Measuring Efflux in MDR *Escherichia coli* using 1,2`DNA and BM27 as Dyes in Real-Time Fluorescence Assays

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Background

Overexpression of efflux pumps in multi-drug (MDR)-resistant gram-negative bacteria contributes to the development and level of resistance against many antimicrobial agents [1,2].

In order to better understand resistance mechanisms and support drug development, accurate efflux measurement is crucial and would overcome the limitations of MIC testing and conventional drug accumulation assays that determine only a net total of influx and efflux. We previously reported a real-time assay using 1,2'-dinaphthylamine (1,2'-DNA) that was able to measure different efflux half times in *E. coli* AcrB mutants [3]. We now extend the findings with this method and report the feasibility of using BM27 as a new alternative dye with this technique.

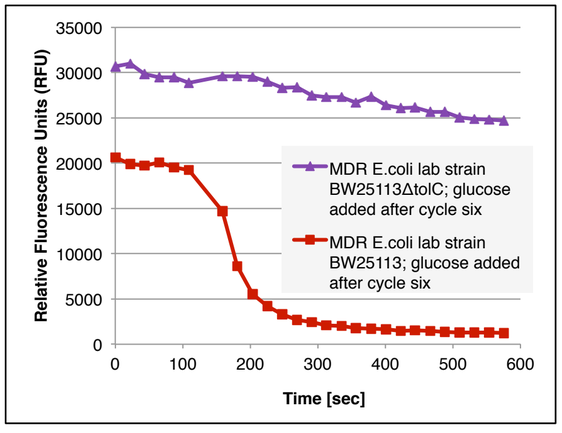

Figure 1: Real-time efflux assay using 50μM BM27 in an MDR *E.coli* wildtype AcrABTolC strain vs. efflux-incompetent Δ*tolC* strain. Fluorescence levels drop significantly after energization of the cells.

References:
1 Nikaido, H et al. 1998
2 Yasufuku, T et al. 2011
3 Bohnert, JA et al. 2011

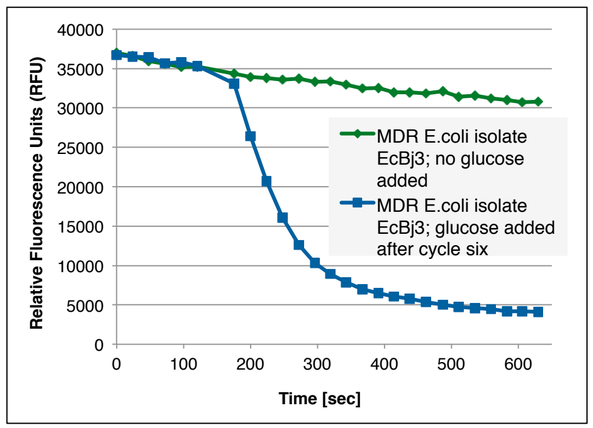

Figure 2: Real-time efflux assay using 32μM 1,2´-DNA in an MDR *E.coli* clinical solate – energized vs. non-energized.

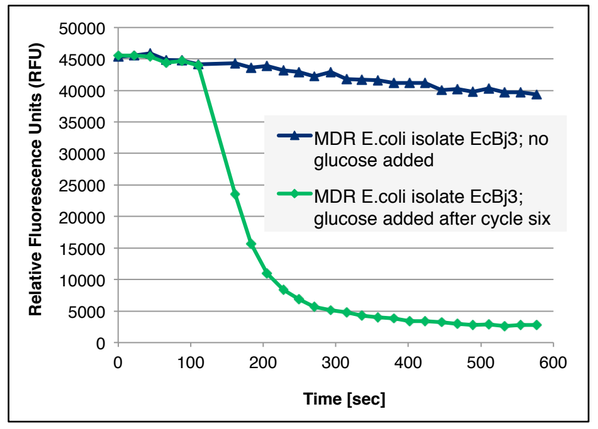

Figure 3: Real-time efflux assay using 50μM BM27 in an MDR *E.coli* clinical isolate (see Fig.2 for 1,2`-DNA efflux in the same strain).

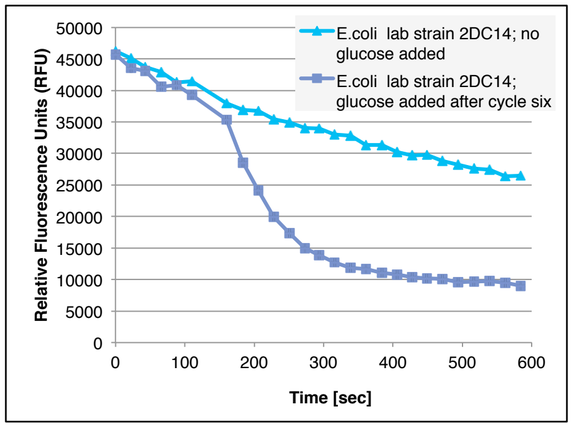

Figure 4: Real-time efflux assay using 50μM BM27 in an *acrEF*-overexpressing Δ*acrAB* *E.coli* strain. Occasionally, pre-energization efflux was observed as seen here.

Methods

A series of well-defined laboratory *E. coli* strains included *tolC* and *acrB* inactivation mutants and MDR clinical isolates.

- overnight growth in LB medium; adition of the proton-motive force inhibiting agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) to deenergize the cells and avoid preenergization efflux
- loading of the energy-depleted cells with fixed concentrations of either 1,2'-DNA or BM27 dyes and further incubation [3]
- removal of CCCP and the dyes from the medium before starting the measurement on a 96-well plate in a microplate reader (excitation: 370nm, emission: 420nm)
- after a scanning period of ~150 seconds, addition of glucose (final concentration of 50mM) to trigger efflux; fluorescence measurement continued for 500 seconds
- active efflux was considered when post-energization fluorescence levels dropped significantly.

Results

Both Δ*tolC* and Δ*acrB* strains accumulated the two dyes but showed no efflux when energized. *E.coli* lab strain BW25113 with constitutive expression of *acrB* showed low efflux capacity for 1,2'-DNA but high capacity for BM27 (Fig.1).

BM27 was shown to be a substrate of AcrF and YhiV in experiments with an Δ*acrAB* strain with *acrEF* overexpression (Fig.4) and YhiV expressing strain respectively.

In a collection of 33 MDR *E. coli* clinical isolates with differing MDR efflux and porin gene expression levels, we found high BM27 efflux capacities (example Fig.3) in most isolates, but there was no consistent relationship between efflux capacity and multipanel MIC levels, susceptibility to efflux pump inhibitors (EPIs) or gene expression profiles.

The $AUC_{non\text{-}energized}/AUC_{energized}$ ratios in the reference strains ATCC25922 and ATCC35218 were 2.4 ±0.4 and 2.4 ±0.3, respectively. Values ≥3.0 were observed in 17/33 isolates, values ≤2.0 in 3/33 isolates, respectively.

Conclusion

Both dyes may be useful to screen for and measure efflux capacity in enteric bacteria. BM27 - when confirmed in subsequent studies - may have the advantage to be a substrate of several RND-type pumps. Whether and how chemical library screening for EPI activity can be integrated into the new real-time efflux assays remains to be determined.

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