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Original Research Article

Interaction of ethidium and tetraphenylphosphonium cations with *Salmonella enterica* cells

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ABSTRACT

Background and objective: One of the main causes of bacterial resistance to antimicrobials is multidrug resistance induced by the increased efficiency of the efflux pumps. In this study we analyzed how the conditions of assay affect the efflux of indicator substrates ethidium (Et^+) and tetraphenylphosphonium (TPP^+) in *Salmonella enterica* ser. Typhimurium cells. Impact of the outer membrane permeability barrier, composition and temperature of the medium on accumulation of the indicator compounds also was analyzed.

Materials and methods: The fluorescence of Et^+ and Nile Red was measured using 96-well plates and a plate reader. In parallel to traditional studies of fluorescence we applied a constructed selective electrode to follow the accumulation of Et^+ in *S. enterica* cells. Simultaneously with monitoring of Et^+ concentration in the cell incubation medium, electrochemical measurements of TPP^+ accumulation were performed. Furthermore, Et^+ and TPP^+ were used within the same sample as agents competing for the interaction with the efflux pumps. An inhibitor phenylalanyl-arginyl-β-naphtylamide (PAβN) was applied to evaluate the input of RND-family pumps in the total efflux of these indicator compounds.

Results: *S. enterica* cells with the intact outer membrane (OM) bound very low amounts of Et^+ or TPP^+. Cells with the permeabilized OM accumulate considerably higher amounts of the indicator compounds at pH 8.0, but only Et^+ was considerably accumulated at pH 6.5. At conditions of electrochemical monitoring accumulation of Et^+ by the permeabilized cells at 37 °C was considerably faster than at 23 °C, but at the higher temperature most of the cell-accumulated Et^+ was extruded back to the medium. The fluorescence of Et^+ in suspension of cells incubated in 400 mmol/L Tris buffer was about twice higher compared to 100 mmol/L one. The inhibitory action of TPP^+ on Et^+ efflux was evident only in 400 mmol/L Tris although PAβN effectively increased Et^+ fluorescence at both buffer concentrations.

Conclusions: Results of our experiments indicate that ionic strength of the incubation medium influence the selectivity, the medium temperature and the assay conditions impact the kinetics of efflux. The lower accumulated amount and the weaker fluorescence of Et^+ registered in slightly acidic medium indicate that $\Delta\Psi$ plays a role in the accumulation of this

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indicator cation. The bound amount of Et^+ to the de-energized or permeabilized cells considerably varies depending on the conditions and methods of de-energization or permeabilization of cells. Tris/EDTA permeabilization of the cells does not inhibit the efflux.

1. Introduction

Extrusion of drugs from cells by efflux pumps (EPs) is one of the main reasons for bacterial resistance to antibiotics [1,2]. EPs are able to eject different lipophilic and amphiphilic compounds, such as dyes, detergents, lipids, quorum sensing signal molecules. EPs are also associated with virulence [3–5] and biofilm formation [6]. Therefore, information about the efflux is vital for the effective usage of available antibacterial drugs and the discovery of new ones.

Lipophilic cations, such as ethidium (Et^+) and tetraphenylphosphonium (TPP^+), are well known EP substrates [7–11]. Membrane voltage (transmembrane difference of electrical potential, $\Delta\Psi$, negative inside) drives the accumulation of these cations in bacterial cytosol. The specific feature of Et^+ is its affinity to DNA. Intercalation into the double helix increases fluorescence of this indicator compound [12]. Intracellular components bind low amount of the TPP^+ [13] and, therefore, this compound can be used as an indicator of $\Delta\Psi$ in bacteria and mitochondria.

Fluorimetric assay of cell-accumulated Et^+ is one of the most popular method to study EP activity in bacteria [14–17]. The fluorescence is related to the amount of Et^+ bound to bacterial DNA and the latter depends on the intracellular concentration of this indicator. Measurements of Et^+ fluorescence are very convenient for studying the competition between efflux substrates [18] because it is possible to use several compounds in the same sample. However, a gradual decrease in Et^+ fluorescence during the monitoring period was observed in experiments with *Pseudomonas aeruginosa* cells although the cell-bound Et^+ is not destructed [14]. Beside this, Martins and colleagues [15] have reported about variations in the energy-dependence of Et^+ efflux from *Enterobacter aerogenes* cells in media of different pH.

In this study we analyzed how the assay conditions affect the efflux of Et^+ from *Salmonella enterica* ser. Typhimurium cells. To follow the accumulation of Et^+ in cells, in addition to traditional measurements of fluorescence we applied potentiometric monitoring of the concentration of this indicator by the constructed selective electrode. In parallel to Et^+ measurements accumulation of TPP^+ in the cells was also assayed. Beside this, Et^+ and TPP^+ were used simultaneously within the same sample as agents competing for the interaction with EPs and the efflux inhibitor phenylalanyl-arginyl-β-naphtylamide (PAβN) was applied to evaluate the input of RND-family pumps. Results of our experiments indicate that ionic strength of the incubation medium influence the selectivity, the medium temperature and the assay conditions impact the kinetics of efflux.

2. Materials and methods

2.1. Bacteria cultivation and preparation for experiments

Salmonella enterica ser. Typhimurium strain SL1344 wild type cells were obtained from Prof. Séamus Fanning (Institute of Food and Health, University College Dublin, Ireland). Overnight culture of cells was grown in Luria-Bertani broth, containing 0.5% NaCl (Sigma–Aldrich, Munich, Germany), diluted 1:50 in fresh medium, and the incubation was continued until the OD_{600} reached 1.0. The cells were collected by centrifugation at 4 °C for 10 min at 3000 *g* (Heraeus™ Megafuge™ 16R, Thermo Scientific, Germany). The pelleted cells were re-suspended in 100 or 400 mmol/L Tris-hydroxyaminomethane (Tris)/HCl (Roth, Karlsruhe, Germany), pH 8.0, to obtain $\sim 4 \times 10^{10}$ cells/mL. To permeabilize the outer membrane (OM), the cells were at 37 °C 10 min incubated in 100 mmol/L Tris/HCl containing 10 mmol/L ethylene diamine tetra-acetic acid (EDTA; Sharlau, Barcelona, Spain), pH 8.0, then pelleted and re-suspended as described above. Concentrated cell suspensions were kept on ice until used, but not longer than 4 h. Heat treatment of the cells was performed incubating 1 mL of the concentrated suspension in a 1.5-mL Eppendorf tube for 10 min in a boiling water bath.

2.2. Fluorescence measurements

Stock solutions of ethidium (Et^+) bromide (Acros Organics, New Jersey, USA) or Nile red (NR, Sigma–Aldrich, Munich, Germany) were added to the corresponding concentrations into test-tubes containing Tris buffer solution with 0.1% glucose (Sharlau, Barcelona, Spain). After mixing, stock solutions of phenylalanyl-arginyl-β-naphtylamide (PAβN) hydrochloride (Sigma–Aldrich, Munich, Germany) and/or tetraphenylphosphonium (TPP^+) chloride (Fluka, St. Gallen, Switzerland) were added. Then the concentrated cell suspension was added to obtain OD_{600} of 1.0, the samples were mixed and within 2 min transferred into a 96-well flat-bottom black plate, 120 μL per well ($n = 3$). Relative intensity of the fluorescence (excitation 535 nm or 485 nm, emission 612 nm or 535 nm for Et^+ or NR, respectively) was monitored in "TECAN GENios Pro™" (Männedorf, Switzerland) plate reader, thermostating the plate at 23 °C or 37 °C. The plate was shaken 5 s before each registration point. The representative curves from 3 independent experiments are presented.

2.3. Potentiometric measurements

TPP^+ and Et^+ concentrations in the incubation media were potentiometric monitored using selective electrodes as

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