A high-throughput, homogeneous, FRET-based assay to detect bacterial membrane-bound enzyme (MraY) activity

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- Inhibitors of peptidoglycan synthesis represent antibacterial drug targets
- A FRET-based assay was developed to find MraY inhibitors
- The simultaneous dual emission detection capability of the PHERAstar® FS enhances high-throughput

Introduction

Peptidoglycan is an important structural component of the bacterial cell wall. The continual synthesis of peptidoglycan and remodelling of the cell wall is essential for most bacteria. Since the enzyme phospho-N-acetyl-muramoylpenta-peptide translocase (MraY) catalyzes one of the last cytoplasmic steps in the peptidoglycan biosynthesis, it represents a target for antibacterial drugs. This integral membrane protein catalyzes the attachment of soluble UDP-N-acetyl-muramolympentapeptide (UNAM-pp) to the lipid undecaprenyl phosphate (C55P) which is membrane bound.

Assay Principle

In this application note we want to present a novel homogeneous FRET-based assay to monitor the activity of MraY. A donor fluorophore (BODIPY-FL) is attached to UNAM-pp [B-UNAM-pp] while the acceptor fluorophore-labelled 1,2-dipalmitoyl-sn-glycerol-3-phosphothanolamine-N-(lissaminerhodamine B sulfonyl) fluorphore-labelled 1,2-dipalmitoyl-sn-glycerol-3-phosphothanolamine-N-(lissaminerhodamine B sulfonyl) is embedded in micelles which also contain MraY and the lipid substrate C55P (Figure 1).

Preparation of UNAM-pp and cloning, expression of MraY in E. coli as well as subsequent preparation of membranes from E. coli overexpressing MraY is described in the literature. A 6 µL mixture containing 0.06% Triton X-100, C55P and E. coli membranes containing MraY was preincubated for 30 min. Addition of 3 µL of B-UNAM-pp +/- UMP initiated the reaction. Reaction buffer consisted of (final concentrations): 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 50 mM MgCl2, 1 mM dithiothreitol and 0.05% Triton X-100. Final concentration of reactants: 20 mM C55P, 50 µg/mL membrane protein, 2 µM B-UNAM-pp with 0-24 µM LRPE and 0 or 5 mM UMP. Triplicate MraY assays were performed at room temperature. Fluorescence was excited and simultaneous dual-emission measured using 20 flashes for each reading at a focal height 10.6 mm. Measurements were made every minute for 1 hour and triplicate time courses averaged.

Results & Discussion

When the reaction is exposed to fluorescent excitation at 485 nm a time-dependent decrease in 520 and increase in 590 emission is observed (data not shown). Ratio-metric measurements were used as they have less noise than individual fluorescence intensity measurements (Figure 2). This is due to the elimination of fluctuations that equally affect both measurements. The ratio change exhibits an increase in a time and LRPE dependent manner.

Materials & Methods

- PHERAStar FS microplate reader (BMG LABTECH)
- Optic module [Ex:485 nm; Em: 520/590]
- Chemicals were obtained from commercial sources
- 384 well, low-volume, black, polystyrene plates (Matrix Tech)
The sensitivity of this assay to inhibition was studied using tunicamycin (Figure 4). The results show that IC₅₀s measured using ∆(F₅₉₀/F₅₂₀) are lower and exhibited less of an increase with reaction time than those measured with ∆F₅₂₀.

**Conclusion**

This assay has several advantages over those previously reported. First: it uses no radioisotopes so no special training, handling and disposal are required. Second: it uses a donor fluorophore with an excitation wavelength in the visible rather than UV part of the spectrum. This reduces interference from test compound auto-fluorescence and absorption. Finally: a FRET assay using the ratio of the emission of acceptor and donor fluorescence intensities upon donor excitation yields greater precision.

The use of ∆(F₅₉₀/F₅₂₀) over ∆F₅₂₀ is advantageous due to greater sensitivity to inhibition, resistance to loss of sensitivity to inhibition and a higher signal-to-noise ratio. Furthermore a Z’ of greater than 0.7 indicates that this approach will be suitable for high throughput screens.