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Rapid and Reliable Detection of Antimicrobial Peptide Penetration into Gram-Negative Bacteria Based on Fluorescence Quenching[▽]

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In this paper, we describe a rapid flow cytometry method to identify antimicrobial peptides that are internalized into bacterial cells and differentiate them from those that are membrane active. The method was applied to fluorescently labeled $Bac7_{1-35}$ and polymyxin B, whose mechanisms of action are, respectively, based on cell penetration and on membrane binding and permeabilization. Identification of peptides with the former mechanism is of considerable interest for the intracellular delivery of membrane-impermeant drugs.

Antimicrobial peptides (AMPs) are effectors of innate immunity that have evolved different mechanisms to inactivate bacteria (3, 25). Most of them display amphipathic scaffolds that allow interaction with, and damage of membranes (6); others, such as the proline-rich peptides, kill bacteria without lytic effects and through interaction with intracellular targets (15, 16, 22). This mode of action implies that nonlytic AMPs have the capacity to be internalized by cells and are therefore cell-penetrating peptides (CPPs).

Although a vast literature has been produced on CPP entry of and localization in eukaryotic cells (9, 12, 14), little direct evidence of nonlytic peptide internalization into bacteria has been produced so far. The study of localization of AMPs in bacteria has relied on immunoelectron microscopy (16), which is incompatible with high-throughput screening, and on fluorescence confocal microscopy (13, 17), which is not quantitative.

To provide an effective tool to assay the internalization of CPPs in bacteria, here we propose a flow cytometric approach based on fluorescently labeled peptides and the use of the cell-impermeant quencher trypan blue (TB). To this aim, we investigated in parallel the internalization of $Bac7_{1-35}$, a nonlytic proline-rich peptide that penetrates gram-negative bacteria without membrane disruption (4, 7), and of polymyxin B (PMB), a cyclic peptide antibiotic that binds to the bacterial surface, causing membrane permeabilization (5, 18).

An N-terminal active fragment of Bac7, $Bac7_{1-35}$, was synthesized by linking the dipyrromethene boron difluoride (BODIPY) fluorophor to a C-terminal cysteine residue (19), while BODIPY-labeled polymyxin B (PMB-BY) was purchased from Invitrogen. This probe was chosen since it leaves unchanged the net charge of the conjugated molecule, a crucial feature for the antimicrobial properties of the peptides. In addition, it is insensitive to pH and polarity (2), so that its spectral features are not influenced by the environment. The antibacterial activity of $Bac7_{1-35}$-BY and PMB-BY was determined on *Escherichia coli* HB101 and *Salmonella enterica* serovar Typhimurium ATCC 14028 (1). The addition of BODIPY to $Bac7_{1-35}$ did not change its potency, with MICs of 0.5 to 1 μM for both of the bacterial strains. Conversely, the presence of BODIPY caused a fourfold MIC reduction (1 versus 0.25 μM) in the activity of PMB, an effect likely due to the higher BODIPY/PMB size ratio.

We initially checked the effect of the peptides on membrane permeabilization by measuring the percentage of propidium iodide (PI)-positive cells by flow cytometry as previously reported (16). Loss of membrane integrity, in fact, would allow TB to enter bacterial cells, thereby biasing the results. Based on these assays (Fig. 1), $Bac7_{1-35}$-BY and PMB-BY were used at nonpermeabilizing concentrations of, respectively, 0.25 and 0.1 μM.

E. coli cells were then incubated for 10 min with $Bac7_{1-35}$-BY or PMB-BY and analyzed by flow cytometry. Both peptides

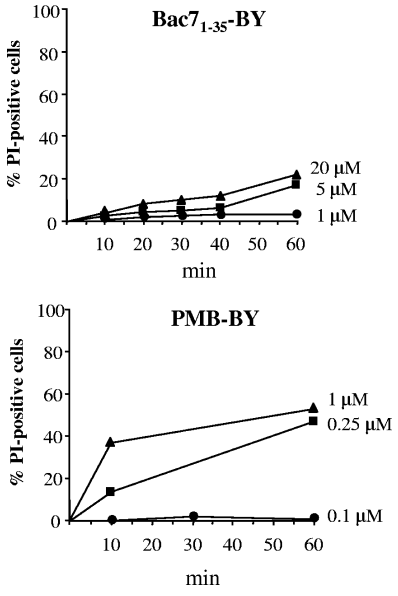

FIG. 1. Flow cytometric evaluation of membrane integrity after treatment with $Bac7_{1-35}$-BY and PMB-BY. Percentage of PI-positive cells after incubation of *E. coli* HB101 (1×10^6 CFU/ml) with 1, 5, or 20 μM $Bac7_{1-35}$-BY or 0.1, 0.25, or 1 μM PMB-BY is shown. The background level of permeabilized cells, obtained by using non-peptide-treated samples, was always lower than 3% and was subtracted from the corresponding peptide-treated sample.

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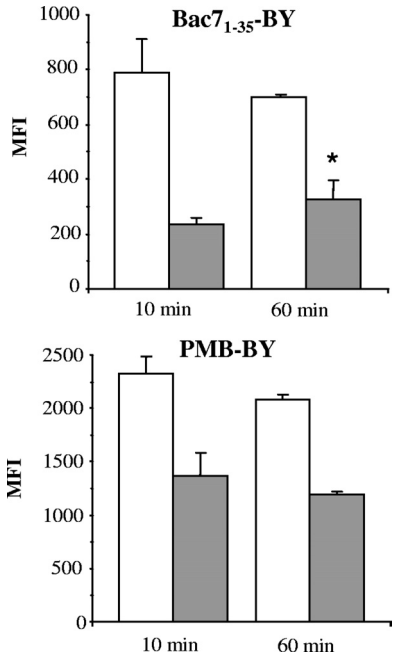

FIG. 2. Binding of Bac7$_{1-35}$-BY and PMB-BY to *E. coli* HB101. Bacterial cells (1×10^6 CFU/ml) were incubated with 0.25 μM Bac7$_{1-35}$-BY or 0.1 μM PMB-BY for 10 or 60 min and then analyzed by flow cytometry without washing (empty histograms) or after washing with high-salt solution (gray filled histograms). Analyses were performed by using a Cytomics FC 500 (Beckman-Coulter, Inc.) equipped with an argon laser (488 nm, 5 mW) and detectors for filtered light set at 525 nm for BODIPY detection. Statistical analysis was performed by the unpaired *t* test. The average MFI (mean fluorescence intensity) ± standard deviation of four independent experiments is shown. *, $P \leq 0.05$ for 60-min treatment versus 10-min treatment.

bound efficiently to the cells (Fig. 2), in agreement with previous results for obtained with Bac7$_{1-35}$ (8) and PMB (20, 21). When the cells were washed with high-salt solution, to ensure that only peptide molecules strongly associated with the surface and/or internalized by cells were left, the cell-associated fluorescence decreased significantly. When the incubation time was extended to 60 min, the amount of Bac7$_{1-35}$-BY strongly associated with the cells increased significantly, while that of PMB-BY remained unchanged or slightly decreased, indicating faster binding kinetics and rapid saturation of binding sites by the latter peptide (Fig. 2).

To analyze the peptides' penetration of bacterial cells, peptide-treated cells were incubated with TB, which is excluded from the interior of intact cells and which should quench only the extracellularly accessible BODIPY dye. TB has been used for many years for phagocytosis studies (10, 23) and in studies of CPP translocation into eukaryotic cells (11). However, to our knowledge, it was never used to investigate the uptake of peptides into bacterial cells. Peptide-treated HB101 cells were analyzed with a flow cytometer after addition of 1 mg/ml TB for 10 min at room temperature. The fluorescence intensity due to cell treatment with Bac7$_{1-35}$-BY for 10 min was not significantly reduced by the quencher (<5% reduction), while TB caused a highly significant reduction of fluorescence intensity (over 40%) in PMB-BY-treated cells (Fig. 3). Prolonging the incubation time to 60 min or increasing the peptide concentration (data not shown) caused a proportional increase in fluorescence intensity specifically for Bac7$_{1-35}$-BY while leaving the percentage of quenching unchanged. This effect was not observed with PMB-BY and is consistent with enhanced uptake of Bac7$_{1-35}$-BY within the cells. In addition, when *E.*

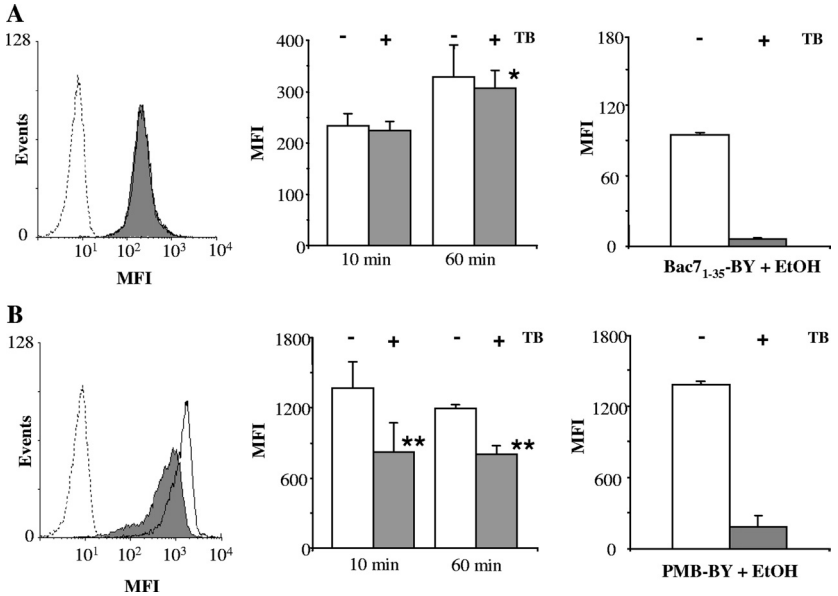

FIG. 3. Fluorescence quenching in *E. coli* cells exposed to Bac7$_{1-35}$-BY or PMB-BY. *E. coli* cells (1×10^6 CFU/ml) were incubated with 0.25 μM Bac7$_{1-35}$-BY (A) or 0.1 μM PMB-BY (B), washed, and analyzed by flow cytometry with (gray histograms) or without (empty histograms) incubation with TB for 10 min at 37°C. (Left panels) Representative experiments with *E. coli* cells treated for 10 min with the indicated peptide. Non-peptide-treated cells are shown by the dotted line. (Central panel) Fluorescence of cells treated with the peptides for 10 or 60 min. (Right panel) Fluorescence of cells treated with the peptides for 10 min and then permeabilized with ethanol (30 min at −20°C) before flow cytometry analysis. The average MFI (mean fluorescence intensity) ± standard deviation of four independent experiments is shown. *, $P \leq 0.05$ for 60-min treatment versus 10-min treatment. **, $P \leq 0.01$ for TB-treated cells versus non-TB-treated cells. EtOH, ethanol.

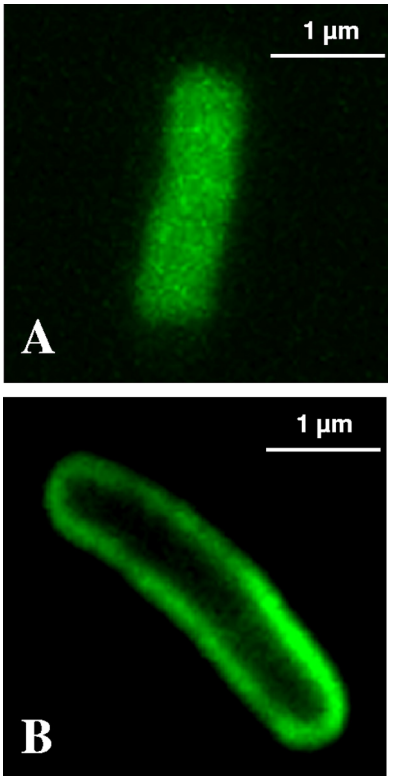

FIG. 4. Confocal microscopy images of *E. coli* cells exposed to BODIPY-labeled peptides. *E. coli* HB101 cells were analyzed after incubation with 0.25 μM $Bac7_{1-35}$-BY (A) or 0.1 μM PMB-BY (B) for 60 min. Treated cells were washed four times with high-salt solution, and 10 μl was placed between two cover glasses to obtain an unmovable monolayer of cells. Unfixed bacterial cells were examined with a Nikon C1-SI confocal microscope with an oil immersion objective lens. All of the images are representative sections from the middle of the bacterial cell. Many fields were examined, and for each experiment, over 95% of the cells displayed the patterns of the respective representative cells shown here.

coli cells were treated with $Bac7_{1-35}$-BY at 4°C, peptide fluorescence was not observed, indicating that membrane translocation was prevented (data not shown). Comparable results were obtained with *S. enterica* serovar Typhimurium ATCC 14028 (data not shown), indicating that this method may also be applied to gram-negative species other than *E. coli*.

To exclude the possibility that the nonquenchable fluorescence fraction was due to factors other than peptide internalization, aliquots of bacterial cells were completely permeabilized with ethanol after incubation with the labeled peptides (24) and then incubated with TB. Alcohol treatment restored full accessibility to TB (Fig. 3, right panel), irrespective of the peptide used, indicating that the labeled peptides become fully accessible after ethanol treatment.

The cellular localization of $Bac7_{1-35}$-BY and PMB-BY in *E. coli* HB101 cells was confirmed by examining the cells in parallel by confocal scanning laser microscopy. $Bac7_{1-35}$-BY appeared to be homogeneously distributed into the bacterial cytoplasm (Fig. 4A) without accumulation in the membranes. On the contrary, the fluorescence associated with PMB-BY was clearly present only on the cell surface (Fig. 4B). These data emphasize the difference in cellular distribution between $Bac7_{1-35}$-BY and PMB-BY and fully support the quenching results obtained by flow cytometry.

Overall, these results are consistent with the uptake and internalization of $Bac7_{1-35}$ by cells and are in agreement with previous observations obtained by immunoelectron microscopy (16). Conversely, the fluorescence intensity of *E. coli* and *S. enterica* serovar Typhimurium cells exposed to PMB-BY decreased by approximately 50%. This percentage is only slightly lower than that shown with a fluorescein-labeled antibody directed to the *E. coli* surface O and K antigens of lipopolysaccharide. In this case, over 70% of the fluorescence was quenched after TB addition (unpublished data). An accessibility of PMB-BY to the quencher similar to that of the surface-localized anti-lipopolysaccharide supports a surface localization of PMB. Interaction of this peptide with a compartment less accessible to TB, such as the lipid bilayer, might explain the small difference observed.

In summary, the method described here is suitable to rapidly screen the cell-penetrating capacity of peptides in bacteria and is useful in structure-activity relationship studies when many samples have to be compared. The assay can also be easily coupled to the PI uptake assay to investigate the permeabilizing capacity of the peptide under scrutiny (16). This would allow rapid determination of whether or not the mechanism of action of a specific AMP depends on membrane permeabilization. Finally, this method may also be applied to other nonpeptidic antibiotics to screen their cell-penetrating properties once they have been labeled with a suitable fluorophor.

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