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Envelope permeability to H^+ of *Escherichia coli* cells exposed to acid stress

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Changes in the buffering capacity and envelope permeability to H^+ of exponential-phase *E. coli* cells grown in a rich medium in response to acidity were studied. An acid pulse technique was used to measure both parameters. Our results have showen that the buffering capacity and the envelope permeability to H^+ of *E. coli* are influenced by the sublethal acid stress (pH 4.5), which induces adaptive tolerance response (ATR) and protects cells from a subsequent strong acid challenge. Both of these parameters are higher in cells exposed to acidic pH compared to those grown at pH 7.4. Changes in both parameters could result from changes in cell surface and membrane properties developed during the adaptation process and contribute to survival after a strong acid stress.

Key words: *E. coli* acid tolerance response (ATR), acid pulse, envelope permeability, buffering capacity

INTRODUCTION

Enteric bacteria often encounter acid stress conditions in a variety of pathogenic and natural situations. *E. coli* have developed systems that enable bacteria to survive extremely acidic conditions, such as those present in the human stomach. Bacteria that are able to survive under such conditions have to endure a pH difference of 4–5 pH units either by secreting the excess of protons or blocking extracellular protons from the cytoplasm [1]. A sublethal environmental acid stress (pH 5.5–4.5) induces an adaptive tolerance response in many bacteria and provides protection towards subsequent exposure to a lethal stress (pH < 4.0) by a mechanism known as acid tolerance response (ATR) [2]. The ATR phenomenon has been studied in a variety of bacterial species, both gram-negative and gram-positive [3]. ATR in *E. coli* is efficiently developed in exponentially-phase cells grown either in rich or minimal medium and protects bacteria form strong acid (pH 2.5) challenge [4]. Paul and Hirschfield have recently demonstrated that exposure of exponential-phase *E. coli* to mild acidity is accompanied by the synthesis of specific acid shock proteins which are thought to be responsible for ATR development [5]. The roles of these proteins remain largely unsolved. These proteins could function in different compartments of bacteria from the cell surface through the cytoplasm. We have shown that the glutamate-dependent AR system one of the known *E. coli* molecular systems, which provide acid resistance (AR) to stationary-phase cells, also contributes to ATR of exponential-phase *E. coli* grown in minimal medium. None of the acid resistance systems tested were responsible for ATR of *E. coli* grown in rich medium, indicating that the molecular components of ATR are dependent on growth conditions [4].

Among the potential contributors to *E. coli* ATR, molecular factors changing envelope permeability to protons are considered [3]. Cellular envelopes are the first barriers which are thought to protect bacteria from different environmental stresses, and changes in their composition may represent adaptive mechanisms against the stress exerted by acid pH [6]. However, the understanding of how the excess of protons could pass through the relatively impermeable membranes, how changes in membrane composition during acid adaptation affect their proton permeability and, most important, how it responses to cell survival during acid stress is very incomplete.

The aim of the present study was to investigate whether a mild acid stress, which induces acid tolerance response of exponential-phase *E. coli,* changes the buffering capacity of bacterial suspension and the cell envelope permeability to H^+.

MATERIALS AND METHODS

Bacterial strains and growth conditions and acid pulse experiments. The *E. coli* K-12 strain used was MG1655 (F^- lambda- *ilvG- rfb*-50 *rph*-1). *E. coli* cells were grown in LBG (Luria–Bertani supplemented with 0.4% glucose) medium pH 7.4.

Acid pulse experiments were performed as described elsewhere [7] with the following modifications. *E. coli* MG1655 cells from overnight cultures were diluted 1:1000 with a LBG medium pH 7.4 and grown at 37 °C with rotary aeration to 6×10^8 CFU/ml. To induce acid adaptation, cells were resuspended in the same volume of LBG medium, adjusted by HCl to pH 4.5, and incubated at 37 °C with rotary aeration. After adaptation the cells were collected by centrifugation at 20 °C, washed and resuspended in 300 mM KCl (5×10^9 CFU/ml) containing 56 mM potassium thiocyanate (KSCN). The external pH of the cell suspension during different experiments was adjusted to 5.0, 6.0 or 7.0 pH units by adding 100 mM HCl or 300 mM NaOH. After adjustment, the cell suspensions were preincubated at room temperature for 1h.

The measurements were carried out simultaneously on two magnetically stirred 7 ml samples in 10 ml glass vials thermostated at 28 °C. The acid pulse was performed by adding 40 µl of 100 mM HCl. In control experiments envelopes, of the cells were permeabilized adding Polymyxin B to 100 µg/ml final concentrations and Gramicidin D to 5 µg/ml. Polymyxin B and Gramicidin D were purchased from Sigma.

The pH electrodes (HI1131, Hanna instruments) were connected to the electrode potential-amplifying system based on an ultra low input bias current operational amplifier AD549JH (Analog Devices, USA). The amplifying system was connected to a computer through the data acquisition board AD302 (Data Translation, Inc., Malboro, USA).

RESULTS AND DISCUSSION

The cellular envelope of enterobacteria represents the first barrier to protect the cell from environmental acidity. The lipid bilayer of the membranes is relatively impermeable to H^+ and this feature contributes to constitutive cellular pH-homeostasis. However, to cope with strong environmental acidity (pH < 4.0), additional inducible molecular mechanisms are needed to protect cellular structures and repair damages of biomolecules [3]. It has been proposed that adaptive responses to acid of *E. coli* and *S. entherica* ser. Typhimurium involve changes in the cell surface properties in addition to the enhancement of intracellular pH homeostasis, and this could be a mechanism for increasing microbial survival [8–10].

To investigate how adaptation to a mild acidity, which induces acid tolerance response, could change the properties of *E. coli* envelope, we examined the flux of H^+ through the envelope of acid-adapted and unadapted MG1655 cells using the acid pulse technique (see Materials and Methods). In the conditions of equilibrium distribution of H^+ across the cell envelope, a small amount of HCl was added. The interaction of added H^+ ions with the cells was registered by monitoring the pH of the suspension. Addition of HCl led to a rapid acidification of the cell suspension, which was followed by a slow alkalization indicating a rather efficient neutralization of the acid by the cells (Fig. 1A). No alkalization phase was registered when before the pulse the unadapted cells were permeabilized by the membrane-active antibiotics Polymyxin B and Gramicidin D (Fig. 1). The final levels of pH after the pulse were nearly equal in the cases of permeabilized cells and intact ones. These results indicate that a low permeability of the cell envelope to H^+ is responsible for the slow neutralization of the acid added. However, the effect of membrane-active antibiotics on the permeability of the adapted cells was considerably weaker (Fig. 1 B). A lower sensitivity of the adapted cells to Polymyxin B (data not shown) could be the main reason for this difference.

The difference between the initial and the final equilibrium pH was used to estimate the buffering capacity of the cell suspensions (Table). The adapted cells neutralized the acid more effectively than did the unadapted ones, and the final level of pH after the pulse in the case of adapted cells remained by about 0.2 pH units higher. These results indicate that the acid-adapted exponential-phase *E. coli* cells develop a mechanism allowing them

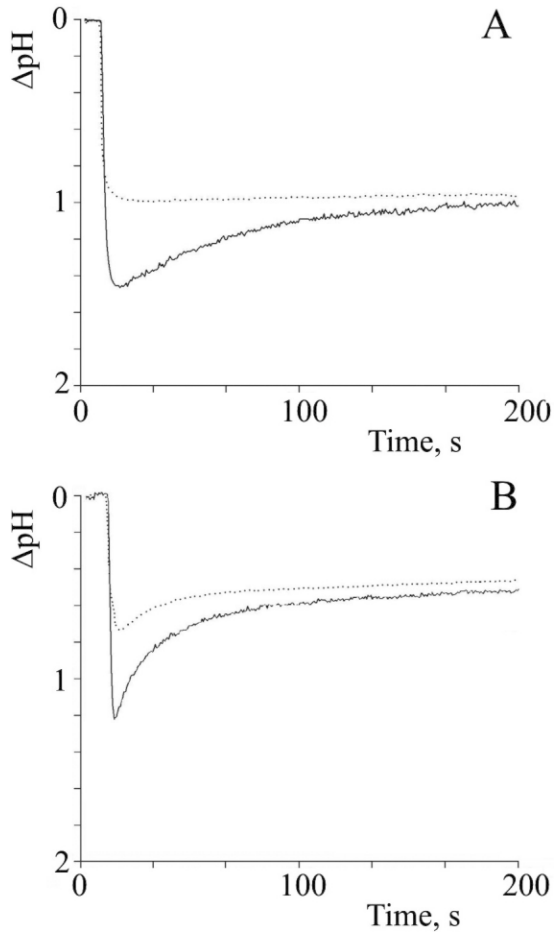

Fig. 1. Changes of pH of *E. coli* MG1655 cell suspension after acid pulse addition
Unadapted (A) and adapted (B) cells. In control experiments (dotted lines) the cells were permeabilized by Polymyxin B and Gramicidin D before the pulse (see Materials and methods). Results of the typical experiment are shown.

to neutralize H^+ from the external medium more effectively. This task could be fulfilled by the proteins with basic properties or polyanions accumulated in different cell compartments, e.g., cell surface, membranes, periplasm during the adaptation period. In a recent study Maurer et al. have showen [11] that the envelope and periplasmic systems of *E. coli*, which consume and export protons, are induced by acid (pH 5.0). Our results on measurements of the buffering capacity of *E. coli* cell suspensions well agree with these observations.

Results of our experiments showed that the initial pH of the adapted cells suspensions, washed and resuspended in the same medium to the same cell concentration, was by ~0.3 pH units lower compared to the unadapted ones. Therefore we examined the buffering capacity and the envelope permeability to H^+ of cell suspensions adjusted to different pH values. These experiments indicated (Figs. 2 and 3) that the cell suspensions adjusted to pH 5.0 or pH 6.0 had considerably higher buffering capacities than the suspension adjusted to neutral pH (pH 7.0). Again, at all medium pH studied the buffering capacity of *E. coli* cells adapted to acid was considerably higher compared to unadapted ones (Table). Similar results have been observed by other authors comparing the buffering capacities of different bacterial species at different external pH [12, 13]. This was also true in the case of another enterobacteriaceae, *S. typhimurium,* indicating that both bacteria during exponential growth show similar acid response strategies [12].

Table. **Buffering capacities of *E. coli* cell suspensions adjusted to different external pH**

Initial pH of bacterial suspension	ΔpH of unadapted cells	ΔpH of adapted cells
pH 5.0	0.88*	0.64
pH 6.0	0.76	0.65
pH 7.0	1.71	1.54

* Difference between the initial and the final equilibrium pH is shown.

It is generally believed that bacterial response to acid changes the membrane permeability to protons [3]. Brown et al. have shown [14] that acid-adapted *E. coli* contain increased levels of cyclopropane fatty acids (CFA) in their membranes. Chang and Cronan have demonstrated that this postsynthetic modification of the lipids plays an important role in the protection of *E. coli* cells from acid shock [15]. They suggested that such modification should decrease the proton permeability of the mem-

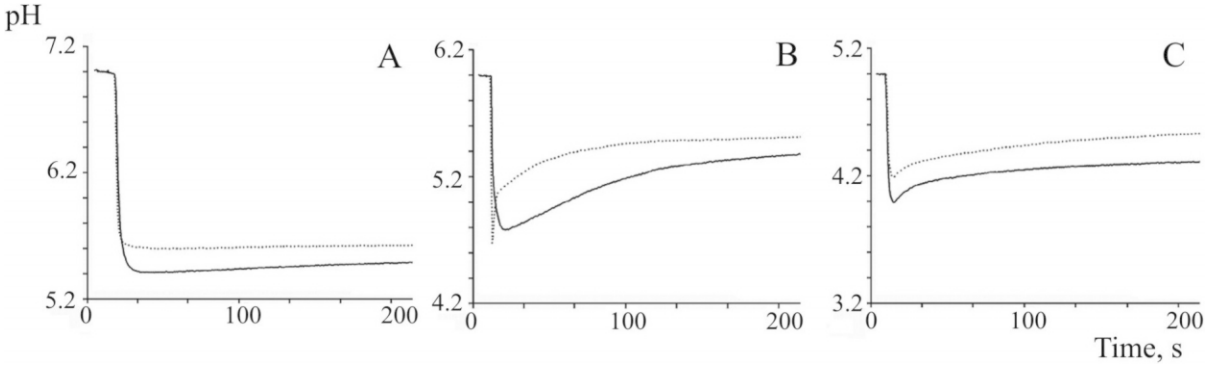

Fig. 2. Acid pulse-induced changes of pH of MG1655 cell suspensions at different external pH
Unadapted (solid line) or adapted (dotted) cells. External pH of cell suspensions were adjusted to 7.0 (A), 6.0 (B) or 5.0 (C).

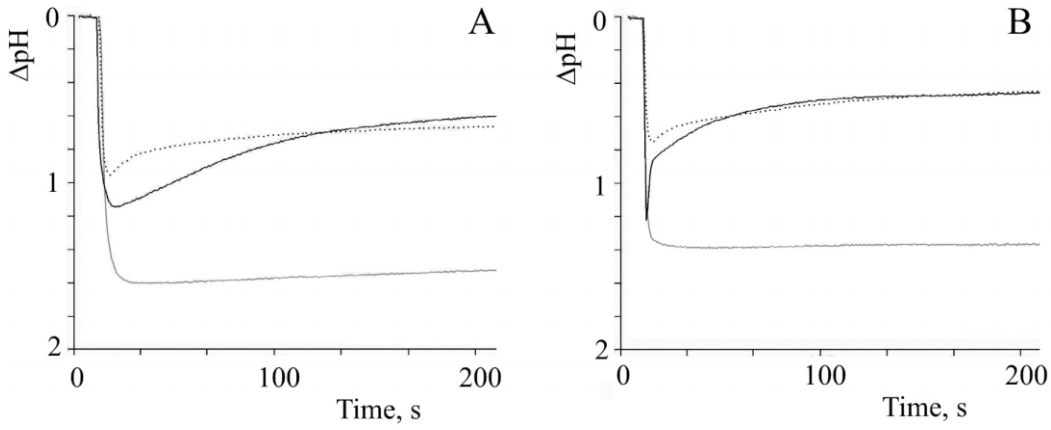

Fig. 3. Analysis of the initial external pH effects on acid pulse-induced pH changes of unadapted (A) and adapted (B) MG1655 cell suspensions
Data are taken from experiment presented in Fig. 2. Initial pH of the cellular suspensions was 7.0 (grey), 6.0 (black) or 5.0 (dotted).

branes. Yuk and Marshall reported [16] a significant decrease in the percentage of unsaturated fatty acids in membranes and an increase of saturated ones when *E. coli* cells were acid-adapted at pH 5.0. Authors speculate that such a change in fatty acid composition under acidic growth conditions, leading to a decreased membrane fluidity, would also lead to a decreased proton permeability of the membranes. In accordance to this proposition, Jordan et al. showed a correlation between the increased acid tolerance of *E. coli* and the decreased permeability of the cell envelope to protons [17].

However, in contrast to the observations discussed above, results of our experiments show that exponential-phase *E. coli* cells, grown in LBG and adapted to acid in the same medium at pH 4.5, at all external pH values studied neutralize H^+ ions at a higher rate compared to unadapted ones (Fig. 2). It should also be mentioned that envelope permeability to H^+ for both types of cells was higher in media with lower pH values. In our experiments, acid-adapted *E. coli cfa* mutant cells, defective in the ability to produce cyclopropane fatty acids, did not differ in the ability to accumulate H^+ ionsas compared to the wild type parent MG1655 (data not shown). These observations indicate that the lack of the factor responsible for this lipid modification is not essential for the membrane permeability to H^+ of acid-adapted exponential-phase *E. coli.*

Although results on the analysis of H^+ permeability of acid-adapted *E. coli* cells obtained in this study disagree with the observation of Jordan and colleagues [17], results of earlier investigations on H^+ permeability of bacterial envelopes of various gram-negative and gram-positive species are comparable to our findings. Thus, Rius et al. found that H^+ conductance of *E. coli* and *S. typhimurium* cells are higher at the acidic pH than at neutral one [12]. Results of our experiments indicate that the increase of the buffering capacity of exponential-phase *E. coli* cells is the main factor of adaptation to acid. Possibly *E. coli* are able to use different strategies to cope with an excess of protons in the media not only by increasing barriers to H^+ or extruding them via proton pumps, but also by allowing H^+ ions to reach the cell sites where they can be neutralized most effectively.

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RŪGŠTINIO STRESO SUKELTI *ESCHERICHIA COLI* APVALKALĖLIO LAIDUMO VANDENILIO JONAMS POKYČIAI

Santrauka

Tiriant atsako į rūgštinį aplinkos stresą molekulinius mechanizmus bei jų reguliaciją, iškelta prielaida, kad bakterijų membranų struktūrinių ir funkcinių savybių pokyčiai yra svarbus bakterijų adaptacijos rūgštiniam stresui fiziologijos aspektas. Buvo tiriamos atsparumą rūgščiai po adaptacijos (ARA) įgijusių eksponentinės augimo fazės *E. coli* ląstelių membranų savybės. Rūgštinio pulso metodu buvo analizuotas membranų laidumas vandenilio jonams ir ląstelių suspensijų buferinis talpumas. Mūsų tyrimų duomenimis, adaptacija esant silpnam rūgštiniam stresui (pH 4,5), lemianti didesnį bakterijų išgyvenimą vėlesnio stipraus rūgštinio streso (pH 2,5) sąlygomis, didina tiek ląstelių buferinį talpumą, tiek membranų laidumą H^+ jonams. Taip pat nustatyta, kad abu šie parametrai yra didesni esant rūgštiniam nei neutraliam ląstelių suspensijos pH. Diskutuojama, kad didesnis adaptuotų *E. coli* ląstelių buferinis talpumas galėtų būti reikšmingas veiksnys bakterijoms išgyvenant stiprų rūgštinį stresą.