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The role of sulfides in stress-induced changes of Eh in *Escherichia coli* cultures

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ABSTRACT

Real-time monitoring of the state of bacterial cultures is important in both experiment and biotechnology. Using Eh and sulfide sensors, we demonstrated that the abrupt reversible reduction in Eh (Eh jump), occurring during transition of *E. coli* from exponential growth to starvation and antibiotic-induced stresses, is the result of sulfide excretion from the cells. Changes in the potential of sensors had a two-phase mode. The potential reduced within 10–15 min and returned within 10–30 min. In the parental strain, maximum amplitudes of Eh jumps (ΔEh) were 25 ± 2 mV, 57 ± 6 mV and 36 ± 7 mV under isoleucine starvation, glucose depletion and ciprofloxacin exposure that corresponded to 43 ± 3 nM, 96 ± 5 nM and 140 ± 1 nM of sulfide, respectively. In the glutathione-deficient mutant (Δ*gshA*), ΔEh values and sulfide concentration increased 1.5–4 times compared to the parent. Stress-induced sulfide excretion occurred in the background of inhibition of growth and respiration and a decrease in the membrane potential. The formation of sulfide caused by cysteine desulfurization may be related with maintaining of cysteine homeostasis under conditions of slow metabolism. There was a close relationship between transmembrane fluxes of sulfide, cysteine and glutathione.

1. Introduction

The abrupt changes in the oxidation-reduction potential to negative values (Eh jumps) measured by the platinum electrode in aerobic cultures of *Escherichia coli* and other bacteria, growing in a minimal medium were previously detected when growth ceased due to depletion of carbon or nitrogen [1]. A characteristic feature of these Eh jumps was the absence of direct connection with changes in the levels of dissolved oxygen (dO_2) and pH of the medium, which were also observed after cessation of growth. In the Eh jumps two phases can be distinguished. There was a rapid decrease in Eh immediately after the growth stopped in the first phase and a slower return to the baseline in the second phase. Studies that are more detailed have shown that the profile of the Eh jump (amplitude and duration of the phases) depends on strains, pH and the ionic composition of the medium. Eh jumps in *E. coli* cultures were also detected under other stress conditions, including acidification of the cytoplasm, heat shock, exposure to antibiotics and uncouplers [1–3].

It is interesting that in mixed cultures of *E. coli* and *S. marcescens*, growth cessation caused by glucose exhaustion resulted in Eh increase, in contrast to its decrease in pure cultures. This inversion of the Eh jumps was observed even when the share of *S. marcescens* in the mixed population did not exceed 1% [1]. This phenomenon makes it possible to use Eh registration for early detection of contamination of pure cultures. In gram-positive bacteria *Bacillus subtilis* and *B. megaterium*, characteristic Eh jumps to negative values upon growth cessation after glucose exhaustion were also observed [1]. In these bacteria, unlike *E. coli*, second phase of the jump was absent, i.e., the Eh drop was irreversible until the growth was resumed after the addition of glucose. This observation suggests that the jump profile may be not only strain-specific, but also species-specific.

Although the parameters of the Eh jumps and the conditions for their induction have been well described, little is known about the factors that cause a shift in the redox potential when inhibiting the growth of bacteria. Some data suggest that the Eh jumps under the situations described may be associated with the increase in the level of low molecular weight thiols in the medium. This is confirmed by the ability of sulfhydryl reagents to restore Eh to the basal level at any phase of the jump. In addition, a tight correlation was found between the starvation-induced Eh changes and the level of low molecular weight thiols in the medium [1]. On the other hand, it is known that *E. coli* accumulate in the medium thiol glutathione (GSH) [4] and possess transport systems

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for its efflux and uptake [5–7]. The presence of these transporters allows aerobically growing *E. coli* to carry out continuous transmembrane cycling of endogenous glutathione between cells and the medium. Due to the establishment of a dynamic balance between GSH efflux and uptake, a constant extracellular concentration of GSH counting per biomass unit is maintained. The transmembrane GSH cycle functions only in respiring cells and disturbed under changes in $\Delta\mu H^+$ and ATP levels [8]. *E. coli* also possesses an inducible L-cysteine/L-cystine shuttle system, which supplies periplasm with reducing equivalents [9]. Finally, it was reported that in certain situations in *E. coli* cultures an increase in extracellular H_2S occurs [10,11].

In this work, the role of the above sulfur-containing substances in the generation of Eh jumps under starvation and antibiotic-induced stresses in *E. coli* was studied.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A parental strain of *Escherichia coli* BW25113 (wt) and a knockout mutant JW2663 (Δ*gshA*), deficient in glutathione (GSH) synthesis, were used. Bacteria were grown in M9 minimal glucose (0.15%) medium [12]. To adapt *E. coli* to aerobic conditions, cells grown overnight were precultured in 100 ml of fresh medium at 37 °C in 250-ml flasks with shaking at 150 rpm from OD_{600} of 0.1 to OD_{600} of 0.5. Then cultures were diluted with prewarmed fresh medium to OD_{600} of 0.1 and were further grown under the same conditions. Samples for analysis were removed in mid-log phase (OD_{600} of 0.4) without stopping rotation of flasks.

2.2. Real-time monitoring of Eh, dissolved oxygen (dO_2), pH and ions of extracellular sulfide (S^{2-}) and potassium (K^+)

Redox potential (Eh) in the cell-free medium and *E. coli* cultures was continuously measured directly in the flasks using platinum and reference electrodes and Mettler Toledo SevenCompact™ pH/Ionmeters S220.

Dissolved oxygen (dO_2) and pH in *E. coli* cultures were continuously measured directly in the flasks using a Clarke oxygen electrode InPro 6800 (Mettler Toledo) and a combined pH electrode InLab Expert Pro-ISM (Mettler Toledo), respectively. The dO_2/pH controller of a BioFlo 110 fermentor (New Brunswick Scientific Co., USA) was used for data recording.

Extracellular sulfide levels were detected directly in the flasks using the system of sulfide-specific ion-selective XC-S^{2-}-001 (Sensor Systems Company, Russia) and reference electrodes and a computer pH/ion meter cpX-2 (IBI Pushchino, Russia).

Changes in the levels of extracellular K^+ were continuously registered directly in the flasks using the system of K^+-selective (ELIS-121K) and reference electrodes and a computer pH/ion meter cpX-2 (IBI Pushchino, Russia). For K^+ measurements, *E. coli* cells were grown as described above, except that the medium contained a low K^+ concentration (0.1 mM).

2.3. Determination of sulfur-containing substances

H_2S levels were estimated using lead acetate [Pb(Ac)2], which reacts specifically with H_2S to form a brown lead sulfide stain. Lead acetate–soaked paper strips were affixed in culture flasks above the level of the liquid culture for 20 min. Spots were photographed and quantified using ImageJ2x. Results were expressed as arbitrary units.

Extracellular sulfite was assayed in 2.5-ml samples taken by rapid filtration through 0.45 μm-pore-size membrane filters (Vladipor, Russia). The filtrates were then mixed with 400 μl of freshly prepared fuchsin reagent [13] and measured at OD 580 nm. The results were quantified using a calibration curve.

For the determination of intracellular cysteine, 40 ml samples of cell culture was harvested by centrifugation (8000 × *g* for 5 min) at different time points, suspended in 4 ml of cold 20 mM EDTA and lysed by sonification at 0 °C, using a 30 s pulse for six cycles. Perchloric acid (the final concentration 0.5 mM) was added to the lysate to precipitate proteins. After 30 min, the suspension was centrifuged (8000 × *g* for 5 min), supernatant was adjusted to pH 8.5 with KOH, frozen, centrifuged to eliminate the potassium perchlorate and evaporated using a rotary evaporator RV 10 (IKA) at 65 °C to 0.5 ml and then treated with 0.5 ml of dithiothreitol (50 mM) for 10 min. Extracellular L-cysteine was determined in 30-ml samples taken by rapid filtration through 0.45 μm-pore-size filters. The filtrates were concentrated to 0.5 ml using a rotary evaporator and then treated as described above to precipitate protein and reduce cystine to cysteine. The amount of L-cysteine in reduced supernatants was determined according to the method of Gaitonde [14]. Standard curves were prepared with known amounts of cysteine, which were treated as samples of cell suspensions.

Extracellular glutathione was determined in 2.5-ml samples taken by rapid filtration through 0.45 μm-pore-size filters. For the determination of intracellular glutathione, 10 ml samples of cell culture was harvested by centrifugation (8000 × *g* for 5 min) at different time points and prepared as described previously [8]. GSH was measured using the DTNB-glutathione reductase recycling method [15] modified as described previously [8].

2.4. Study of membrane potential and ATP determination

Changes in the membrane potential ($\Delta\Psi$) were evaluated using $\Delta\Psi$-sensitive fluorescent dye $DiBAC_4(3)$ [16] as described previously [17]. Samples of log-phase cells treated with the protonophore carbonyl cyanide *m*-chlorophenyl hydrasone (CCCP, 20 μM) were used as positive control. Fluorescent cells were counted using a Leica DM2000 microscope as earlier described [17]. Total cell number was counted in transmitted light. About 1000 cells were counted for every sample and all experiments were conducted 3–6 times on separate days.

ATP concentration was measured using the luciferin-luciferase ATP monitoring kit (“Lumtek”, Moscow). For ATP extraction, 50 μl of the cell suspension was mixed with 450 μl of cell disruption reagent (DMSO). The extraction was completed within 0.5–1 min and the ATP concentration was determined according to the manufacturer's instructions.

2.5. Statistical analysis of the data

Each result is indicated as the mean value of three to five independent experiments ± the standard error of the mean (SEM). Significant difference was analyzed by Student's *t*-test. A P-value of 0.05 was used as the cut-off for statistical significance. Results were analyzed by means of the program packet Statistica 6 (StatSoft Inc. 2001).

3. Results

3.1. Determination of sensitivity of Eh and sulfide sensors to extracellular sulfur-containing reductants

Determinations of the sensitivity of Eh (platinum electrode) and sulfide sensors to various sulfur-containing substances, such as sulfide (Na_2S), sulfite (Na_2SO_3), cysteine (Cys), and glutathione (GSH) were performed under the same conditions that were used to bacterial growth studies. Both sensors and reference electrodes were placed in shaking (150 rpm) 250-ml flasks containing 100 ml of the cell-free M9 medium with glucose (1.5 g/l) at 37 °C. Compared to other sulfur-containing reductants, the addition of sulfide caused the greatest change in the potential of both Eh and sulfide sensors within a range of concentrations from 20 nM to 5 μM (Fig. 1A, B). For both sensors, the same lower threshold of sensitivity to sulfide (about 20 nM) was