and 8-hydroxy-2′-deoxyguanosine in the substrate DNA [19]. Introduction of SSBs converts the supercoiled pUC19 plasmid to relaxed nicked circular forms. As double strand breaks accumulate, the circular DNA molecules are converted to linear forms. Further degradation occurs as damage levels increase and DNA becomes increasingly degraded. Figure 4a shows that plasmid relaxation and formation of linear molecules occurs as a function of exposure time, and extensive degradation of the plasmid is seen after 60 min of treatment. When purified Sub1 protein is added to the assay, it protects the DNA from oxidation in a dose-dependent manner (Fig. 4d). At 25 and 50 ng/μl, Sub1 is seen to partially protect the DNA from oxidation. By contrast, addition of a control protein, BSA, has no effect on DNA protection when it is present at a concentration of 50 ng/μl. While this does not rule out the possibility that the transcription functions of Sub1 may contribute to oxidative stress resistance, it clearly demonstrates that Sub1 protein can directly protect DNA from oxidative damage, a feature consistent with its DNA-binding activity, the high concentrations of Sub1, especially after induction, and its nuclear localization.

Discussion

In summary, we found that Sub1 protects DNA from oxidative damage in yeast and in vitro. Sub1 is an abundant nuclear protein, suggesting it can protect the genomic DNA from oxidative damage and therefore it may be important in maintaining genomic stability [20].

Sub1 family of proteins has been proposed to function in many processes: they are recruited to transcription complexes [8, 9], DNA repair complexes [11, 21], double-strand DNA breaks [22, 23], and replication complexes [24, 25]. Since Sub1 binds to DNA with a strong preference for unpaired double-stranded DNA regions and single-stranded DNA [9–11, 20], and our results indicate that it prevents oxidative DNA damage, this suggests a common function for this family of proteins in all of these processes. In each of the above Sub1-DNA interactions, the DNA is devoid of histones and other potentially protective proteins and is partially unwound, exposing ssDNA regions, and/or
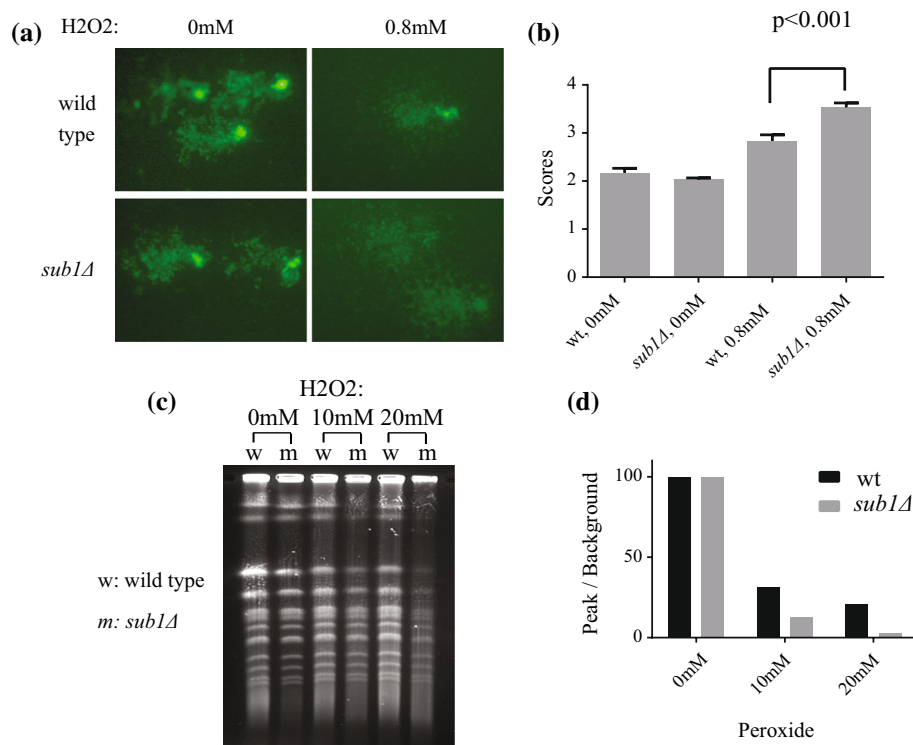
Fig. 3 Peroxide produces more DNA breaks in the *sub1Δ* mutant. **a** Analysis of SSBs by alkaline comet assay (wild-type, MVY101; *sub1Δ*, MVY105). Shown are representative microscopic pictures. **b** Numeric scores were assigned to 30 comets in each group (see methods) and the mean sores with standard errors were shown for each group. The nonparametric Wilcoxon test was used to test the difference in the wild-type and mutant after peroxide treatment. **c** Production of DSBs examined by PFGE. Cells (wild-type, MVY101; *sub1Δ*, MVY105) were treated with different amounts of hydrogen peroxide as indicated and chromosomes are separated by PFGE. *w* wild-type, *m sub1Δ* mutant. **d** Analysis of the signal intensity of the lanes in the PFGE gel by using ImageJ (Version 1.47), presented are the ratios of peak and background normalized to untreated lanes

unpaired dsDNA regions. The function for Sub1 in all of these processes may be to prevent oxidative damage when DNA is most vulnerable to attack by oxidative agents.

The precise chemical mechanism of oxidation protection by Sub1 remains to be determined. Sub1 shares some weak similarities in function and structure to the bacterial Dps protein, a well-conserved oxidation resistance protein found only in prokaryotes. Dps binds DNA nonspecifically and protects DNA from oxidation through three modes of actions: DNA shielding, iron sequestration, and its ferroxidase activity [26, 27]. DNA shielding appears to play a role in Sub1's antioxidant activity, because Sub1 binds to DNA nonspecifically and stably and its DNA-binding activity is required for oxidation resistance [11]. The co-crystal of Sub1 with DNA indicates that a single molecule of Sub1 can bind to an 8 base loop of ssDNA, or 4 base pairs of unpaired dsDNA [28]. Full protection from peroxide damage was attained in vitro at a ratio of 1 Sub1 molecule to 3 base pairs of DNA (Fig. 4d), suggesting Sub1 can protect the DNA when the naked DNA is fully covered by Sub1. In human cells, the estimated nuclear concentration of Sub1 is 1 µM [20], roughly 1 Sub1 molecule per 3000 base pairs. However, it is possible that the Sub1 protein maximally binds to the exposed naked DNA in the

nucleus and preferentially protects such regions of the genome from oxidation. It remains to be determined whether Sub1 shares with Dps protein the ability to directly detoxify ROS, or sequester iron in the cell. It also remains possible that the transcriptional functions of Sub1 contribute to oxidation resistance in addition to its direct DNA protective effects.

Oxidative stress is the underlying cause of cancers and many other diseases. Because Sub1 can specifically protect the nuclear DNA, it may be an important player in cancer prevention. Mutations in Sub1 may reduce its ability to prevent oxidative DNA damage. In fact, studies have shown that Sub1 maps to chromosome locus 5p13 where loss of heterozygosity frequently occurs in bladder and lung tumors [29], suggesting a potential protective role for Sub1 against cancers.

Materials and methods

Yeast strains and plasmid

Yeast strains used in this study are listed in Table 1. All yeast knockout strains were created by PCR-based gene replacement methods [30, 31].
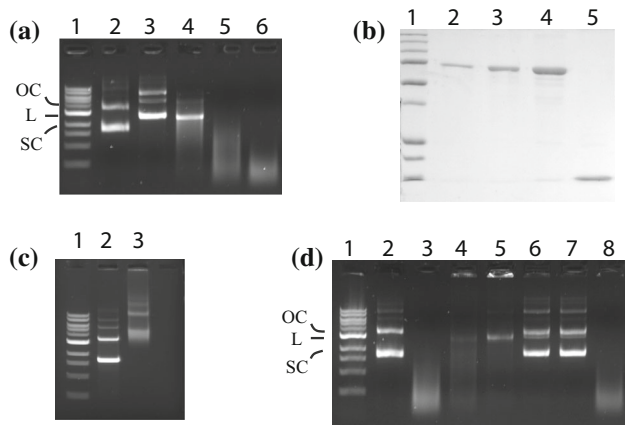
Fig. 4 Sub1 protects DNA from oxidation in vitro. **a** Oxidation of the pUC19 plasmid by the MCO system. Lanes are: *1* NEB 1 kb DNA standard; *2* pUC19 DNA; *3–6* pUC19 DNA oxidized by the MCO system for 15, 30, 45, 60 min. *SC* supercoil form; *L* linear form; *OC* open-circle form. **b** Sub1 is purified to homogeneity. *Lanes* are 5 μL of: *1* Bio-Rad precision plus all blue protein standard; *2* 100 ng/μL BSA; *3* 200 ng/μL BSA; *4* 500 ng/μL; *5* purified Sub1 at 1 μg/μL. **c** Purified Sub1 binds to DNA, indicating it is functional. *Lanes 1* NEB 1 kb DNA standard; *2* pUC19 DNA; *3* pUC19 DNA plus Sub1. **d** Sub1 protects DNA from oxidation in vitro. The MCO reactions were carried out at 37 °C for one hour. Lanes as: 1: NEB 1 kb DNA standard; 2: pUC19 DNA; 3: pUC19 oxidized by the MCO system; *4–7* pUC19 oxidized in the presence of Sub1 at concentrations: 25, 50, 100, 200 ng/μL; *8* pUC19 oxidized in the presence of 50 ng/μL BSA. *SC* supercoil form; *L* linear form; *OC* open-circle form

Analysis of peroxide induced SUB1 gene expression

Cells are grown in 20 ml YPD liquid medium at 30 °C overnight, resuspended in 75 ml YPD liquid medium, and incubated for 4 h. Cell density is adjusted to an OD_{600} of 0.8, and hydrogen peroxide is added at the indicated concentrations. Cells are harvested after 30 min. RNA is extracted and measured by northern blotting as described elsewhere [32, 33].

Reverse transcription quantitative PCR (RT-QPCR)

Total RNA was reverse transcribed by using random hexamers and the ImProm reverse transcription system (Promega) to produce cDNA. Quantitative PCR of SUB1 and RND18 was performed using the SYBR green method as described previously [34]. Primer 316 (CTGTACCCA CACTTCAAGCTAA) and primer 332 (TCATTTCAGCTTC

CAAGCTTTG) were used to amplify *SUB1* cDNA. Primer 870 (GTGCTGGCGATGGTTCATTC) and primer 871 (CCTTGGATGTGGTAGCCGTT) were used to amplify the *RND18* cDNA.

Analysis of SSB damage by the comet assay

The yeast comet assay is as described in [17]. To quantify the SSB, we assign numeric values to the comets: 2 for comets with bright comet heads and nondiffused tails, 3 for comets with diffuse tails and a small but visible head, and 4 for very diffused tails and no visible heads. At least 30 comets were quantified in each group, and nonparametric Wilcoxon test was used to determine the significant level of the difference between groups.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis of DNA is as described in [35]. Cells are grown to log phase and treated with hydrogen peroxide at the specified concentrations for 10 min. The gel plugs containing the chromosomal DNA are electrophoresed in 0.5X TBE at 14 °C for 24 h with 6 V/cm voltage using an initial/final switching time of 60/120 s.

Sub1 protein purification

Recombinant Sub1 was purified from the *E. coli* strain MV4996 expressing full-length human Sub1 (pMV801) as described previously [18] with modifications. Briefly, MV4996 is incubated in LB liquid medium with 100 μg/ml ampicillin and induced with IPTG (1 mM) for 3 h. Cells are then resuspended in BC300 [18], sonicated, and the cell lysate loaded onto a Heparin-Sepharose column. After washing extensively with BC300, Sub1 is eluted with BC500 (without EDTA) and loaded into a P11 phosphocellulose column (Whatman Inc. Piscataway, NJ), washed with 10 column volumes of BC500 (without EDTA), and eluted with BC1000 (without EDTA). The final Sub1 eluate is dialyzed against 500 ml of 25 % glycerol for 3 h, followed by dialysis against 1 L of 25 % glycerol overnight. The dialyzed protein is quickly frozen in liquid nitrogen and then stored at −80 °C.

Table 1 Yeast strains used in this study	Strain	Original name, genotype (annotation)	Reference
	MVY101	FY833, Mata *ura3-52 leu2Δ trp1Δ63 his3Δ200 lysΔ202*	[1]
	MVY105	MVY101 with *sub1Δ*::hisG	[1]
	MVY653	MVY105 with pMV1327 (GFP-SUB1 fusion in pUG36)	This study
	MVY809	MVY610 with SUB1::SUB1-GFP	This study

MCO assay for oxidative damage

The MCO assay is as described previously [19] with some modifications. The reaction contains 5 μl of 100 μM FeCl$_3$, 5 μl of 100 mM DTT, 5 μl of 200 mM Hepes (pH = 7.0), 2.5 μl of 230 ng/μl pUC19 DNA, and water to a final volume of 50 μl. FeCl$_3$ is added in the last step to initiate the reactions. Sub1 (1 μg/μl stock) or BSA (500 ng/μl stock) is added as indicated (Fig. 4d). Reactions are incubated at 37 °C for one hour unless otherwise indicated. After the incubation, 2 μl of 0.5 M EDTA is added to stop the reactions and 52 μL phenol (pH = 8.0) is added to remove the proteins. After centrifugation, 10 μl of the aqueous fraction is loaded onto the agarose gel and electrophoresed at 5 V/cm for 30 min. The gel is then stained with ethidium bromide and photographed (KODAK Gel Logic 2000).

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