DNA FRAGMENTATION IN MICROORGANISMS ASSESSED IN SITU

Running title: DNA fragmentation in microorganisms

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Chromosomal DNA fragmentation may be a direct or indirect outcome of cell death. Unlike research in higher eukaryotic cells, DNA fragmentation in microorganisms is rarely studied. We report an adaptation of a diffusion-based assay, developed as a kit, which allows for simple and rapid discrimination of bacteria with fragmented DNA. Intact cells were embedded in an agarose microgel on a slide, incubated in a lysis buffer to partially remove the cell walls, membranes, and proteins, and then stained with a DNA fluorochrome, SYBR Gold. Identifying cells with fragmented DNA uses peripheral diffusion of DNA fragments. Cells without DNA fragmentation only show limited spreading of DNA fiber loops. These results have been seen in several Gram-negative and Gram-positive bacteria, as well as in yeast. Detection of DNA fragmentation was confirmed by fluoroquinolone treatment and by DNA Breakage Detection-Fluorescence In situ Hybridization (DBD-FISH). *Proteus mirabilis* with DNA spontaneously fragmented during exponentially and stationary growth, or *Escherichia coli* with DNA damaged after exposure to hydrogen peroxide or antibiotics such as ciprofloxacin or ampicillin, were clearly detected. Similarly, fragmented DNA was detected in *Saccharomyces cerevisiae* after amphotericin B treatment. Our assay may be useful for a simple and rapid evaluation of DNA damage and repair as well as cell death, either spontaneous or induced by exogenous stimuli, including antimicrobial agents, or environmental conditions.

**Key words:** DNA fragmentation; DNA damage; cell death; quinolones; DBD-FISH
INTRODUCTION

Chromosomal DNA fragmentation, resulting from massive DNA double-strand breaks, is a hallmark of cell death. In higher eukaryotic cells, this may be a consequence of active programmed cell death (PCD), i.e., apoptosis, where DNA is cleaved by an activated endonuclease (26). Otherwise, DNA fragmentation may occur passively through necrotic cell death. Passive DNA fragmentation is likely in microorganisms killed by various causes. Recent studies suggest, however, a possible PCD in unicellular bacteria and yeast (38). In fact, bactericidal antibiotics may trigger a PCD response. It has been reported that bactericidal antibiotics can stimulate the production of hydroxyl radicals that contribute to cell death (20). Tolerant bacterial cells, that are resistant to the bactericidal action of some antibiotics, may be cells with a disabled PCD. Ecological pressure, differentiation processes, starvation and certain DNA damaging agents, may activate PCD involving DNA fragmentation (1, 6, 18, 22, 35). DNA fragments released during autolysis may be absorbed by other bacteria, contributing to antigenic variation and the spread of antibiotic resistance (12). Bacterial autolytic mechanisms have been described primarily with reference to the cell wall level. Nevertheless, active DNA fragmentation has only partially been addressed.

The presence of DNA breakage is usually evaluated using biochemical or molecular procedures such as alkaline unwinding, DNA elution, gel electrophoresis, sucrose gradient sedimentation, melting curve analysis, viscoelastometry, or light-scattering (2, 37). Unfortunately, evaluating DNA fragmentation within an individual cell is not possible nor is it possible to identify important low level intercellular variations. In situ procedures allow for cells with fragmented DNA to be discriminated from those without 

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DNA fragmentation. However, in situ procedures have been developed primarily for use in higher eukaryotic cells. DNA breaks can be enzymatically labelled by attaching modified nucleotides that can be visualized by direct and indirect methods (10). Usually this process involves the *E. coli* DNA polymerase I in the In Situ Nick Translation (ISNT) assay or end-labelling with the Klenow fragment of DNA polymerase I, or the terminal deoxynucleotidyl transferase (TdT) in the TUNEL procedure (10). In each of these, a free and accessible 3′-OH group at the end of the break is necessary as substrate for extension, but the ends of DNA breaks may be chemically modified by many DNA-damaging agents.

In situ detection of DNA breaks in higher eukaryotes can also be seen through the single-cell gel electrophoresis (SCGE) or comet assay (27). In this technique, the cells are trapped in an inert agarose microgel on microscope slide, deproteinized by incubation with a lysis solution, and then electrophoresed. DNA staining with a fluorochrome reveals a comet, with a head and a tail of chromatin in the direction of the positive pole of the electric field. Cells with DNA breaks have a higher tail and/or greater DNA concentration in the tail. In contrast to enzymatic labelling, cells in the SCGE assay are unfixed, fully accessible to lysis, and the DNA migration is not dependent on the chemical nature of the breaks. An image analysis system is habitually used for evaluation. Nevertheless, electrophoresis is not necessary to identify cells with fragmented DNA. After lysis in the agarose microgel, cells with fragmented DNA can be identified because they produce a peripheral halo of diffused DNA fragments in the agarose matrix. This is the underlying principle of the diffusion assay to detect cells with DNA fragmentation (33, 36).
Regarding microorganisms, the TUNEL assay has been used to determine DNA fragmentation in spheroplasts from yeast, but only one paper has described its use in bacteria, specifically in *E. coli* and in the archaeon *Halofex volcanii* (31). The procedure was time-consuming, requiring fixation, centrifugation and permeabilization steps. Moreover, the evaluation used a flow cytometer, requiring a high number of cells. These technical factors make this procedure impractical for routine assessment of DNA fragmentation in the standard microbiology laboratory. For the comet assay, only one paper has described its use for *E. coli* (34). The procedure was also lengthy, requiring lysozyme digestion of the cell wall prior to incubation in the lysis solution and in conjunction with prolonged proteinase K digestion. The resulting images are difficult to interpret.

Despite its importance from both a basic research and a clinical point of view, DNA fragmentation has not been assessed in the microbiology laboratory. This may be due to the lack of a simple and rapid evaluation procedure. Most current techniques are complex, technically demanding, and are not specifically adapted for the different microorganisms. Here we present a diffusion-based assay to identify DNA fragmentation in bacteria and yeast, using fluorescence microscopy. This assay is assembled as a kit in order to implement a simple, fast, reproducible and accurate method for studying DNA fragmentation in microorganisms.
MATERIALS AND METHODS

Microorganisms and cultures. Chromosomal DNA fragmentation was assayed in Gram-negative and Gram-positive bacteria (Table 1). Gram-negative bacteria were grown in Luria Bertani (LB) broth (1% Bacto Tryptone, 0.5% yeast extract, 0.5% NaCl) or on LB agar, at 37°C in aerobic conditions. Gram-positive bacteria were grown on Trypticase Soy Agar (TSA) plates (Diagnostics Systems, Sparks, MD, USA). *Candida albicans* and *S. cerevisiae* yeast were grown in yeast extract/peptone/dextrose (YPD) broth and plates. Cell growth in liquid cultures was monitored with the spectrophotometer (Unicam 8625, Cambridge, UK). In amphotericin B experiments, viability was determined by colony-counting after sequential dilutions and plating.

Experiments. Five different experiments illustrated the use of the procedure to determine chromosomal DNA fragmentation. In the first experiment, aliquots of exponentially growing cultures of the *E. coli* strain TG1, were exposed to ciprofloxacin (5 µg/ml and 0.012 µg/ml), for 40 min in LB broth, at 37°C. Fluoroquinolone is an inducer of DNA fragmentation; this experiment validated the assay. In the second experiment, *P. mirabilis* was incubated in LB medium at 37°C in aerobic conditions. The initial optical density in the spectrophotometer, measured at 600 nm, was 0.05. *P. mirabilis* in exponentially growing phase and in stationary phase were analyzed for frequency of cells with fragmented DNA and for membrane permeability, in aliquots that were removed periodically along 106 h. These were batch cultures, where growth uses an unsupplemented amount of nutrients, so the nutrients will decrease in time, together with an increase in metabolites. In a third experiment, TG1, either exponentially growing or in stationary phase, was incubated with 10mM hydrogen...
peroxide for 10 min at room temperature in LB broth. Immediately the cells were processed with the DNA fragmentation kit, as described later. The purpose of this experiment was two-fold: 1) to assess the DNA damage induced by hydroxyl radicals, and 2) to explore the influence of the growth phase on the effect of hydrogen peroxide on DNA. In the fourth experiment, exponentially growing cultures of TG1 were exposed to ampicillin (300 µg/ml) for 40 min in LB broth, at 37°C. This compared the effect on DNA of incubation with an antibiotic with a different mechanism of action to that of quinolones. Finally, exponentially growing cultures of *S. cerevisiae* were incubated with increasing doses of amphotericin B (0, 0.5, 1, 2, 4, 8 and 16 µg/ml), at 30°C, for 3 and 24 h, in suspension, in YPD broth. After incubation, the cells were processed with the DNA fragmentation kit, as described later. 1000-5000 microorganisms were scored per treatment.

**Membrane permeability.** A mixture of SYBR Green II (21) and propidium iodide (Molecular Probes, Eugene, OR, USA) was prepared in PBS, at 333X and 0.17 µg/ml, respectively. A 4µl aliquot of microorganisms growing in liquid medium was diluted in 16 µl of culture medium and was incubated for 5 min with 4 µl of dye mixture in the dark. If the microorganisms were at a very low density, the dye mixture was added to a 20 µl aliquot of cell culture without dilution. 5 µl of the stained cell suspension were dropped onto a glass slide, covered with a coverslip, and examined by fluorescence microscopy. Permeable cells that did not exclude the propidium iodide appeared red, whereas those “alive” only fluoresced green. In *S. cerevisiae*, non-permeable cells appeared unstained.
Determination of DNA fragmentation. The commercial sperm-Halomax® kits (Halotech DNA SL, Madrid, Spain) used to determine DNA fragmentation in different mammalian spermatozoa were evaluated (13, 17, 30). Using sperm-Halomax® as a reference, a prototype Micro-Halomax® kit was developed for microorganisms (Halotech DNA SL, Madrid, Spain). While Gram-negative bacteria could be directly processed, a previous brief cell wall digestion step was necessary for Gram-positive bacteria. *S. agalactiae* was incubated with mutanolysin (0.1 mg/ml), *E. faecalis* with a mix of mutanolysin (0.1 mg/ml) and lysozyme (4 mg/ml), and *S. aureus* with a mix of lysostaphin (0.05 mg/ml) and lysozyme (0.25 mg/ml). *S. pyogenes* was incubated with lysozyme (1 mg/ml). To this purpose, bacteria were scrapped from the culture plate and resuspended in 0.25 ml of LB medium or phosphate buffer saline (PBS) pH 6.88, in 0.5 ml snap cap micro-centrifuge tubes. The enzyme was added at the desired final concentration and was incubated for 15 min at 37°C. *C. albicans* and *S. cerevisiae* were digested with lyticase, 2.5 U/ml for 15 min and 0.07 U/ml for 10 min, respectively, in 1M sorbitol, 1M EDTA and 15mM betamercaptoethanol, pH 7.5, at 30°C. All enzymes were purchased from Sigma (St Louis, MN, USA).

An aliquot of each sample was diluted to a concentration of 5-10x10⁶ microorganisms/ml in the broth culture medium specific for each microorganism. The yeast was centrifuged and resuspended in the lyticase buffer without the enzyme. 0.5 ml snap cap micro-centrifuge tubes containing gelled aliquots of 60 µl of low-melting-point agarose (Pronadisa, Laboratorios Conda, Madrid, Spain) in distilled water are provided with the Micro-Halomax® kit. The tube was placed in a water bath at 90-100°C for 5 min to melt the agarose and then transferred to a water bath at 37°C (Memmert, Schwabach, Germany). After 5 min incubation, to allow for equilibration to 37°C, 25 µl
of the diluted sample containing the microorganism was added to the tube and was mixed with the melted agarose. Aliquots (20 µl) of the sample-agarose mixture were pipetted onto a precoated slide provided with the kit and were covered with a 22 x 22 mm coverslip. The coating of the slides consists of a dried agarose layer prepared with 1% standard agarose in water and drying in an oven at 80ºC (Memmert, Schwabach, Germany). The slide was placed on a cold plate in the refrigerator (4ºC) for 5 min, to allow the agarose to solidify producing a microgel with the intact cells trapped inside. The coverslip was gently removed, and the slide was immersed in 10 ml of lysis solution provided in the Micro-Halomax® kit, previously tempered to 37 ºC, for 5 min, in a closed try at 37ºC. This solution contains 2% SDS, 0.05M EDTA and 0.1M DTT, pH 11.5. The slide was always placed in horizontal position to prevent DNA stretching. After washing horizontally for 3 min in a tray with abundant distilled water, the slide was dehydrated by incubation horizontally in cold (-20ºC) ethanol baths, first 70%, then 90% and finally 100%, for 3 min each, followed by air-drying in an oven (Memmert, Schwabach, Germany). DNA staining with the fluorochrome SYBR Gold (Molecular Probes, Eugene, OR, USA) (7), could be performed immediately after drying. Before staining, the dried slide must be incubated in a microwave oven (Whirlpool, Norrköping, Sweden) at 750 W for 4 min to promote the attachment of DNA to the slide. The slide may also be placed in an oven at 80ºC for 1h to overnight. The slide was then stained with 25 µl of SYBR Gold diluted 1:100 in TBE buffer (0.09M Tris-Borate, 0.002M EDTA, pH 7.5), covered with a plastic coverslip, and incubated for 5 min in the dark. The slide was briefly washed and mounted in TBE. Fluorescence microscopy must be performed immediately after staining to avoid drying. If needed, the slide may be stored at 4ºC for hours in a
self-made humid box in the dark to prevent drying. If dried, the coverslip may be removed by incubation in PBS and, after a brief wash, may be re-stained again. If immediate evaluation is not necessary, dried slides may be left overnight or a couple of days in a high temperature oven (80°C) and then stored in a tightly closed box, in the dark, at room temperature, for several months, before staining.

Fluorescence microscopy allows for 10x to 100x magnification, but 100X is necessary for a precise visualization of the small spots from nucleoids with fragmented DNA. Three microgels can be placed on a same slide, including a control sample if required, so that all microgels are simultaneously processed under the same conditions.

DNA Breakage Detection-Fluorescence In Situ Hybridization (DBD-FISH) (14, 15). To confirm the presence of DNA breakage in cells with diffused DNA spots, the DBD-FISH procedure was used in *E. coli*, *P. mirabilis*, *Acinetobacter haemolyticus*, *Staphylococcus aureus* and *S. cerevisiae* nucleoids. The cells were immersed in agarose microgels and were lysed as described. They were then washed in 0.9%NaCl and were incubated in an alkaline unwinding solution (0.03M NaOH) for 2.5 min at 22°C. The gels were neutralized in 0.4M TrisHCl, pH 7.5, washed in distilled water, dehydrated in increasing ethanol baths (70-90-100%) for 2 min each, and air-dried.

A DNA probe to label the total DNA from the microorganism was prepared. DNA from each microorganism was isolated using standard procedures, and was labeled with biotin-16-dUTP, using a nick translation kit, according to the manufacturer’s instructions (Roche Applied Science, San Cugat del Vallés, Spain). The DNA probe was mixed at 4.3 ng/µl in the hybridization buffer (50% formamide / 2xSSC, 10%
dextran sulfate, 100mM calcium phosphate, pH 7.0) (1xSSC is 0.015M NaCitrate, 0.15M NaCl, pH 7.0). The probe in hybridization buffer was denatured by incubation at 80°C for 8 min and was then incubated on ice. This solution (30 µl) was pipetted onto the dried slide, covered with a glass coverslip (22x60mm) and incubated overnight at room temperature, in the dark, in a humid chamber. The coverslip was removed, and the slides were washed twice in 50% formamide/2xSSC, pH 7.0, for 5 min, and twice in 2xSSC pH 7.0, for 3 min, at room temperature. The slides were incubated with 80 µl of blocking solution (4XSSC, 0.1% Triton X-100, 5%BSA) for 5 min, covered with a plastic coverslip, in a humid chamber, at 37°C. This solution was decanted, and the bound probe was detected by incubation with 80 µl of streptavidin-Cy3 (Sigma Chem, St Louis, MN, USA) in 4XSSC, 0.1% Triton X-100, 1%BSA (1:200), covered with a plastic coverslip, in a humid chamber at 37°C. After washing in 4XSSC, 0.1% Triton X-100, three times, 2 min each, slides were counterstained with 20 µl of DAPI (2 µg/ml) in Vectashield (Vector, Burlingame, CA).

**Fluorescence Microscopy and Digital Image Analysis.** Images were viewed with an epifluorescence microscope (Nikon E800), with a 100x objective and appropriate fluorescence filters for FITC-SYBR Gold (excitation 465-495 nm, emission 515-555 nm), PI-Cy3 (excitation 540/25 nm, emission 605/55 nm) and DAPI (excitation 340-380 nm, emission 435-485 nm). The images were captured with a high-sensitivity CCD camera (KX32ME, Apogee Instruments, Roseville, CA). Groups of 16 bit digital images were obtained and stored as .tiff files. Image analysis used a macro in Visilog 5.1 software (Noesis, Gif sur Yvette, France). This allowed for thresholding, background subtraction, and measurement of the total fluorescence intensity (surface area, in pixels x mean fluorescence intensity, in grey level) of the signals. In the
experiment concerning ciprofloxacin exposure, the surface area of diffusion of the DNA fragments from nucleoids, in number of pixels, was established, for control, 0.012 µg/ml, and 5 µg/ml. Since the data were not normally distributed, as ascertained by the Kolmogorov-Smirnov test, a non parametric Mann-Whitney U test was performed to compare between doses.
RESULTS AND DISCUSSION

Technical implications. Here we present an adapted single-cell diffusion assay, initially used to identify DNA fragmentation in mammalian sperm cells (13, 17, 30), for assessing chromosomal DNA integrity in microorganisms, with relatively small genomes. Intact unfixed microorganisms were immersed in an agarose microgel on a slide, lysed, and stained with a DNA fluorochrome. In higher eukaryotic cells, cells without DNA fragmentation only release DNA loops around a central core, but cells with fragmented DNA produce a large halo of diffusion of DNA spots or fragments.

Given the relatively small genome size of microorganisms, classical fluorochromes such as propidium iodide, DAPI, Hoechst, etc., are not suitable for staining. In order to visualize DNA fragments, it is necessary to use a highly sensitive fluorochrome such as one from the SYBR family. SYBR Gold provides excellent sensitivity and photostability in comparison with other fluorochromes from the same family, giving an accurate visual assessment under the fluorescence microscope (7). Antifading solution was not used since it diminishes the contrast between the small DNA dots and the background.

Validation of the assay: ciprofloxacin treatment and DBD-FISH. Ciprofloxacin is a fluoroquinone that induces DNA double-strand breaks by trapping DNA gyrase and topoisomerase IV on DNA (19). DNA breaks have been shown by several methodologies including viscosity measurements of cell lysates (24, 25). After processing with the Micro-Halomax® kit and SYBR Gold staining, bacteria from untreated control cultures showed nucleoids with DNA loops spreading from a central
core, which corresponds to the residual bacterium, with a compact, microgranular surface, extended peripherally to may branches (Fig. 1a, b). Remarkably, a few bacteria, 0.4%, spontaneously had a very big halo of DNA spots radiating from the residual central core (Fig. 1a, 5a). These images were similar to those visualized in higher eukaryotic cells with fragmented DNA, with the diffusion-based assay (13, 17, 30, 36). After treatment with 5 µg/ml ciprofloxacin, all the nucleoids appeared similar to nucleoids with extensive diffusion of DNA spots as observed occasionally in the control cultures. Thus, ciprofloxacin demonstrates that 1) our procedure confidently detects DNA fragmentation, and that 2) images of nucleoids with a big halo of diffusion of DNA spots indicate extremely fragmented DNA. Quantitative analysis of digital images revealed that the mean surface of the nucleoids was 11.5-fold higher than of the untreated control bacteria (Fig. 1d; Table 2).

Interestingly, the effect of ciprofloxacin on DNA integrity was increased with respect to untreated control cultures, at the minimum inhibitory concentration (MIC), i.e., 0.012 µg/ml (Fig. 1c). After this low dose, the DNA damage was less and was constant among the bacteria. In fact, the nucleoids appeared more spread, with their average surface being 3.9-fold higher than the untreated control cells (Table 2) and having larger peripheral DNA fragments than after the high dose. As postulated by Drlica et al. (11), ciprofloxacin at low doses like MIC and short incubation times may block growth without killing the cells, suggesting the formation of reversible complexes. At higher doses, like 5 µg/ml, the DNA is extremely fragmented, as here observed, perhaps causing rapid death. The experiment demonstrates the sensitivity and potential value of our procedure for determining the activity of quinolones, both in basic and in clinical research. This approach is currently under investigation in our laboratory.
To further confirm the presence of DNA breaks in nucleoids with diffused DNA fragments in control *E. coli* cultures, the DBD-FISH technique was employed (14, 15). This procedure uses the same microgel as the diffusion assay, allowing simultaneous or sequential visualization of the nucleoids with or without fragmented DNA and labelling of DNA breaks. DBD-FISH is a powerful procedure that involves microgel-embedding, lysis, and incubation with a limited alkaline DNA unwinding step (3, 32). This final step transforms DNA breaks into limited single-stranded DNA (ssDNA) segments generated from the ends of the breaks, which hybridize to fluorescent DNA probes. As DNA breaks increase, more ssDNA is produced, increasing probe hybridization and fluorescence intensity. Fluorescence may be quantified using image analysis software. When hybridizing with a whole genome probe, DNA breaks in the entire genome are assessed. Damage within specific DNA sequence areas may be evaluated by hybridizing specific DNA probes.

The DBD-FISH procedure was applied to *E. coli* (Fig. 2) and other microorganisms lysed in the microgel. Nucleoids with diffused spots were strongly labelled, further confirming massive DNA breaks.

Three kinds of experiments demonstrated the potential of the procedure to determine chromosomal DNA fragmentation: 1) analysis of cells with spontaneous fragmented DNA in culture, 2) reactive oxygen species (ROS) induced DNA damage, and 3) analysis of antibiotic and antifungal agent effects.
**Batch cultures.** *P. mirabilis* was incubated in liquid LB medium for 106 h, monitoring turbidity and removing aliquots periodically to determine membrane permeability and DNA fragmentation. Bacteria with fragmented DNA were easily separated from nucleoids without fragmented DNA (Fig. 3a). The frequency of bacteria with DNA fragmentation was established using the Micro-Halomax® kit. This evaluated nucleoids with diffused DNA fragments in 1000-5000 cells per experimental point. Membrane permeability was determined by SYBR Green II and propidium iodide staining. All cells were permeable to SYBR Green II, but propidium iodide is a vital dye, so only cells with membrane permeability, which do not exclude the dye, appear red under the propidium iodide filter set of the microscope. The frequency of propidium iodide permeable cells was established in 1000-5000 microorganisms, per experimental point (Fig. 4).

The culture changed from exponentially growing to stationary phase after 9 h. The percentage of cells permeable to propidium iodide increased at 48 h from 0.5% to 5%, and then progressively rose through the end of the experiment to 88%. The proportion of bacteria with fragmented DNA significantly increased after 81 h from 0.5-1% to 9.5%. It remained constant at 35% from 99 to 103 h and then rose to 52 % 3 h later.

These results suggest that membrane permeability does not indicate the presence of fragmented DNA, being independent parameters related to different initial targets and not correlated in time. Moreover, the stationary phase seems not to be steady in the frequency of bacteria with fragmented DNA. In the initial period of the stationary phase, the proportion of bacteria with DNA fragmentation did not increase over the exponential phase. The percentage increased later, probably reflecting a progressive...
change in the turnover rate between dead and dividing cells. Perhaps with the accumulation of metabolites and the depletion of nutrients, the fraction of cells with fragmented DNA should further increase.

**Hydrogen peroxide treatment.** The effect of ROS on DNA integrity was evaluated. Hydrogen peroxide decomposes into hydroxyl radicals (OH) through catalysis by low valence transition metal ions in a Fenton-Haber-Weiss reaction. These oxidizing agents strongly reacted with macromolecules. OH attack on DNA results in a variety of base damages and DNA breaks (8, 9). In the only report using the TUNEL assay in bacteria (31), labelling of DNA breaks was detected in exponentially growing cultures of *E. coli*, after exposure to extremely high doses of H$_2$O$_2$ (86 mM for 30 min). Surprisingly, in stationary phase cultures, even doubling the H$_2$O$_2$ dose (172 mM) did not result in DNA breakage. This suggested that H$_2$O$_2$ does not directly cause DNA breaks, which could be transient intermediates in DNA repair produced by the DNA repair enzymes (31).

We tested this hypothesis using our DNA fragmentation assay. The percentage of *E. coli* cells with fragmented DNA was 0.4% and 37.6% in untreated control cells, growing exponentially or in stationary phase, respectively (Fig. 5a, c). Using a lower dose for a shorter time than in the previous report (10mM, 10 min), 100% of nucleoids showed extensively fragmented DNA, either in exponential or in stationary growth phase (Fig 5b, d). This result suggests a higher sensitivity in the microgel-based assay compared with TUNEL in bacteria and illustrates a difficulty with enzymatic procedures for labelling DNA breaks. In the case of TdT, a free 3’-OH group at the terminus of the DNA break is essential as substrate in order to polymerize the nucleotides (10). Attack by agents like H$_2$O$_2$ does not produce “clean” DNA termini, but
rather chemically modified ends, such that direct DNA breaks could be undetectable to enzymes (9). To allow for DNA repair, Exonuclease III removes blocking groups at the 3’ terminus (9). Labelling by TdT should therefore be possible. The absence of enzymatic labelling in stationary phase cultures could be explained if end-processing is impaired at this stage. Nevertheless, H$_2$O$_2$-induced DNA breaks are visible with our assay, since it is independent on the chemical nature of the DNA break. Overall, our diffusion assay identifies DNA damage by OH, both in exponentially and stationary growth phases.

**Ampicillin incubation.** To evaluate the influence of ampicillin treatment on chromosomal DNA, exponentially growing cultures of TG1 were exposed to 300 µg/ml ampicillin for 40 min or 24 h. This dose was much higher than the MIC of 3 µg/ml. In contrast to ciprofloxacin, that affects DNA, the cell wall is the primary target for ampicillin; it inhibits peptidoglycan synthesis after binding to penicillin binding proteins and activating autolysins (5, 16).

Contrary to ciprofloxacin, 40 min treatment with ampicillin barely increased the frequency of cells with fragmented DNA or with appearance of DNA damage. The nucleoids were similar to those from untreated control cells. When incubated for 24 h, the density of bacteria, and correspondent nucleoids, was reduced, but had a uniform background of DNA spots that were probably from spontaneously lysed cells that released the DNA fragments to the medium (Fig. 6). This suggests that cell death initially appears to be independent on DNA damage, but may evolve late in DNA degradation.
Amphotericin B incubation in yeast. A presumed PCD has been described in *S. cerevisiae*, following acidic, oxidative, or osmotic stress, and after ultraviolet exposure (23). This has also been reported for *Candida albicans* after acetic acid, H$_2$O$_2$, or amphotericin B treatment (28, 29). Apoptotic cells were very significantly increased after 200 min incubation with 4 µg/ml amphotericin B (28). Apoptotic cells were considered those not growing and not permeable to propidium iodide. DNA fragmentation was not assessed. After a dose of 16 µg/ml, practically all *Candida* cells did not grow, appearing 10% propidium iodide impermeable that were assumed apoptotic, whereas the propidium iodide permeable cells were presumed necrotic.

An image of *C. albicans* showing DNA fragmentation is presented in figure 3b. Nevertheless, we assessed the possible induction of DNA fragmentation by amphotericin B in *S. cerevisiae*. In untreated control cultures, no cells with fragmented DNA were detected in 6000 yeasts. There was no evidence of fragmented DNA with any dose of the antifungal agent when incubated for 3 h. After 24 h incubation with amphotericin B, yeast cells with fragmented DNA were recorded in a dose-dependence manner (Fig. 7). The frequency of cells with fragmented DNA was lower than that with propidium iodide permeable membrane. In fact, with the highest dose assayed, 5% of the cells contained fragmented DNA, whereas 85% were propidium iodide permeable.

This decouples membrane permeability from DNA fragmentation as being indicative of death in these microorganisms, at least after treatment with antifungal agents that target to yeast cell membrane, like amphotericin B (4). A substantial and proportional decrease in viability with dose, assayed 48 h after treatment, was demonstrated (Fig. 7), suggesting that DNA fragmentation after amphotericin B treatment is either a rare phenomenon or a late response.
Conclusion. The experiments presented here illustrate the ability of the technique, assembled as a kit, to determine the presence of fragmented DNA in microorganisms. Its simplicity, short assay time (50 min) and efficacy, makes this technique useful for the routine determination of DNA fragmentation and intercellular variation. Applications may be extensive in both basic and clinical research. Only a fluorescence microscope is required. Though direct visual identification is quite sharp, the scoring process may be partially automated by adapted image analysis software. This automation could be more complete by integrating a microscope with a motorized plate and focus, a CCD camera for image capture, and image analysis software. This could be useful when scoring many thousands of microorganisms, resembling the flow-cytometer facilities.

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FIGURES

Figure 1. Images after application of the Micro-Halomax® kit to *E. coli* cultures. Cells were embedded in an agarose microgel, lysed, and stained with SYBR Gold. a: Nucleoids from control untreated cells, showing spread of DNA loops from a central core. One has highly diffused DNA spots (asterisk). b: A detailed image of an undamaged nucleoid, showing an uneven microgranular surface and multibranched appearance. c: Ciprofloxacin treatment at the MIC dose, 0.012 µg/ml, for 40 min, resulted in DNA fragments. d: These fragments increased after 5 µg/ml treatment; nucleoids showing a big halo of diffusion of DNA spots, similar to the image marked by asterisk in (a). Bar: 5 µm in a, c, d; 2.5 µm in b.

Figure 2. DBD-FISH detected DNA breaks in nucleoids from control cultures of *E. coli*. Cells in an agarose microgel were lysed and treated with an alkaline unwinding treatment to transform DNA breaks into restricted single-stranded DNA motifs detected by hybridization with a whole genome probe, Cy-3 labeled (red). The central nucleoid with diffused DNA spots appears intensely labelled, confirming the presence of spontaneous massive DNA breaks (asterisk). Nucleoids without fragmented DNA were visible with DAPI (blue). Bar: 5 µm.

Figure 3. Images after the application of Micro-Halomax® to cultures of *P. mirabilis* (a) and *C. albicans* (b), processed as indicated in the Materials and Methods. Nucleoids without DNA fragmentation released DNA loops around a central core from the residual cell. Otherwise, nucleoids with fragmented DNA were clearly identified by a big halo of DNA spots (asterisks). Bar: 5 µm.
**Figure 4.** Kinetics of the frequency of *P. mirabilis* cells with fragmented DNA and with propidium iodide permeable membrane. Identification of bacteria with fragmented DNA was performed using the Micro-Halomax® kit, as indicated in the Materials and Methods.

**Figure 5.** Exponentially growing cultures from *E. coli* control cells (a) and exposed to 10mM hydrogen peroxide for 10 min (b), evaluated with the diffusion-based assay, using the Micro-Halomax® kit. a: bacterial nucleoids from control cultures only show spreading of DNA loops. Some nucleoids had a big halo of diffused DNA spots, as indicated in the image (asterisk). b: all nucleoids observed after H$_2$O$_2$ treatment reveal a halo of DNA spots. In stationary phase cultures, untreated cells (c) showed similar images to (a), with a higher proportion of background nucleoids with diffused DNA spots (asterisks), whereas those treated with H$_2$O$_2$ (d) were similar to (b). Bar: 5 µm.

**Figure 6.** *E. coli* cultures processed with the Micro-Halomax® kit after ampicillin treatment, 300 µg/ml, 24 h. Nucleoids from residual cells appear more relaxed, accompanied by a dense background of DNA fragments. Bar: 5 µm.

**Figure 7.** *S. cerevisiae* cells with fragmented DNA (right scale), propidium iodide (PI) permeable membrane, and viability (left scale), after incubation for 24 h with increasing doses of amphotericin B.
REFERENCES


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DNA damage induced by ionizing radiation and hydrogen peroxide in CHO cells. Int. J.

remove 3’ blocks from DNA synthesis primers in H2O2-damaged Escherichia coli.


<table>
<thead>
<tr>
<th>Gram Negative Bacteria</th>
<th>Gram Positive Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td><em>Enterococcus faecalis</em></td>
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<tr>
<td><em>Enterobacter cloacae</em></td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td><em>Streptococcus agalactiae</em></td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td><em>Streptococcus pyogenes</em></td>
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<tr>
<td><em>Salmonella spp.</em></td>
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</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
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<td><em>Acinetobacter haemolyticus</em></td>
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<td><em>Acinetobacter baumannii</em></td>
<td></td>
</tr>
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<td><em>Klebsiella oxytoca</em></td>
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<tr>
<td><em>Klebsiella pneumoniae</em></td>
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<tr>
<td><em>Citrobacter freundii</em></td>
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</tbody>
</table>

**Table 1.** Bacteria assessed with the Micro-Halomax® kit.
<table>
<thead>
<tr>
<th>Dose (µg/ml)</th>
<th>n</th>
<th>Surface Area (mean ± SD)</th>
<th>Relative Increase in Surface</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>150</td>
<td>7411.9 ± 2173.8</td>
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<tr>
<td>0.012</td>
<td>145</td>
<td>29198.8 ± 12747.9</td>
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</tr>
<tr>
<td>5.0</td>
<td>146</td>
<td>85156.8 ± 19301.1</td>
<td>11.5</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 2. Data of digital image analysis of nucleoids from *E. coli* treated with ciprofloxacin, 40 min, in LB broth, and processed with the Micro-Halomax® kit (n = number of nucleoids examined; surface area is in number of pixels; P = significance level obtained with Mann-Whitney U test).