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Use of resazurin to detect mefloquine as an efflux-pump inhibitor in *Pseudomonas aeruginosa* and *Escherichia coli*

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ABSTRACT

Multi-drug-resistant bacteria can cause serious infections that are extremely difficult to treat. Bacterial efflux pumps are known to contribute to multi-drug resistance and, thus, constitute a promising target for novel antibacterial agents. Resazurin is widely used to monitor bacterial growth because resazurin is reduced to the fluorescent resorufin by live cells. We have shown by flow cytometric analysis and by accumulation studies with wild type and efflux deficient strains that resazurin is a substrate of efflux pumps in *Escherichia coli* and *Pseudomonas aeruginosa*. Our investigations showed that the conversion rate of resazurin to resorufin is affected by efflux pumps. This finding was used to design an assay useful to detect efflux pump activity and to find potential efflux-pump inhibitors in a microtiter plate format. Mefloquine was detected as efflux-pump inhibitor when a panel of selected chemical compounds was tested for assay validation purposes.

1. Introduction

The emergence of pan-resistant, glucose–non fermenting Gram-negative bacteria such as *Pseudomonas aeruginosa* or *Acinetobacter baumannii* is posing a major threat to successful antimicrobial chemotherapy (Obritsch et al., 2002). Many Gram-negative bacteria express efflux pumps of the Resistance-Nodulation-Division (RND) family. These pumps contribute to multi-drug resistance because they efficiently expel various chemicals, including different classes of antibiotics (Dreier 2007; Livermore 2002; Piddock, 2006a,b; Poole, 2000, 2004, 2005; Schweizer 2003). Thus, understanding multi-drug efflux systems could lead to innovative ways to restore the activity of antibiotics whose activities are compromised by efflux.

Resazurin is a blue, non-fluorescent compound that is reduced by living cells to resorufin, which is pink and highly fluorescent. Fluorescence and/or absorption can be read as a measure of cell viability or cell density. Resazurin is also sold as a solution under the trade name Alamar Blue, which can be used to monitor cell proliferation and viability of bacterial and eukaryotic cells in a simple, fast and cheap way (O'Brien et al., 2000).

Here, we report results from studies with *P. aeruginosa* and *Escherichia coli* suggesting that resazurin is a substrate of RND efflux pumps. Active efflux had an impact on the use of resazurin for the measurement of *P. aeruginosa* and *E. coli* growth kinetics. The finding could be used to monitor active drug efflux in bacteria and to detect substrates and inhibitors of RND efflux pumps.

2. Materials and methods

2.1. Bacterial strains

P. aeruginosa PAO750 was obtained from H. Schweizer at Colorado State University (Kumar et al., 2006). *P. aeruginosa* strains PAO1 Δ*mex*B and PAO1 Δ*mex*R were constructed following the methods described by (Hoang et al., 1998). *E. coli* BW25113 *acr*AB:Kan^r was constructed from BW25113 (*E. coli* stock center at Yale University New Haven, CGSG strain No. 7739) using a published method (Datsenko and Wanner, 2000). The relevant characteristics of the strains are listed in Table 1. Minimum inhibitory concentrations (MIC) were determined according to the guidelines of the CLSI.

2.2. Chemicals and media

Alamar Blue was bought from Invitrogen (Carlsbad, CA, USA). The resazurin concentration of the Alamar Blue solution was determined

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Table 1
Bacterial strains used in this study.

Strain	Relevant characteristics
E. coli	
BW25113	wt
BW25113 *acr*AB:Kan^r	Inactivated *acr*AB
P. aeruginosa	
PAO1	wt
PAO1 Δ*mex*B	Δ*mex*B
PAO1 Δ*mex*R	Overexpression of *mex*AB-*opr*M
PAO750	Δ(*mex*AB-*opr*M), Δ(*mex*CD-*opr*J), Δ(*mex*EF-*opr*N), Δ(*mex*JK), Δ(*mex*XY), Δ*opm*H

to be 460 μM by absorption measurements at 600 nm. Alamar Blue could be replaced by 100 μg/ml resazurin in phosphate-buffered saline (PBS) in our assays. Ciprofloxacin was obtained from ICN Biomedicals (Irvine, CA, USA), glucose from Merck (Darmstadt, Germany), glycerol and ethidium bromide (EtBr) from Acros Organics (Geel, Belgium). Luria Bertani broth (LB, Difco) and Müller–Hinton broth were obtained from Becton Dickinson Biosciences (San Jose, CA, USA). Phe–Arg–β–naphthylamide (PAβN), 1-(1-naphthylmethyl)-piperazine (NMP), carbonyl-cyanide-3-chlorophenylhydrazone (CCCP), berberine, resazurin, reserpine, chloroquine, optochin and PBS were purchased from Sigma (St. Louis, MO, USA). Mefloquine was from the Basilea compound collection.

2.3. Growth measurements

Bacteria were grown in 96-well microtiter plates in 100 μl of Müller–Hinton broth supplemented with resazurin as described by the provider. Further additions are specified in the figure legends.

2.4. Resazurin accumulation assay

Bacterial colonies from Luria agar plates were resuspended in PBS + 0.4% glucose to an OD_{600} of 1. Test compounds were added as specified in the text. 10 μl of a 100 μg/ml resazurin solution in PBS were added to 90 μl of cell suspension. Fluorescence signals ($\lambda_{ex} = 530$ nm and $\lambda_{em} = 590$ nm) were measured with a SpectraMax M2e microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA). All measurements were done at 20 °C.

2.5. EtBr efflux assay

The assay was set up as an adaptation of a published method (Hsieh et al., 1998). A culture of *E. coli* BW25113 in LB was grown to stationary phase, harvested by centrifugation (Eppendorf centrifuge 5810R, 3500 rpm, 5 min) and taken up in assay buffer (PBS + 0.4% glycerol) to an OD_{600} of 2. Cells were incubated for 30 min at 37 °C with 10 μg/ml EtBr and test compound where indicated. Cells were pelleted by centrifugation (Eppendorf 5417R centrifuge, 3500 rpm, 4 min, 4 °C) and taken up in fresh assay buffer. EtBr fluorescence was measured at 20 °C as a function of time (SpectraMax M2 microtiter plate reader, $\lambda_{ex} = 530$ nm, $\lambda_{em} = 610$ nm).

2.6. Flow cytometric analysis

Homogeneous suspensions of *P. aeruginosa* in PBS + 0.4% glucose of $OD_{600} = 1$ were treated with resazurin (10 μl of a 100 μg/ml solution in PBS). PAβN was added to a final 32 μg/ml where indicated. Cell populations were then analyzed with a FACSCalibur reader (Becton Dickinson, San Jose, CA, USA) measuring light scattering and fluorescence ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 585$ nm).

3. Results

3.1. Determination of the cell density of P. aeruginosa cultures with resazurin

The growth of *P. aeruginosa* cultures was monitored by the conversion of resazurin into the fluorescent resorufin. Growing PAO1 caused a substantial increase of resorufin fluorescence over time (Fig. 1). Almost no change of resorufin fluorescence was detected in control experiments without bacteria. Ciprofloxacin had an MIC of 0.125 μg/ml for PAO1. We observed the expected strong reduction of resorufin production in the experiment with 64 μg/ml ciprofloxacin. Phe-Arg-β-naphthylamide (PAβN) had an MIC of > 512 μg/ml and led to an apparent acceleration of resorufin generation at 100 μg/ml. This was highly interesting because PAβN was described as a broad spectrum efflux-pump inhibitor (Lomovskaya et al., 2001). Thus, the increased rate of resorufin production could be due to increased resazurin and/or resorufin accumulation as a consequence of efflux-pump inhibition. We tested this hypothesis with PAO750 (Kumar et al., 2006). This strain was derived from PAO1 by deletion of the genes coding for the main efflux systems (Table 1). PAO750 produced resorufin at an increased rate compared to PAO1 (Fig. 1). OD_{600} measurements in parallel to the fluorescence readings showed that the different resorufin curves were not simply a reflection of altered growth rates (data not shown).

3.2. Resorufin production in bacterial cells

Resorufin accumulation experiments were done with suspensions of bacteria in PBS + 0.4% glucose. Optical density measurements indicated that the bacteria did not grow under these conditions during the experiment (data not shown). We observed a significantly increased rate of resorufin production by PAO1 Δ*mex*B compared to PAO1 (Fig. 2). Deletion of the *mex*B gene inactivates the MexAB-OprM efflux pump. Thus, our results suggested that resazurin was a substrate of MexB. Deletion of *mex*R leads to increased efflux-pump

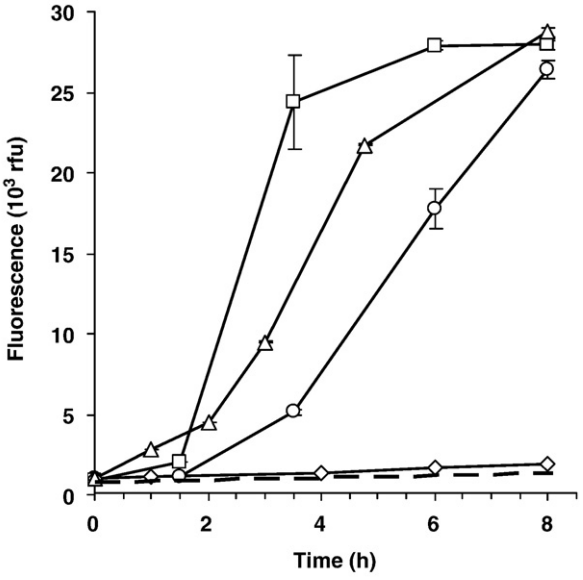

Fig. 1. Measurement of bacterial growth with resazurin. *P. aeruginosa* cultures were grown in Müller–Hinton broth supplemented with resazurin. Cultures were started at $OD_{600} = 0.125$ and cell density changes were monitored by measuring the fluorescence intensity of the produced resorufin. Circles: PAO1, diamonds: PAO1 + 64 μg/ml ciprofloxacin, squares: PAO1 + 100 μg/ml PAβN, triangles: PAO750. The dashed line shows resorufin generation in Müller–Hinton broth without bacteria.