BIOLOGIJA. 2006. Nr. 2. P. 24–27
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Use of tetraphenylphosphonium ions for studies of activity of tetracycline-extruding pumps

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One of the factors determining the resistance of bacteria to tetracycline are TetA(B) pumps extruding this antibiotic from the cells in a protonmotive force-dependent way. Tetraphenylphosphonium (TPP^+) ions, an indicator of membrane voltage in bacteria, were used to determine the activity of these Tn*10*-encoded structures. Loading of the pumps with tetracycline in a concentration-dependent manner caused an additional accumulation of TPP^+ in *Escherichia coli* cells. Comparison of the amount of tetracycline added to the additional amount of TPP^+ accumulated by the cells led to the conclusion that affinity of TetA(B) pump to TPP^+ is considerably higher compared to that of tetracycline. Our experiments showed that registration of TPP^+ concentration in bacterial suspensions could be used for real-time studies of activity and efficiency of TetA(B) pumps.

Key words: tetracycline resistance, efflux pump, Tn*10*

INTRODUCTION

Multiple drug resistance (MDR) of pathogenic bacteria is a worldwide problem. It is important to develop new express-methods for identifying the presence of antimicrobial compounds-extruding pumps and estimating their efficiency in pathogenic bacteria as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and enterobacteria. Several methods based on measurement of accumulation of fluorescencent probes (ethidium bromide, acriflavine and pyronin Y) or radio-labeled compounds (antibiotics, tetraphenylphosphonium (TPP^+) ions) [1, 2] are commonly used to determine the role of MDR pumps in antibiotic resistance of bacteria.

One of the best known cases of bacterial resistance to antibiotics is the resistance to tetracyclines. Efflux pumps, which maintain the intracellular concentration of this antibiotic at a low level, are the main mechanism of resistance to tetracycline in pathogenic bacterial strains [3]. Resistance genes of non-tetracycline-producing bacteria are often located in mobile genetic elements such as transposons, conjugative plasmids or conjugative transposons [4]. Transposon Tn*10* encodes the tetracycline efflux pump TetA(B), which belongs to the major facilitator superfamily [5]. Efflux pumps of this family utilize the protonmotive force to ensure the transport of sugars, intermediate metabolites and drugs [6, 7]. Tn*10*-encoded tetracycline efflux pumps were analyzed for their ability to extrude out of the cell tetracycline and its analogues [5, 8], but there are no data in the literature on the use of indicator compounds (TPP^+, ethidium bromide, etc.) for the studies of this pump activity. We performed a series of experiments to examine whether TPP^+ ions can be used as a substrate of TetA(B) efflux pumps. Results of these experiments allowed us to introduce an alternative method for estimation of activity of tetracycline-extruding pumps – real-time measurements of TPP^+ accumulation in bacteria using selective membrane electrodes. Such measurements can be used for the registration of activity of tetracycline-extruding pumps and for the search of their inhibitors. Another positive feature of this method is that neither expensive radio-labeled antibiotics nor aggressive compounds, such as ethidium bromide, are used for studies.

MATERIALS AND METHODS

Bacteria growth and viability testing conditions

Cells of *Escherichia coli* MC4100 strain and its derivate *E. coli* IQ86 were grown in Luria–Bertani (LB) medium containing 0.9% of NaCl at 37 °C with aeration. Overnight bacterial culture was grown in 25 ml of LB. In the case of IQ86 cells, 10 μg/ml of tetracycline hydrochloride (Serva) was added to the cultivation medium. The final cell batch was grown from a 100 times diluted overnight culture in the absence of tetracycline to a concentration of $\sim 4 \times 10^8$ cells/ml. Exposure of the cells to tetracycline was performed when the concentration of cultivated cells reached $\sim 3 \times 10^8$ cells/ml, adding this antibiotic to the final concentration of 100 μg/ml and incubating for additional 15 min at 37 °C with aeration. Cells were harvested by centrifugation and resuspended in 100 mM Tris-HCl buffer

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(pH 8.0) to obtain 4–5×10^{10} cells/ml. The concentrated cell suspension was kept on ice until used (maximally for 5 h).

Tris-EDTA-treated cells were prepared as described previously [9]. The harvested cells were resuspended in 100 mM Tris buffer containing 10 mM EDTA (pH 8.0) to 1/10 of the original volume, shaked at 37 °C for 10 min and pelleted by centrifugation.

For viability measurements the obtained cell suspension was diluted in LB medium to ~4×10^8 cells/ml and incubated for 10 min at 37 °C with aeration. Then a proper concentration of tetracycline was added and cells were incubated for additional 20 min at 37 °C with aeration. To determine the number of colony-forming units (CFU), after incubation the cells were diluted in 0.9% NaCl and spread on agar lawn containing 1% of tryptone, 0.5% of yeast extract, 0.5% of NaCl and 2% of agar. The experiments were duplicated and the mean values were calculated. All experiments were repeated at least twice, to make certain that the results obtained are reproducible.

Measurements of TPP$^+$ fluxes

Concentrations of TPP$^+$ in the media were monitored using selective electrodes as described previously [10]. Briefly, the concentrated cell suspension was added to 5 ml of 100 mM Tris-HCl buffer, pH 8.0, in the reaction vessels thermostated at 37 °C to obtain the final cell concentration of ~8×10^8 cells/ml. The cell suspensions were aerated by magnetic stirring. Calibration of the measuring system using standard amounts of TPP$^+$ chloride (Aldrich) was carried out at the beginning of every measurement. To induce ion-permeable pores in the cell envelope and depolarize the plasma membrane, at the end of experiments polymyxin B sulfate (Sigma) was added to the final concentration of 100 μg/ml. This led to an efflux of TPP$^+$ ions from the cell to the incubation media.

The construction and characteristics of the TPP$^+$-selective electrode were described elsewhere [11]. Ag/AgCl reference electrodes (Orion Research, Inc., model 9001) were indirectly connected to the vessels through agar salt bridges. The electrodes were connected to a potential-amplifying system based on an AD549JH ultralow input bias current operational amplifier (Analog Devices, USA).

RESULTS AND DISCUSSION

^{3}H-labeled TPP$^+$ ions are commonly used in studies of bacterial multidrug resistance, because MDR pumps can extrude this compound from the cytosol [2]. We decided to use home-made TPP$^+$-selective electrodes [11] to follow the interaction of this lipophilic cation with *E. coli* cells of different susceptibility to tetracycline. Two *E. coli* strains were chosen for experiments: IQ86 – carrying the transposon Tn*10*, which encodes efflux pump-mediated resistance to tetracycline, and its parental strain *E. coli* MC4100. As expected, MC4100 cells were more sensitive to tetracycline compared to IQ86 ones (Fig. 1) at used experimental conditions (see Materials and Methods). In the case of MC4100 cells, the bactericidal effect of tetracycline was detected at concentrations of 10–20 μg/ml and only ~5% of the cells were able to form colonies after treatment with 200 μg/ml of tetracycline. Cells of the tetracycline-resistant strain IQ86 showed a considerably higher capability to form colonies after the treatment: ~5% of the cells were able to form colonies even after incubation with 600 μg/ml of tetracycline.

IQ86 cells accumulated a lower amount of TPP$^+$ compared to the tetracycline-sensitive MC4100 ones when EDTA-permeabilized cells were incubated in Tris buffer containing this indicator cation (Fig. 2). Addition of tetracycline to the incubation medium caused an additional accumulation of TPP$^+$, which correlated with the amount of tetracycline added. The final amounts of TPP$^+$ accumulated were a bit different at low concentrations of tetracycline added, but became almost equal when the concentration of this antibiotic was 200 μg/ml or higher.

An equilibrium distribution of TPP$^+$ between bacterial cytosol and incubation medium depends on the level of $\Delta\psi$ (negative inside), the cell envelope permeability to this cation, and the efficiency of lipophilic compound-extruding pumps. The lower initial amount of TPP$^+$ accumulated by IQ86 cells could be explained by a contribution of the spontaneously expressed Tn*10*-encoded tetracycline efflux pump TetA(B) which is absent in MC4100 cells. Loading of all cellular efflux pumps (TetA(B) and MDR) with tetracycline resulted in an increment of the cell-accumulated amount of TPP$^+$. However, at high tetracycline concentrations all these pumps are saturated with this antibiotic and the contribution of TetA(B) pumps in the equilibrium distribution of TPP$^+$ becomes negligible.

In more detail, the capability of the pumps to extrude TPP$^+$ was studied in experiments with cells which during cultivation were for a short period exposed to tetracycline (see Materials and Methods). The initial amount of TPP$^+$ accumulated by tetracycline-exposed IQ86 cells was less than 50% compared to the control (cells not exposed to tetracycline) (curves 2 and 3, Fig. 3). After addition of 400 μg/ml of tetracycline the amount of TPP$^+$, additionally accumulated by the exposed IQ86 cells, was ~60% higher compared to unexposed ones. However, the

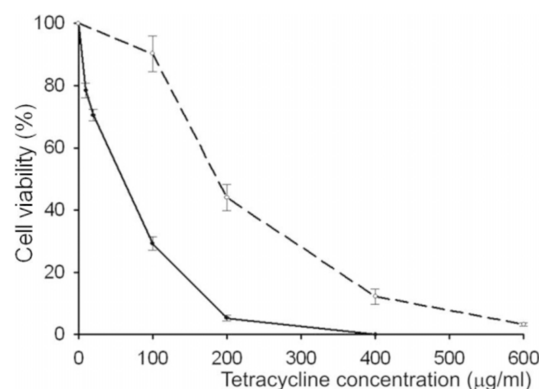

Fig. 1. Sensitivity to tetracycline of *E. coli* IQ86 (○) and MC4100 (●) cells. Bars indicate SE

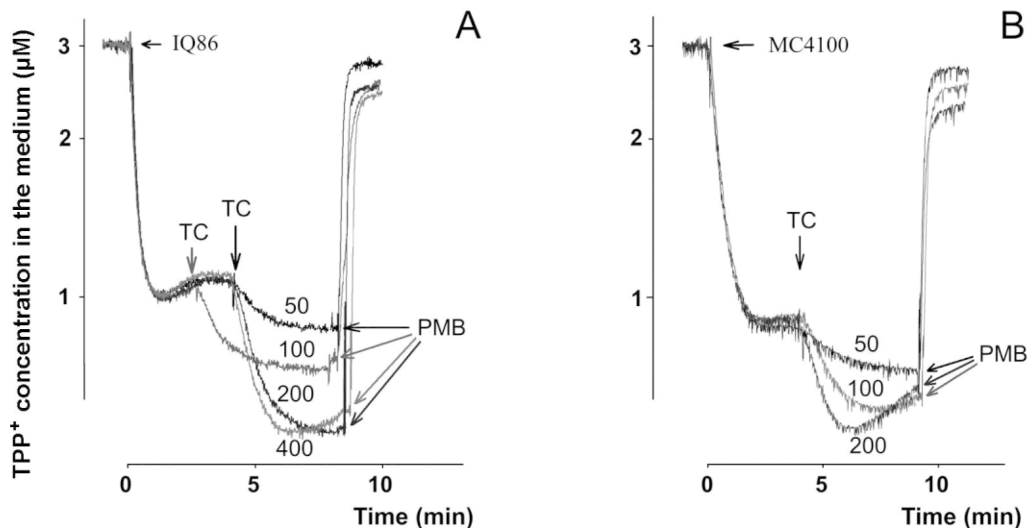

Fig. 2. Effects of tetracycline on the accumulation of TPP^+ by IQ86 (A) and MC4100 (B) cells. Experiments were performed in 100 mM Tris-HCl buffer, pH 8.0, at 37 °C. Concentration of Tris/EDTA-treated cells was 8×10^8 cells/ml, tetracycline (TC) was added to the final concentrations indicated next to the curves, Polymyxin B (PMB) was added to the final concentration of 100 μg/ml. The cells were not exposed to tetracycline during cultivation

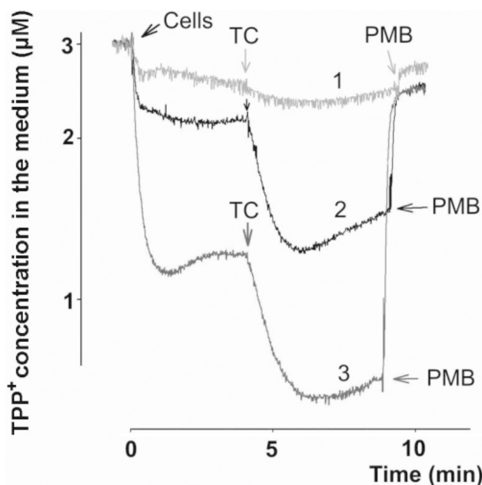

Fig. 3. Effects of tetracycline on the accumulation of TPP^+ by tetracycline-exposed *E. coli* MC4100 (curve 1) and IQ86 (curve 2) cells. Experiments were performed as described in Fig. 2, except that TC in all cases was added to the final concentration of 400 μg/ml. Curve 3 is taken from Fig. 2A

final amount of TPP^+ accumulated by the exposed cells remained lower compared to unexposed ones, and the further increase of the amount of tetracycline added did not lead to additional accumulation TPP^+. At higher than 400 μg/ml concentrations tetracycline depolarized the plasma membrane and induced an efflux of accumulated TPP^+ (results not shown). Tetracycline-exposed MC4100 cells accumulated a considerably lower amount of TPP^+ (compare Fig. 2B and curve 1 in Fig. 3), most probably because of the damage of membrane voltage-generating systems and depolarization of the plasma membrane.

These results indicate that tetracycline-induced pumps, first of all the Tn*10*-encoded tetracycline resistance-mediating pump TetA(B), effectively extrude TPP^+ ions from the cells as one of their substrates. Our results have shown that a spontaneous expression of the TetA(B) pump in *E. coli* IQ86 is rather weak compared to the situation when expression of the pump is induced by addition of tetracycline to the cultivation medium.

When expressed in micromoles per litre, the additional accumulation of TPP^+-inducing concentrations of tetracycline are ~100 times higher compared to TPP^+ concentrations in the medium. Beside this, when the concentration of tetracycline in the medium was increased from 100 to 400 μg/ml, accumulation of TPP^+ by the tetracycline-exposed IQ86 cells increased only by some ~45% (results not shown). These data allow to conclude that tetracycline has a considerably lower affinity to the TetA(B) pump compared to TPP^+. Our data indicate that a weak selectivity is characteristic not only of MDR pumps, but also of the specialized, tetracycline-extruding ones.

Our experiments showed that using selective membrane electrodes potentiometric measurements of TPP^+ concentration in the medium could be used to monitor the activity of tetracycline-extruding pumps. This electrochemical method serves as an alternative to the other methods of quantification of the activity of MDR and Tet A(B) pumps, such as the use of radiolabelled antibiotics, $[^3H]TPP^+$ or hazardous fluorescent compounds like ethydium bromide.

ACKNOWLEDGEMENTS

Lithuanian State Science and Study Foundation supported this work (Grant C–20/2005).

Received 12 February 2006
Accepted 7 May 2006

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TETRAFENILFOSFONIO JONŲ PANAUDOJIMAS TETRACIKLINĄ IŠMETANČIŲ SIURBLIŲ AKTYVUMUI ĮVERTINTI

S a n t r a u k a

Vienas veiksnių, lemiančių bakterijų specifinį atsparumą tetraciklinui, yra TetA(B) siurbliai, kurie naudoja protonovaros jėgą tetraciklinui iš ląstelės išmesti. Šių transpozone Tn*10* koduojamų siurblių veikla tiriama naudojant radioaktyviais izotopais žymėtą tetracikliną bei įvairius jo analogus. Savo tyrimuose siurblių aktyvumui įvertinti pasirinkome tetrafenilfosfonio (TPP^+) jonus, plačiai naudojamus registruojant bakterijų membranos įtampą. *Escherichia coli* ląstelių apvalkalėlyje esančių siurblių apkrovimas vienu jų substratu – tetraciklinu – sąlygojo kito substrato – TPP^+ jonų – papildomą sugėrimą. Siurblių apkrovimui pridėto tetraciklino kiekio ir ląstelių papildomai sukaupto TPP^+ jonų kiekio palyginimas leidžia teigti, kad TetA(B) siurblys nėra atrankus tik tetraciklinams, o TPP^+ jonų giminingumas šiems siurbliams pasirodė esąs netgi didesnis. Mūsų eksperimentai rodo, kad galima nepertraukiamai registruoti TetA(B) siurblių veiklą selektyviais elektrodais matuojant TPP^+ jonų koncentracijos pokyčius bakterijų suspensijose.