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Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells

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Abstract

The differentiation between live and dead bacterial cells presents an important challenge in many microbiological applications. Due to the persistence of DNA in the environment after cells have lost viability, DNA-based detection methods cannot differentiate whether positive signals originate from live or dead bacterial targets. We present here a novel chemical, propidium monoazide (PMA), that (like propidium iodide) is highly selective in penetrating only into 'dead' bacterial cells with compromised membrane integrity but not into live cells with intact cell membranes/cell walls. Upon intercalation in the DNA of dead cells, the photo-inducible azide group allows PMA to be covalently cross-linked by exposure to bright light. This process renders the DNA insoluble and results in its loss during subsequent genomic DNA extraction. Subjecting a bacterial population comprised of both live and dead cells to PMA treatment thus results in selective removal of DNA from dead cells. We provide evidence that this chemical can be applied to a wide range of species across the bacterial kingdom presenting a major advantage over ethidium monoazide (EMA). The general application of EMA is hampered by the fact that the chemical can also penetrate live cells of some bacterial species. Transport pumps actively export EMA out of metabolically active cells, but the remaining EMA level can lead to substantial loss of DNA. The higher charge of PMA might be the reason for the higher impermeability through intact cell membranes, thus avoiding DNA loss.

Keywords: Differentiation live vs.dead; Bacterial pathogens; Quantitative PCR

1. Introduction

Differentiation of live and dead cells is an important challenge in microbial diagnostics. Metabolic and reproductive activity and, in the case of pathogenic microorganisms, the potential health risk are limited to the live portion of a mixed microbial population. Due to the relatively long persistence of DNA after cell death in the range between days to 3 weeks (Josephson et al., 1993; Masters et al., 1994), DNA-based diagnostics tend to overestimate the number of live cells.

The most important criterion for distinguishing between viable and irreversibly damaged cells is membrane integrity. Sorting out noise derived from membrane-compromised cells helps to assign metabolic

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activities and health risks to the intact and viable portion of bacterial communities. Live cells with intact membranes are distinguished by their ability to exclude DNA-binding dyes that easily penetrate dead or membrane-compromised cells. This principle is routinely applied for microscopic live–dead discrimination and increasingly in flow cytometry (Nebe-von-Caron et al., 2000,1998; Shapiro and Nebe-von-Caron, 2004). The most common membrane-impermeant dye is propidium iodide (PI).

In the last few years, EMA-PCR was reported to be an easy-to-use alternative to microscopic or flow cytometric distinction between live and dead cells (Nogva et al., 2003; Rudi et al., 2005a,b; Wang and Levin, 2006). This diagnostic DNA-based method combines the use of a live–dead discriminating dye with the speed and sensitivity of real-time PCR. Ethidium monoazide (EMA) is a DNA-intercalating dye with the azide group allowing covalent binding of the chemical to DNA upon exposure to bright visible light (maximum absorbance at 460 nm). Cells are exposed to EMA for 5 min allowing the dye to penetrate dead cells with compromised cell walls/membranes and to bind to their DNA. Photolysis of EMA using bright visible light produces a nitrene that can form a covalent link to DNA and other molecules (Coffman et al., 1982; DeTraglia et al., 1978; Hixon et al., 1975). Photo-induced cross-linking was reported to inhibit PCR amplification of DNA from dead cells. In a recent publication, it was shown that EMA cross-linking to DNA actually rendered the DNA insoluble and led to its loss together with cells debris during genomic DNA extraction (Nocker and Camper, 2006). The unbound EMA, which remains free in solution, is simultaneously inactivated by reacting with water molecules (DeTraglia et al., 1978). The resulting hydroxylamine is no longer capable of covalently binding to DNA (Kell et al., 1998). DNA from viable cells, protected from reactive EMA before light exposure by an intact cell membrane/cell wall, is therefore not affected by the inactivated EMA after cell lysis. EMA treatment of bacterial cultures comprised of a mixture of viable and dead cells thus leads to selective removal of DNA from dead cells. The species tested were *Escherichia coli* 0157:H7 (Nogva et al., 2003), *Salmonella typhimurium* (Nogva et al., 2003), *Listeria monocytogenes* (Nogva et al., 2003; Rudi et al., 2005a,b), *Campylobacter jejuni* (Rudi et al., 2005a) and *Vibrio vulnificus* (Wang and Levin, 2006).

Though this technique is promising, the use of EMA prior DNA extraction was found to suffer from a major drawback. In the case of *E. coli* 0157:H7, though the entire genomic DNA from dead cells was removed, the treatment also resulted in loss of approximately 60% of the genomic DNA of viable cells harvested in log phase (Nocker and Camper, 2006). We observed in this study that EMA also readily penetrates viable cells of other bacterial species resulting in partial DNA loss. The lack of selectivity and of overall applicability made us test a newly developed alternative chemical: propidium monoazide (PMA). PMA is identical to PI except that the additional presence of an azide group allows cross-linkage to DNA upon light exposure. As PI is highly membrane impermeant and generally excluded from viable cells, it has been extensively used to identify dead cells in mixed populations. Upon penetrating compromised cell membranes, PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye molecule per 4–5 base pairs of DNA (Waring, 1965).

The higher charge of the PMA molecule (two positive charges compared to only one in the case of EMA) and the fact that selective staining of nonviable cells with propidium iodide has been successfully performed on a wide variety of cell types gave us confidence that the use of PMA might mitigate the drawbacks observed with EMA.

In this study, we examined the suitability of PMA to selectively remove genomic DNA of dead cells from bacterial cultures with defined portions of live and dead cells. Because this is a newly developed molecule, optimization of the method was necessary. Photo exposure time for DNA binding and simultaneous inactivation of free unbound PMA was optimized using purified DNA. PMA concentration and incubation time were further optimized with one gram-negative and one gram-positive organism before applying these parameters to the study of a broad spectrum of different bacterial species and comparing the new chemical with ethidium monoazide.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used for this study comprise four gram-negative and five gram-positive species and are listed with their corresponding media and growth temperatures in Table 1. Single colonies from agar streak plates were typically transferred to 50 ml culture tubes containing about 10 ml of the corresponding medium. The cultures were grown to log phase in a shaker at 180 rpm at the given growth temperature. For experiments involving quantitative qPCR, optical densities of *E. coli* 0157:H7 cultures were adjusted to an OD_{600} of 1 by dilution with LB broth.

Table 1
Bacterial species and growth conditions

Species	Growth medium	Growth temperature (°C)	Strain/origin
Escherichia coli 0157:H7	Luria Bertani	37	Strain 932
Listeria monocytogenes	Brain Heart Infusion	30	MSU Microbiology Dept.
Micrococcus luteus	Brain Heart Infusion	30	MSU Microbiology Dept.
Mycobacterium avium	7H9 broth-OADC	37	Strain W2001
Pseudomonas syringae	King's B	22	Strain CC94, Tarn-et-Garonee/ France (Morris et al., 2000)
Salmonella typhimurium	Luria Bertani	30	MSU Microbiology Dept.
Serratia marcescens	Brain Heart Infusion	25	MSU Microbiology Dept.
Staphylococcus aureus	Tryptic Soy Broth	37	ATCC 25923

2.2. Killing conditions

Cells in 500 µl aliquots were killed by exposure to isopropanol (final concentration 70%) for 10 min. Isopropanol was removed by harvesting cells using centrifugation at 5000×*g* for 5 min prior to resuspension in 500 µl of the corresponding medium. As an alternative to isopropanol treatment, *Mycobacterium avium* and *Micrococcus luteus* cells were killed by heating for 15 min to 85 °C and 72 °C, respectively. To study the effect of PMA on defined ratios of viable and dead cells (Fig. 4), *E. coli* 0157:H7 was heat-killed by exposure to 72 °C for 15 min prior to mixing at defined ratios with untreated cells. Loss of viability was examined by streaking 5 µl of cell suspension on the corresponding agar plates followed by incubation at the optimal growth temperature.

2.3. DNA isolation and quantification

Genomic DNA was extracted using the FastDNA® SPIN Kit for soil (MP Biomedicals, Irvine, CA, USA) following the manufacturer's instructions except from extending the centrifugation time for removal of cell debris to 5 min (instead of 30 s). Cell lysis of pure cultures was achieved by bead beating using a FastPrep machine (MP Biomedicals) for 25 s at a speed setting of 4.5 m/s. DNA was quantified using the PicoGreen quantification solution (Molecular Probes Inc., Eugene, OR, USA) and a TBS-380 fluorometer (Turner BioSystems Inc., Sunnyvale, CA, USA) using genomic DNA from *E. coli* O157:H7 as a standard (with a mid-range GC content). The quantification results obtained with the fluorometer were compared with band intensities of high molecular weight genomic DNA visualized on ethidium bromide stained 1% agarose gels. Seven to ten percent of the total corresponding eluate volumes were loaded on the gels.

2.4. PMA treatment of isolated genomic DNA

Genomic DNA extracted from *E. coli* 0157:H7 was diluted with water to final concentrations of 1 ng/µl and 50 ng/µl DNA. In an initial set of experiments, PMA (phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio)propyl]-6-phenyl dichloride; Biotium, Inc., Hayward, California, dissolved in 20% DMSO) was added to 50 µl of the 1 ng/µl DNA solution to reach final concentrations of 3 and 30 µM followed by light exposure for increasing time periods (Fig. 1). Light exposure was performed using a 650-W halogen light source placed 20 cm from the sample tubes. During exposure, samples were placed on ice to avoid excessive heating. In a second experiment, 49 µl of 3 and 30 µM PMA solutions were light exposed for increasing time periods prior to addition of 1 µl of a 50 ng/µl DNA solution. After addition of DNA, the mixtures were light exposed for another minute to achieve cross-linking of remaining active PMA to the DNA (Fig. 2). In both experiments, 1 µl of the DNA solutions was used as

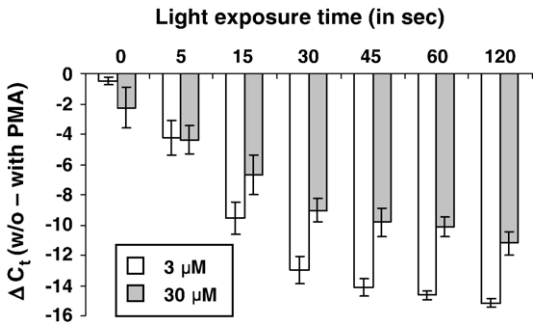


Fig. 1. Effect of increasing light exposure time on inhibition of PCR amplification by PMA. PMA was added at concentrations of 3 and 30 µM to 1 ng/µl genomic DNA extracted from *E. coli* 0157:H7, followed by light exposure up to 2 min. Signal reduction was determined by qPCR detecting relative differences in amplifiable *stx1* gene copies. C_t values derived from PMA-treated samples were subtracted from the corresponding C_t values from identical non-PMA-treated samples. Error bars represent standard deviations from three independent replicates.

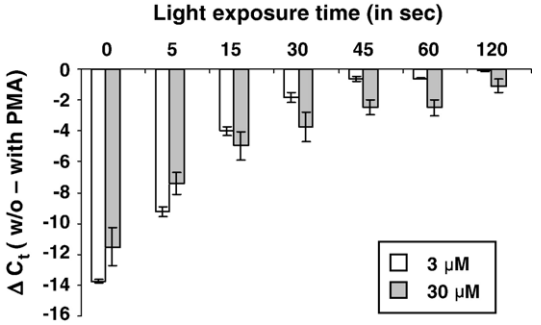


Fig. 2. Effect of increasing light exposure time on PMA inactivation. PMA at concentrations of 3 and 30 μM was light exposed up to 2 min before addition of genomic *E. coli* 0157:H7 DNA (final concentration 1 ng/μl). After mixing, cross-linkage of remaining active PMA to DNA was achieved with an additional light exposure of 1 min. Signal reduction was determined by qPCR detecting relative differences in amplifiable *stx1* gene copies. C_t values derived from PMA-treated samples were subtracted from the corresponding C_t values from identical non-PMA-treated samples. Error bars represent standard deviations from three independent replicates.

template for quantitative PCR (qPCR) using primers and a probe targeting the *stx*1 gene (see below).

2.5. PMA and EMA cross-linking working with bacterial cultures

PMA and EMA (phenanthridium, 3-amino-8-azido-5-ethyl-6-phenyl bromide; Biotium, Inc., Hayward, California) were dissolved in 20% DMSO to create a stock concentration of 20 mM or dilutions of that and stored at −20 °C in the dark. PMA was added to 500 μl culture aliquots to final concentrations of 3, 30 or 240 μM. The DMSO content of PMA dilutions was chosen in a way that the final DMSO concentrations were identical for the exposed cells. Following an incubation period from 1 to 15 min in the dark with occasional mixing, samples were light-exposed for 5 s to 2 min using a 650-W halogen light source placed 20 cm from the sample tubes. During exposure, samples were placed on ice to avoid excessive heating. After photo-induced cross-linking, cells were pelleted at 5000 g for 5 min prior to DNA isolation. After optimization, experiments were completed, PMA was added to a final concentration of 50 μM and incubated for 5 min prior to light exposure for 2 min. The same concentration (50 μM) and procedure was applied to experiments using EMA to make results comparable with the PMA experiments.

2.6. Quantitative PCR

For relative quantification of DNA extracted from *E. coli* 0157:H7 cultures, the *stx1* gene coding for the Shiga-like toxin 1 was used as a genetic target. qPCR was performed in a total volume of 25 μl containing 1 μl extracted genomic DNA and final concentrations of 1× AmpliTaq GOLD buffer (Applied Biosystems, Foster City, CA, USA), 5.5 mM $MgCl_2$, 0.3 μM of primer stx1-forward (5′-GACTGCAAAGACGTATGTAGATTCG-3′; Sharma and Dean-Nystrom, 2003), 0.3 μM of primer stx1-reverse (5′-ATCTATCCCTCTGACATCAACTGC-3′; Sharma and Dean-Nystrom, 2003), 0.15 μM of the stx1-probe (5′-TGAATGTCATTCGCTCTG CAATAGGTACTC-3′; Sharma and Dean-Nystrom, 2003) and 2.5 U of AmpliTaq Gold (Applied Biosystems). The stx1-probe had 6-FAM as the 5′-reporter and BHQ1 as the 3′-quencher. The cycling parameters were: 8 min at 95 °C followed by 45 cycles of 20 s at 95 °C, 30 s at 55 °C and 25 s at 72 °C.

qPCR and data analysis were performed with a SmartCycler II (Cepheid, Sunnyvale, CA). Cycle threshold (C_t) values were automatically calculated by the SmartCycler software using the second derivative method.

2.7. Statistical analysis

Error bars in diagrams represent standard deviations from three independent replicas.

2.8. Fluorescence microscopy

Cells were stained for microscopy by adding 1.25 μl of the dye SYTO9 (3.34 mM in DMSO; Molecular Probes) in combination with 1.25 μl of either PMA or EMA (both 20 mM in 20% DMSO; Biotium Inc.) to 500 μl culture aliquots. SYTO9 generally stains all bacteria in a population green, while PMA and EMA stains them red if the dyes can penetrate the cell walls/membranes. After mixing, samples were mounted on microscope slides. Five minutes after addition of the dyes, photomicrographs were taken on a Nikon E800 microscope using a 100 × 1.4 NA oil objective and FITC and TRITC fluorescence filter sets (ex480/30, DM505, em535/40 and ex546/10, DM575, em590, respectively). The software used for visualization was MetaVue version 6.1 (Universal Imaging, Downington, PA, USA).

3. Results

3.1. Optimization of PMA/DNA cross-linking using pure DNA

PMA was added to a solution of genomic DNA extracted from *E. coli* 0157:H7 followed by light exposure for increasing time periods ranging from 0 s to 120 s. 1 μl

of the 1 ng/μl DNA solution was used as template for quantitative PCR (qPCR) using primers and a probe targeting the *stx1* gene. Increasing light exposure time led to higher threshold cycles (C_t values), meaning more cycles were necessary for detecting a signal above the background. This increase in C_t values indicated increasing inhibition of PCR amplification. Fig. 1 illustrates the signal reduction of PMA-treated DNA as a function of light exposure time compared to the amplification of non-treated DNA. Following a strong initial increase in signal reduction, the inhibition curve flattened off with longer exposure times. Identical tendencies were observed for PMA concentrations of both 3 and 30 μM. The extent of inhibition with different PMA concentrations varied with the Mg^{2+} concentrations used in the PCR reaction (data not shown). PMA concentrations of 60 μM and higher resulted in complete inhibition of the PCR reaction independent of the light exposure time (data not shown).

The efficiency of photolysis (i.e. the inactivation of free PMA not bound to DNA) was examined by light-exposing PMA solutions of 3 and 30 μM for increasing time periods prior to addition of DNA. After addition of DNA, the mixture was light exposed for another minute to achieve cross-linking of remaining active PMA to the DNA followed by qPCR. Increasing inactivation times of free PMA resulted in decreasing signal reduction relative to the identical samples without PMA (Fig. 2). In the case of 3 μM, an inactivation period of 120 s diminished the difference between the PMA and the non-PMA containing samples.

Considering the above results, a 120 s light exposure time was chosen to guarantee efficient binding of PMA to DNA and at the same time to achieve efficient inactivation of free PMA that did not bind to DNA. The latter is important when treating cells with PMA to make sure that no active PMA remains in solution, which could bind to DNA originating from viable cells after the cell lysis step.

3.2. Optimization of the PMA assay using cells

E. coli 0157:H7 (as a representative gram-negative bacterium) and *Streptococcus sobrinus* (as a representative gram-positive bacterium) were chosen as model organisms to optimize the PMA concentration and incubation time for treating cells. Log-phase cultures were split, with one-half being subjected to 70% isopropanol resulting in complete loss of culturability. Killing efficacy was examined by plating on the appropriate medium together with a viable positive control. PMA was added to final concentrations of 3, 30 or 240 μM. Whereas 30 μM corresponds to the PI concentration used in the BacLight live–dead staining kit (Molecular Probes), 240 μM equals the concentration of EMA used in previous studies (Nocker and Camper, 2006; Nogva et al., 2003; Rudi et al., 2005a,b). Fig. 3A and B shows the DNA yield of PMA-treated culture aliquots in relation to the DNA yield from the corresponding untreated live or dead aliquots. Whereas 30 and 240 μM efficiently removed the DNA from dead cells, there was still a significant DNA background with 3 μM. The incubation time (1, 5 or 15 min) did not affect the genomic DNA yield to a great extent, although 15 min seemed to result in a moderate DNA loss in the case of exposing live *E. coli* 0157:H7 to 240 μM PMA. For the following experiments, a PMA concentration of 50 μM and an incubation time of 5 min were chosen. This concentration was considered sufficient not only for bacterial suspensions used in this study, but also for cellular aggregates and in biofilms while avoiding potential DNA loss due to excessive PMA concentrations.

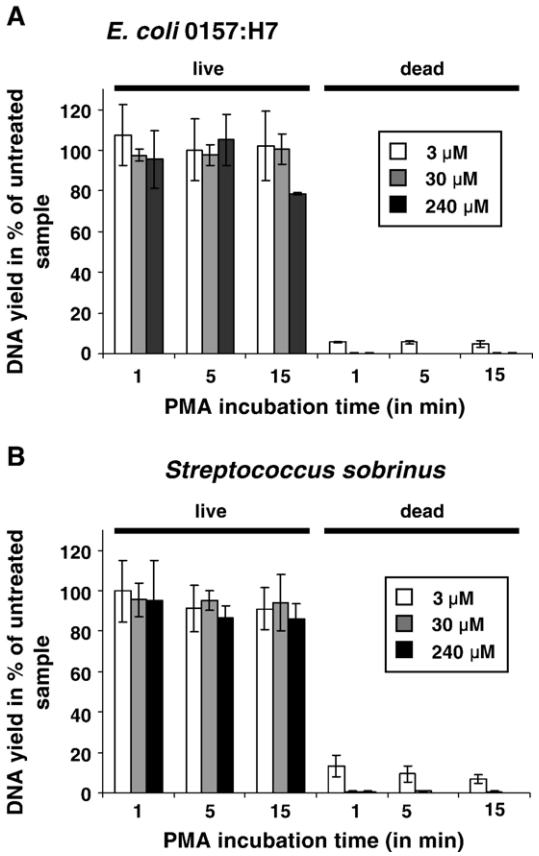


Fig. 3. Effect of increasing PMA incubation times and different PMA concentrations on the genomic DNA yield of live and isopropanol-killed *E. coli* 0157:H7 (A) and *Streptococcus sobrinus* (B). Cells were exposed to PMA at concentrations of 3, 30 and 240 μM for 1, 5 or 15 min. The genomic DNA yield was expressed as a percentage of the corresponding non-PMA-treated samples (live or dead). Error bars represent standard deviations from three independent replicates.

3.3. Effect of PMA on defined ratios of viable and dead cells

To elucidate the relationship between the proportion of viable cells, the DNA yield and the qPCR signals after PMA treatment, mixtures with defined ratios of viable and dead cells were used. An aliquot of *E. coli* 0157:H7 was subjected to heat treatment at 72 °C for 15 min resulting in a decrease in culturable cell counts to zero. Heat-killed cells were mixed with the untreated original culture in defined ratios with culturable viable cells representing 0%, 0.1%, 1%, 10%, 50% and 100% of the total, respectively (Fig. 4A). Whereas the DNA yields from mixtures I to VI were comparable without PMA treatment, increasing proportions of unstressed and viable cells led to a substantial increase in the genomic DNA yield (Fig. 4B and C) and to decreasing C_t values in qPCR (Fig. 4D) after PMA exposure. The highest C_t value, i.e. greatest signal reduction, was observed for sample I containing only heat-killed *E. coli* 0157:H7. A plot of the natural logarithm of DNA yield (in percent of the highest value) versus C_t values revealed a linear correlation with a R^2 value of 0.9742 of the corresponding trendline (Fig. 4E). This suggested that all DNA from live cells seemed to be amplifiable.

3.4. Comparison between PMA and EMA and evaluation of the PMA live/dead assay with more species

The efficient removal of genomic DNA from dead cells by PMA and EMA treatment has been described for *E. coli* 0157:H7 in this and a previous study, respectively (Nocker and Camper, 2006). The difference between the two chemicals lies mainly in their selectivity. Whereas EMA treatment of live *E. coli* 0157:H7 led to a loss of more than 60% of the genomic DNA (10), PMA seems to circumvent this problem. The results were corroborated by microscopic observations using a combination of the dyes SYTO9/PMA or SYTO9/EMA (Fig. 5A). SYTO9 is known to stain all cells green, both with intact and compromised cell membranes. The green color mixes with the red color originating from EMA or PMA when cells take up the corresponding dye. Dead cells stained red with both PMA and EMA. Live cells, in contrast, displayed a difference in that a 5 min exposure with EMA led to red staining, whereas cells were still green after PMA treatment. This suggests that intact *E. coli* 0157:H7 cell membranes can efficiently exclude PMA, but not EMA. This explains the loss of DNA from live cells after EMA treatment, while PMA does not affect the yield.

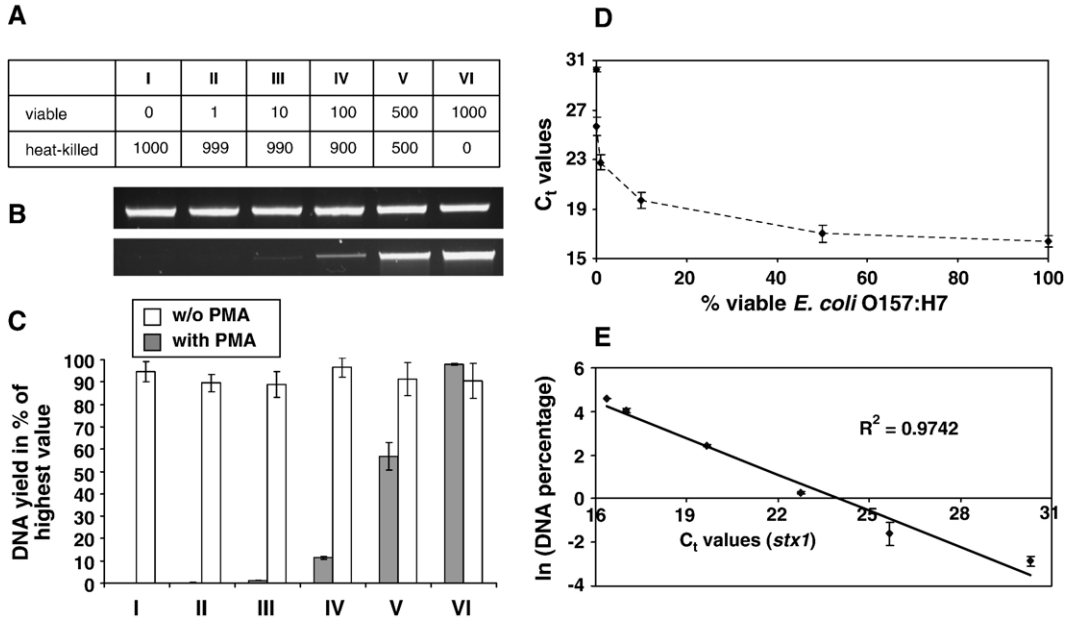


Fig. 4. Effect of PMA treatment on genomic DNA yield and PCR-quantification of defined ratios of viable and heat-killed cells. The error bars represent standard deviations from three independent replicates. (A) Table showing mixing ratios of viable and heat-killed *E. coli* 0157:H7. Numbers represent volumes in microliters. (B) Genomic DNA from non-PMA-treated (top) and PMA-treated (bottom) mixtures visualized on an agarose gel. (C) Genomic DNA yield in percent of the highest value obtained. DNA was quantified using a fluorometer. (D) C_t values of amplified genomic DNA shown as a function of the percentage of viable cells. qPCR was performed using primers specific for the *stx1* gene. (E) Correlation between the natural logarithm of the normalized DNA concentrations and the corresponding C_t values obtained from *stx1* amplification. The R^2 value of the linear trendline is indicated.

To further examine the application of the method to a wider spectrum of bacterial species, we tested the PMA effect (in comparison with EMA) on live and dead cells for the bacteria shown in Table 1. For all species tested, PMA did not affect the DNA yield from live cells, whereas the DNA from dead cells was efficiently removed during the DNA extraction procedure (Fig. 5B). EMA, on the other hand, was equally efficient in removing DNA from dead cells, but also partly reduced the DNA yield from live cells compared to untreated or PMA-treated cells.

Dead cells from all species examined microscopically efficiently stained red with PMA or EMA (data not shown).The images obtained from live cells treated with a combination of SYTO9/PMA or with a combination of SYTO9/EMA showed that PMA did not penetrate live cells in any of the species examined (Fig. 5B). EMA, on the other hand, could partly penetrate both dead and live cells (as seen by the red color) though the staining seemed to be more efficient in the case of dead cells. *Serratia marcescens*, *S. typhimurium* and *Pseudomonas syringae* were the species most resistant to EMA uptake. Live cells of those species stained with SYTO9/EMA appeared green after 5 min.

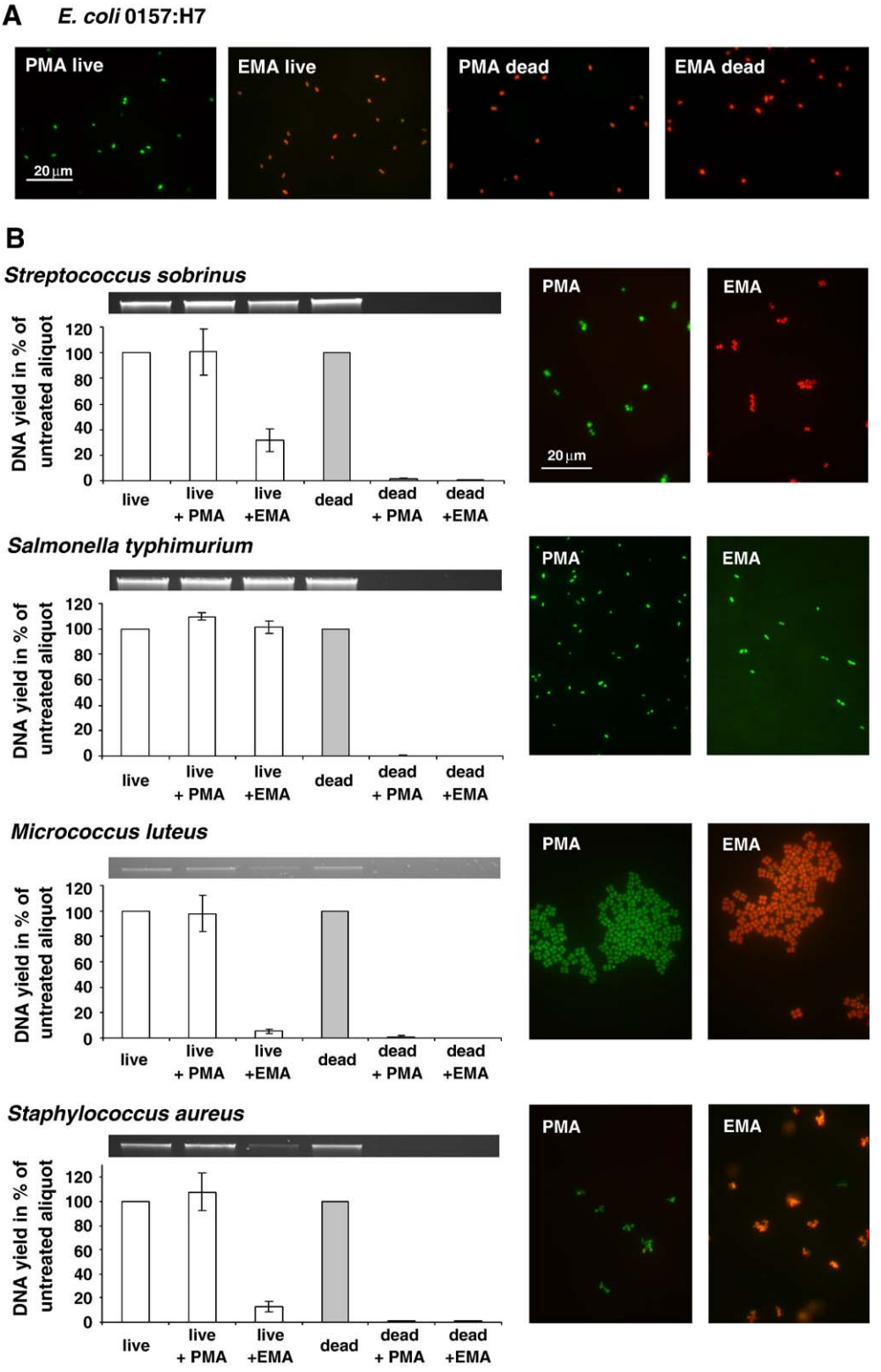
4. Discussion

This study introduces a chemically modified version of propidium iodide with an azide group added to the phenanthridine ring allowing chemical cross-linkage to organic molecules upon short exposure to bright visible light. In analogy to PI, PMA does not seem to penetrate membranes of live cells, whereas it is efficiently taken up by permeabilized cells. Once inside cells, the dye intercalates into double-stranded nucleic acids. Upon binding, photo-induced cross-linkage renders the DNA insoluble and results in its loss during the DNA extraction procedure together with cell debris. In the case of bacterial mixtures containing live and dead cells, the remaining DNA from live cells is amenable to further downstream analyses including quantification, qPCR or presumably microarray analysis. Conclusions from DNA quantifications and hybridization-based assays like microarrays depend on this complete removal of DNA from dead cells.

Although selective analysis of DNA from live cells was first described for EMA using *E. coli* 0157:H7, *S. typhimurium*, *L. monocytogenes*, *Camplylobacter jejuni* and *V. vulnificus* as model organisms (Nogva et al., 2003; Rudi et al., 2005a,b; Wang and Levin, 2006), PMA has the advantage over EMA in terms of increased selectivity. Live cells of the majority of the various bacterial species examined in this study stained red with EMA using an EMA concentration about 4.8-fold below that used in previous studies (Nogva et al., 2003; Rudi et al., 2005a,b). This finding underlines that EMA cannot be considered to be generally membrane impermeable. Although it has been reported to exclusively penetrate only dead or damaged cells (Nogva et al., 2003; Rudi et al., 2005a,b), this may hold true only for selected bacterial species with short exposure times. The data presented here is in agreement with previous results showing that EMA treatment of live *E. coli* 0157:H7 cells leads to a loss of more than 60% of the genomic DNA during the DNA extraction procedure (Nocker and Camper, 2006). Moreover, EMA was recently shown to be unsuitable for live–dead differentiation of *Anoxybacillus* (Rueckert et al., 2005). PCR inhibition was observed after treating viable *Anoxybacillus* with EMA and subsequent DNA extraction indicating that the agent penetrated the membrane of these cells and covalently cross-linked with the DNA during photolysis. We report here, in addition, a significant loss of DNA from live cells for *Staphylococcus aureus*, *L. monocytogenes*, *M. luteus*, *M. avium* and *S. sobrinus* as a result of EMA treatment.

The uptake of EMA by live cells is in agreement with studies examining the uptake of structurally identical ethidium bromide (EB) in wild-type *E. coli*. It was shown that EB is also taken up by viable cells although the net uptake reached only a few percent of that of fully permeabilized cells (Jernaes and Steen, 1994). The reason is seen in the activity of a metabolically driven efflux pump. Metabolic inhibitors were shown to reduce efflux activity. Exposure of cells to cold shock (0° for 30 min) in the presence of TRIS or EDTA led to a net

Fig. 5. Comparison of PMA and EMA membrane permeability characteristics and evaluation of the PMA live/dead assay with nine bacterial species by fluorescence microscopy. (A) Microscopic comparison of dye uptake by live and dead *E. coli* 0157:H7 stained with combinations of SYTO9/PMA and SYTO9/EMA (combined images), respectively. Green color indicates uptake of only SYTO9, red color indicates membrane permeability for PMA or EMA. (B) Effect of PMA and EMA treatment on the relative genomic DNA yield of live (white columns) and dead cells (grey columns) from eight bacterial species and microscopic examination of membrane permeability after staining with either SYTO9/PMA or SYTO9/EMA. DNA yields measured in a TBS-380 fluorometer are shown in relation to the ones from the corresponding non-treated samples (defined as 100%). Genomic DNA was in addition visualized on agarose gels. Dyes were incubated at concentrations of 50 μM (PMA or PMA) and 8.35 μM (SYTO 9) for 5 min in the dark before light exposure for 2 min. Error bars represent standard deviations from three independent replicates.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E. coli 0157:H7
PMA live
EMA live
PMA dead
EMA dead
20 µm
B
Streptococcus sobrinus
Salmonella typhimurium
Micrococcus luteus
Staphylococcus aureus
DNA yield in % of untreated aliquot
0
20
40
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80
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120
live
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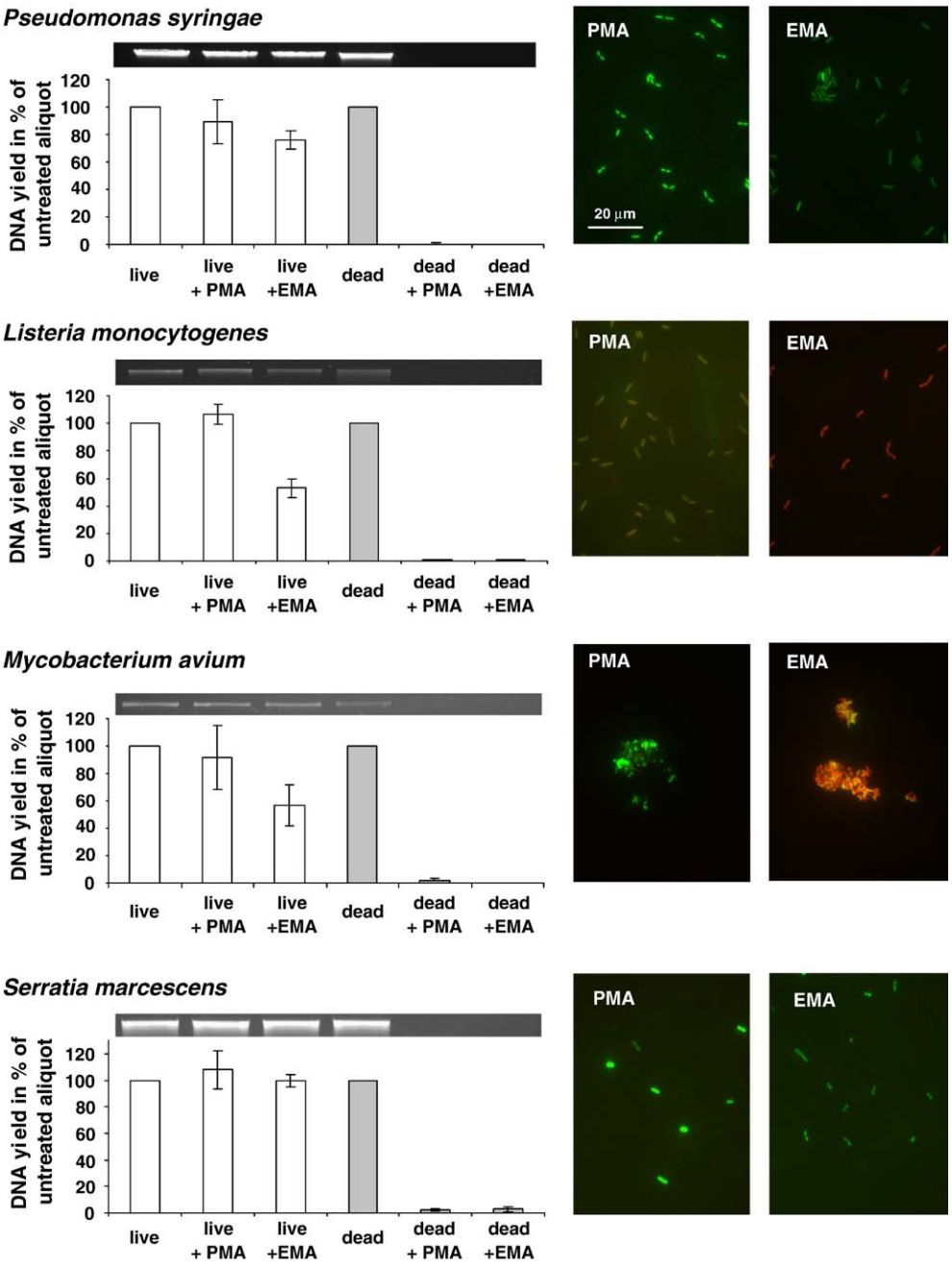

Fig. 5 (*continued*).

uptake of EB similar to that of fully permeabilized cells (Jernaes and Steen, 1994). For this reason, EB is used in flow cytometric studies to identify metabolically active bacteria that are capable of active exclusion of the chemical (Nebe-von-Caron et al., 2000, 1998).

Assuming the same mechanism for EMA, these findings would explain our observation that viable cells from log-phase cultures stained red with EMA. Even with active extrusion of EMA by such export systems, a considerable amount of dye might still remain in the cell. This would explain the significant loss of DNA from EMA-treated live cells compared to untreated or PMA-treated cells. *S. typhimurium* and *S. marcescens* seemed to be the only species of the ones examined that could be

subjected to EMA treatment without any loss of DNA. The reasons for this might be more efficient efflux mechanisms or a less permeable cell membrane. They were also the species most resistant to EMA staining. *P. syringae* seemed to stain very moderately with EMA, correlating with a certain loss of DNA during extraction. The obviously varying membrane permeability of different species, however, impedes a general application of EMA for live–dead differentiation in microbial diagnostics. PMA seems to have the important advantage over EMA of not penetrating live cells. The reason for the significantly higher selectivity of PMA is most probably associated with the higher charge of the molecule (EMA has one positive charge, PMA has two).

Our study suggests that the addition of the azide group to the PI molecule does not change its permeability characteristics. PI is the most commonly used dye for microscopic live–dead discrimination and has been extensively tested with a wide spectrum of bacteria including the ones used in this study. It is widely used in flow cytometry (Nebe-von-Caron et al., 2000, 1998; Shapiro and Nebe-von-Caron, 2004). It is worth mentioning that four physiological states are distinguished using fluorescent stains in flow cytometry: reproductively viable, metabolically active, intact and permeabilized cells (Nebe-von-Caron et al., 2000). Only the latter, the permeabilized cells take up propidium iodide. In addition to not having an intact cell membrane, these cells are depolarized and de-energized. All but the permeabilized cells were shown to be capable of being resuscitated under adequate conditions, whereas cells positive to PI did not recover (Nebe-von-Caron et al., 2000). Excluding the DNA from the latter subpopulation would help limiting the analysis to the cells having the potential of recovery.

Summarizing the above, we present here a novel chemical allowing the removal of genomic DNA from cells with compromised cell membranes. The fast and easy-to-perform pre-treatment of a bacterial population before DNA extraction is compatible with further downstream analyses. Although its application to 'real-world' samples still requires further evaluation, the method could have a great impact on DNA-based diagnostics in various fields, including pathogen diagnostics, bioterrorism and microbial ecology.

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