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Assessment of the activity of RND-type multidrug efflux pumps in *Pseudomonas aeruginosa* using tetraphenylphosphonium ions

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ABSTRACT

Multidrug resistance (MDR) pumps are one of the major causes of antibiotic resistance in *Pseudomonas aeruginosa*. Thus, fast and reliable methods are needed to assay the efficiency of MDR pumps in these bacteria. In this study, it was demonstrated that a membrane voltage ($\Delta\psi$) indicator tetraphenylphosphonium (TPP^+) in combination with the efflux pump inhibitor phenylalanyl-arginyl- β -naphthylamide can be used to monitor the activity of resistance–nodulation–cell division (RND)-type efflux pumps in *P. aeruginosa*. By controlling the outer membrane permeability and $\Delta\psi$, electrochemical measurements of RND pump activity in real time were performed. It was demonstrated that the composition of the medium, the presence of nutrients and the level of aeration affect the efficiency of the TPP^+ -extruding activity of *P. aeruginosa*, urging the standardisation of experimental conditions to obtain quantitative and comparative results.

1. Introduction

Pseudomonas aeruginosa is an exceptionally versatile organism that can adapt to a wide range of ecological niches [1]. A major reason for its prominence as an opportunistic pathogen is its high intrinsic resistance to antimicrobials, including antibiotics and disinfectants. In addition to the low outer membrane (OM) permeability to lipophilic compounds and the highly active periplasmic β -lactamases, multiple efflux pumps are major causes of antibiotic resistance in *P. aeruginosa* [2]. It is of crucial importance to develop rapid methods to evaluate the activity of multidrug resistance (MDR) pumps and to estimate their efficiency in clinical isolates.

Several methods are used to study the activity of MDR pumps. The most popular methods are based on monitoring the accumulation of fluorescent probes, especially ethidium [3]. In addition, radiolabelled antibiotics [4], tetraphenylphosphonium ions (TPP^+) [5] or other lipophilic compounds have been used such assays. However, these methods include procedures (pelleting or filtration of cells, incubation in closed vials) leading to conditions affecting the energy state of bacteria and, therefore, the functioning of the pumps.

Lipophilic cations such as TPP^+ are popular indicators of membrane voltage [transmembrane electrical potential difference ($\Delta\psi$)] in bacteria. By measuring the interaction of TPP^+ with Gram-negative bacteria, it is also possible to detect changes in the permeability of the OM [6]. In 1986, Midgley [7] showed that the level of TPP^+ accumulation in bacteria depends on the efficiency of pumps extruding this indicator cation back to the incubation medium. Further studies revealed that lipophilic cations are the most universal substrates of MDR pumps in prokaryotic [5,8,9] and eukaryotic [10] cells.

Here we describe an electrochemical assay for TPP⁺ accumulation in *P. aeruginosa* cells using selective electrodes. Such monitoring of TPP⁺ accumulation in the absence and presence of phenylalanyl-arginyl- β -naphthylamide (PA β N), a general inhibitor of resistance–nodulation–cell division (RND)-type pumps [11], allows real-time evaluation of the total activity of the RND-type MDR pumps in these bacteria. We demonstrate that the composition of the medium, the presence of nutrients and the level of aeration affect the efficiency of the MDR pump-based TPP⁺-extruding activity of *P. aeruginosa*, urging the standardisation of experimental conditions to obtain quantitative and comparative results.

2. Materials and methods

2.1. Bacterial strains and chemicals

Pseudomonas aeruginosa strain PAO1 was obtained from Prof. Patrick Plésiat (CHU Minjoz, Besançon, France). Gramicidin D (GD), polymyxin B (PMB) sulphate, polymyxin B nonapeptide (PMBN) hydrochloride, PA β N and ethylene diamine tetra-acetic acid (EDTA) were from Sigma (St Louis, MO) and TPP⁺ chloride was from Aldrich (Taufkirchen, Germany).

2.2. Bacterial growth and minimum inhibitory concentration (MIC) determination conditions

Pseudomonas aeruginosa cells were cultivated in Luria–Bertani broth (LB) [12] containing diminished NaCl concentration (0.5%). Cell growth was monitored by measuring the optical density at 550 nm (OD₅₅₀) using a Selecta Clormic digital spectrophotometer (J.P. Selecta,

Barcelona, Spain). MICs of TPP⁺ and PA β N were determined by the microdilution method as described by Wiegand et al. [13].

2.3. Electrochemical measurements

Experiments were performed in 10 mL or 50 mL thermostated vessels at 37 °C with magnetic stirring. Cell cultures were grown to an appropriate OD₅₅₀ and were transferred directly to the vessels or collected by centrifugation for 15 min at 7000 rpm at 4 °C (Sorvall SLA-1500 rotor; Thermo Fisher Scientific Inc., Waltham, MA). Pelleted cells were re-suspended in 100 mM sodium phosphate (pH 8.0) to a density of $1-2 \times 10^{11}$ colony-forming units/mL and kept on ice until used (maximum 4 h).

Measurements of TPP⁺ concentration were performed simultaneously in four reaction vessels as described previously [6,14]. A representative set of curves from three independent measurements is presented.

3. Results

3.1. Media conditions affecting the multidrug resistance pump activities in *Pseudomonas aeruginosa*

Possible reasons for the limited TPP⁺ accumulation in Gram-negative bacteria are: (i) low $\Delta\psi$; (ii) low permeability of the OM; and (iii) high activity of MDR pumps. Both the OM and the pumps prevent TPP⁺ accumulation. Therefore, when assessing pump activity it is crucial to establish conditions warranting high and stable permeability of the OM to TPP⁺ without affecting the $\Delta\psi$ and the activity of MDR pumps.

EDTA treatment is used [6] to increase OM permeability to lipophilic compounds and to rapidly equilibrate TPP⁺ across the cell envelope of Gram-negative bacteria. Addition of 0.4 mM EDTA to *P. aeruginosa* suspension in 100 mM Tris buffer induced complete release of the cell accumulated TPP⁺ (Fig. 1a, red curve). It induced only a transient accumulation of TPP⁺ in 50 mM Tris buffer (Fig. 1a, black curve). Leakage of the accumulated TPP⁺ to the medium and the drop in the suspension turbidity (Fig. 1a, insert) indicated that EDTA rapidly lysed the cells.

Consequently, as EDTA treatment cannot be applied to *P. aeruginosa* cells in Tris buffer, different phosphate buffer concentrations were used for the following experiments (Fig. 1b). At all concentrations studied, except 400 mM, EDTA induced an additional accumulation of TPP⁺, whereas the universal inhibitor of RND-type MDR pumps PA β N induced a strong release of the accumulated TPP⁺ (Fig. 1b). Parallel measurements indicated that EDTA induced a moderate decrease of the cell suspension OD₅₅₀ only when 50 mM phosphate was used. Addition of PA β N had no effect on the culture turbidity (Fig 1b, insert). The channel-forming antibiotic GD induced an additional leakage of TPP⁺, but the suspension OD₅₅₀ considerably decreased only after the addition of PMB (Fig. 1b, insert). Control measurements showed that low OM permeability increasing concentrations of PMB added after PA β N did not induce any additional accumulation of TPP⁺ (not shown, but see Fig. 3). Overall, these data indicate that depolarisation of the plasma membrane (PM) is the principal reason for the PA β N-induced release of TPP⁺. We note that these results are valid only with *P. aeruginosa* and in phosphate buffer without nutrients. Viability measurements revealed that the MIC for TPP⁺ is >8192 μ g/mL, whereas the combination of TPP⁺ with

PA β N decreased it to 512 μ g/mL, both of these values being considerably higher than the 3 μ M (1.12 μ g/mL) concentration used for assay of the pump activities.

If TPP⁺ is a substrate and PA β N is an inhibitor of RND-type pumps, blockage of pump activity should increase the accumulation of TPP⁺. Bacterial MDR pumps are usually activated by nutrients such as glucose [11,15]. However, TPP⁺ accumulation in *P. aeruginosa* was not sensitive to glucose (not shown). In LB medium the cells did not accumulate significant amounts of TPP⁺ (Fig. 1c, blue and red curves). However, when PA β N was added, a strong accumulation of TPP⁺ occurred indicating the suppression of TPP⁺-extruding pump activities. GD had a weak effect on the distribution of TPP⁺ between the cells and the LB medium, but subsequent additions of PMB released most of the cell-associated indicator cations, indicating that accumulation of TPP⁺ was a $\Delta\psi$ -dependent process.

The difference in the initial accumulation of TPP⁺ by the cells in phosphate buffer and LB medium suggests that LB components have a considerable effect on the efficiency of MDR pumps and/or OM permeability to TPP⁺. The $\Delta\psi$ and, consequently, the accumulation of TPP⁺ are medium pH-dependent characteristics of the bacteria (compare Fig. 1b, lilac and red curves; Fig. 1c, blue and red curves). In such a situation, the low buffering capacity of LB complicates the studies of MDR pumps using charged indicator substrates. To increase the buffer capacity, LB was diluted with 400 mM sodium phosphate (1:1). Initial accumulation of TPP⁺ in the buffered LB (Fig. 1c, black curve) proceeded considerably more slowly compared with the measurements in 200 mM phosphate buffer (Fig. 1b, blue curve) or in unbuffered LB medium (Fig. 1c, red curve). A low amplitude of EDTA-induced accumulation of TPP⁺ was common in all the cases when fresh LB-containing medium was

used (see below). Attempts to permeabilise the OM using another popular permeabiliser, PMBN, were unsuccessful. In addition, PMB was less active when added after PMBN (not shown). Despite the low efficiency of the OM permeabilisers, PA β N induced a considerable accumulation of TPP⁺. The following addition of GD had a weak effect, but the subsequent additions of PMB caused an effective release of accumulated TPP⁺. Experiments with heat-inactivated cells (Fig. 1c, green curve) indicated that the sharp initial effect of PA β N on the potential of the TPP⁺-selective electrode has no connection with the activity of MDR pumps.

PA β N-induced TPP⁺ accumulation can be the result of either inhibition of efflux pumps or permeabilisation of the OM, or both. To acquire more information on the mechanism of PA β N action on the capacity of cells to accumulate TPP⁺, gradually increasing concentrations of the inhibitor were used (Fig. 2). In phosphate buffer starting at the lowest concentrations tested, PA β N only released cell-accumulated TPP⁺ (Fig. 2a,c). In phosphate buffer-diluted LB, PA β N-induced changes in the accumulation of TPP⁺ had a more complex nature dependent on the presence or absence of EDTA. In the presence of EDTA, low (>7.5 μ g/mL) concentrations of PA β N induced an additional accumulation of TPP⁺ (Fig. 2b). The second phase of the inhibitor-induced TPP⁺ accumulation was observed at 30 μ g/mL and higher concentrations of PA β N. At very high concentrations of PA β N (>120 μ g/mL) the additional accumulation of TPP⁺ was followed by its release. In the absence of EDTA, PA β N was ineffective up to 30 μ g/mL, but the concentration increase induced additional accumulation of TPP⁺ (Fig. 2d). However, EDTA addition after PA β N induced further TPP⁺ accumulation. This indicates that PA β N is not an effective permeabiliser of the OM. Consequently, the ratio of the cell-accumulated amounts of TPP⁺ after and before addition

of PA β N can be considered as a measure of the total activity of PA β N-sensitive MDR pumps.

3.2. *Impact of aeration on the efficiency of multidrug resistance pumps*

Another experimental factor, frequently varied during studies on MDR pumps, is the aeration of the sample. To assess the role of aeration, experiments were performed in large vessels containing 30 mL of bacterial suspension. The level of aeration was varied by changing the intensity of magnetic stirring [14]. In the case of the cells grown to an OD₅₅₀ of 1, pelleted and transferred to fresh LB medium, accumulation of TPP⁺ was only transient (Fig. 3a). Further addition of EDTA only slightly increased the accumulation. Addition of PA β N induced strong but reversible accumulation of TPP⁺. The extent of reversibility increased with the increase in aeration. PMB at low concentrations induced additional accumulation of TPP⁺, indicating low OM permeability. Such PMB-induced TPP⁺ accumulation was observed even in the presence of 2 mM EDTA (not shown).

The depolarising efficiency of PMB was also related to the intensity of aeration; 10 μ g/mL PMB was enough to induce PM depolarisation at conditions of the highest aeration, but ca. 80 μ g/mL was needed at the lowest aeration level. Noteworthy, further increase in the PMB concentration did not lead to faster depolarisation of the PM. Subsequent addition of GD depolarised the PM at all aeration levels studied (Fig. 3a).

Direct cell samples from exponentially growing culture were less sensitive to the level of aeration (Fig. 3b vs. Fig. 3a); 10 μ g/mL PMB initiated depolarisation of the PM only at the

lowest aeration level (Fig. 3b). This suggests that at conditions of very weak aeration, the EDTA-disrupted barrier of the OM was not restored.

When the same experiment was performed with overnight grown and pelleted cells, only weak dependences of TPP⁺ accumulation and the sensitivity of the cells to PMB on aeration were observed. At these conditions, EDTA was not able to induce any accumulation of TPP⁺ (Fig. 3a vs. Fig. 3c). However, the cells were even less sensitive to differences in aeration levels when direct overnight culture samples were utilised (Fig. 3d). In this case, at all aeration levels PA β N caused a stable TPP⁺ accumulation and low concentrations of PMB did not induce an additional one, indicating that EDTA was able to increase OM permeability to the maximum level.

Based on these results, it appears that the aeration level and the sampling method are important in respect to the efflux pumps' activity measurements. These results also indicate that the change of medium composition can be the main reason for the culture growth phase-related changes of MDR pump activities.

4. Discussion

Here we present results of efflux studies in liquid *P. aeruginosa* cultures where a popular $\Delta\psi$ indicator and a universal substrate of MDR pumps (TPP⁺) in combination with a pump inhibitor (PA β N) are used for electrochemical real-time monitoring of total RND-type efflux pump activity. During the recording, the cell suspension was aerated and thermostated, reagents were added to the reaction mixture and samples were taken for additional analyses without interrupting the experiment or changing the culture conditions.

We discovered that in phosphate buffer, PA β N causes depolarisation of the PM (Fig. 1b). This depolarising effect of PA β N can be prevented by supplementing the buffer with nutrients of the LB medium (Fig. 1c) with the consequence of additional TPP⁺ accumulation. It has been shown [11] that the inhibitory action of PA β N is not caused by dissipation of $\Delta\psi$ but depends on direct interaction with the efflux system. This depolarisation can be due to a very intensive $\Delta\psi$ -exhausting extrusion of PA β N from the cells or to the inhibitor-induced short-circuiting of the PM. It should be mentioned that at very high concentrations PA β N induces TPP⁺ leakage also in the presence of LB (see Fig. 2b,d).

Results obtained in the presence of LB underline the importance of the nutrient-induced intensification of metabolism on the mode of action of MDR pumps. It is possible that the high level of proton-motive force is not enough to keep the H⁺ flux-driven MDR pumps active and it is necessary to guarantee high concentration of protons (low local pH) in the near vicinity of the drug-extruding pumps. This factor could also explain the need for glucose for activation of MDR pumps in enterobacteria [11,15].

This description of the variables affecting *P. aeruginosa* efflux systems emphasises the need to set up accredited standard conditions for efflux pump assays. Experiments indicate that the medium composition and the aeration level are critical parameters affecting accumulation of TPP⁺ in *P. aeruginosa* cells. Additional TPP⁺ accumulation following addition of PA β N is highest at conditions not enabling the reconstruction of the EDTA-disturbed OM barrier, i.e. at low levels of aeration and/or in the exhausted growth medium (Fig. 3). In parallel to repair of the OM barrier, at optimal growth condition cells can respond to inhibition of the main RND-type pumps by activating alternative ones. The impact of

aeration diminishes when overnight cultures are used. Consequently, buffered stationary-phase cell cultures are the most appropriate for comparative studies of multiple samples, e.g. clinical isolates of *P. aeruginosa*.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Influence of media composition on efflux pump activity towards tetraphenylphosphonium ions (TPP^+). Experiments were performed in 5 mL of Tris/HCl (a), sodium phosphate (b) or Luria–Bertani (LB) or LB diluted 1:1 with 400 mM sodium phosphate (NaP) (c) as indicated in the figure. Media pH was 8.0 unless otherwise indicated. A concentrated cell suspension was added to the vessels to obtain an optical density at 550 nm (OD_{550}) of 1. Ethylene diamine tetra-acetic acid (EDTA) was added to a final concentration of 0.4 mM, and phenylalanyl-arginyl- β -naphthylamide ($\text{PA}\beta\text{N}$), gramicidin D (GD) and polymyxin B (PMB) were added to concentrations of 60 $\mu\text{g/mL}$, 4 $\mu\text{g/mL}$ and 60 $\mu\text{g/mL}$, respectively. In (a) and (b), 70 μL samples were taken from the vessels for turbidity measurements throughout the experiments (inserts). In (c, green curve), cells were inactivated by incubating the concentrated suspension at 70 °C for 15 min.

Fig. 2. Influence of phenylalanyl-arginyl- β -naphthylamide ($\text{PA}\beta\text{N}$) concentration and media composition on the capacity of *Pseudomonas aeruginosa* cells to accumulate tetraphenylphosphonium (TPP^+). Experiments were performed in 5 mL of sodium phosphate (a,c) or Luria–Bertani (LB) diluted 1:1 with 400 mM sodium phosphate (b,d). A concentrated cell suspension was added to the vessels to obtain an optical density at 550 nm (OD_{550}) of 1. Ethylene diamine tetra-acetic acid (EDTA) was added to a final concentration of 0.4 mM, and gramicidin D (GD) and polymyxin B (PMB) were added to concentrations of 4 $\mu\text{g/mL}$ and 60 $\mu\text{g/mL}$, respectively. $\text{PA}\beta\text{N}$ was added to the concentrations indicated in the figure ($\mu\text{g/mL}$). In (a) and (b) EDTA was added before $\text{PA}\beta\text{N}$, whereas in (c) and (d) EDTA was added after addition of $\text{PA}\beta\text{N}$.

Fig. 3. Dependence of tetraphenylphosphonium (TPP^+) accumulation on the aeration level of *Pseudomonas aeruginosa* cell cultures. Cells were grown to an optical density at 550 nm

(OD₅₅₀) of 1 (a,b) or overnight (c,d). In (b) and (d) measurements were performed by diluting cultures 1:1 with 400 mM sodium phosphate (pH 8.0), and in (a) and (c) by adding concentrated cell suspension to buffered Luria–Bertani (LB) medium to OD₅₅₀ values of 0.5 and 1, respectively. The intensity of aeration was controlled by magnetic stirring using four levels: ‘high’ (green curves, ++++); ‘intermediate’ (blue, +++); ‘low’ (red, ++); and ‘very low’ (black, +). TPP⁺, ethylene diamine tetra-acetic acid (EDTA), phenylalanyl-arginyl- β -naphthylamide (PA β N) and gramicidin D (GD) were added to the cell suspensions to final concentrations of 3 μ M, 0.4 mM, 60 μ g/mL and 4 μ g/mL, respectively. Polymyxin B (PMB) was added to the concentrations indicated in the figure (μ g/mL).