Microbiological Research 167 (2012) 166–172

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.de/micres

Transmembrane glutathione cycling in growing *Escherichia coli* cells

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ARTICLE INFO

Article history:
Received 11 January 2011
Received in revised form 17 May 2011
Accepted 22 May 2011

Keywords:
Bacteria
Glutathione
Transport
Stresses

ABSTRACT

Glutathione (GSH) plays an important role in bacterial cells, participating in maintenance of redox balance in the cytoplasm and in defense against many toxic compounds and stresses. In this study we demonstrate that in aerobic, exponentially growing *Escherichia coli* culture endogenous reduced glutathione undergoes continuous transmembrane cycling between the cells and medium. As a result of an establishment of a dynamic balance between GSH efflux and uptake, a constant extracellular concentration of GSH counting per biomass unit is maintained. The magnitude of this concentration strictly depends on external pH. GSH cycling is carried out in respiring cells and disturbed by influences, which change the level of $\Delta\mu H^+$ and ATP. Export of GSH is modified by phosphate deficiency in the medium.

1. Introduction

Glutathione (GSH) is the most abundant low-molecular-weight thiol compound in many organisms from bacteria to mammals. In eukaryotic cells, GSH plays an important role in defense against oxidative stress and xenobiotics, in control of enzymes activity and gene expression and in intracellular signal transduction (Meister and Anderson 1983; Filomeni et al. 2002). Its functions in bacterial cells are studied to a lesser extent. Glutathione is contained in millimolar concentrations in most gram-negative and in some gram-positive bacteria (Fahey et al. 1978). In these organisms, GSH is essential in defense against chlorine compounds, methylglyoxal, some antibiotics and heavy metals (Smirnova and Oktyabrsky 2005). As a component of the thiol redox systems, GSH together with glutaredoxins reduces oxidized SH-groups in global regulators OxyR and Fnr (Aslund et al. 1999; Tran et al. 2000). Mutants of *E. coli* lacking glutathione reveal increased sensitivity to hyperosmotic and cold stresses (Smirnova and Oktyabrsky 2005).

More than 20 years ago it has been shown that bacteria *E. coli* and *Salmonella typhimurium* can accumulate reduced glutathione in the growth medium during the exponential phase and then hydrolyze it by γ-glutamyltranspeptidase (GGT) in the stationary phase (Owens and Hartman 1986a; Suzuki et al. 1987). Later a novel type of glutathione transporter encoded by the genes *gsiA*, *-B*, *-C*, *-D* (*yliA*, *-B*, *-C*, *-D*) was found (Suzuki et al. 2005). This transporter imports extracellular glutathione into the cytoplasm in an ATP-dependent manner and belongs to the ATP-binding cassette superfamily. The only known transporter, which exports GSH from the cytoplasm to periplasm in *E. coli*, is the ATP-binding cassette-type transporter CydDC (Pittman et al. 2005). In early studies, it has been shown that an important function of GSH export is to protect the cells from external, potentially toxic, electrophilic compounds (Owens et al. 1986b). Extracellular GSH may also reduce exogenous cystine to cysteine outside the cells to provide its utilization by *E. coli* (Smirnova et al. 2005).

Earlier under various stresses in *E. coli*, we observed reversible changes in intra- and extracellular GSH levels, which were dependent on the type of stress and the degree of adaptation to new conditions (Smirnova et al. 2000; Smirnova and Oktyabrsky 2005). The aim of this work was to investigate the relationship between the level of glutathione in *E. coli* cultures and environmental and intracellular parameters.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Strains of *E. coli* used in this study are K12 (lab. strain collection), SH646 and SH641 *ggt-2* (Hashimoto et al. 1997), AN2342 and AN2343 *cydD*1 (Cruz-Ramos et al. 2004), RI89 *phoR* (Rietsch et al. 1997), NM1101 *gshA* (this study). The strain NM1101 was constructed by introduction of *gshA*::Tn*10* Kan^r into *E. coli* K12 via PI transduction (Miller 1972). Bacteria were grown in M9 minimal glucose medium (Miller 1972). After centrifugation cells grown overnight were resuspended in 100 ml of fresh medium (OD_{600} = 0.1) and then were grown aerobically at 37 °C in 250-ml flasks with shaking at 150 rpm. Cell growth was monitored by measurement of the optical density at 600 nm. For phosphate starvation

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doi:10.1016/j.micres.2011.05.005

experiments, cells were cultivated in 100 mM 3-(N-morpholino) propanesulfonic acid (MOPS) minimal medium (Neidhardt et al. 1974) containing 4 g/l glucose.

2.2. Determination of GSH and GSSG

For the determination of extracellular glutathione, samples of the cell suspensions were passed through 0.45-μm-pore-size membrane filters. One portion of this filtrate was assayed for total glutathione, the other portion after treatment with NEM (2 mM) for 1 h and 7-fold ether extraction to remove GSH was used for the measurement of GSSG. For the determination of intracellular total glutathione, 10 ml samples of cell culture was harvested by centrifugation (8000 rpm for 5 min) at different time points, suspended in 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 rpm for 5 min), supernatant was adjusted to pH 7.5 with KOH, frozen, and centrifuged to eliminate the potassium perchlorate. GSH and GSSG were measured using the DTNB-glutathione reductase recycling method (Tietze 1969). 200 μl of the probe was added to a mixture of 100 mM sodium phosphate/5 mM EDTA buffer (pH 7.5), 0.6 mM DTNB, and 0.3 U of glutathione reductase (final volume 1 ml). The assay was started by the addition of 0.2 mM HADPH and was performed on a Shimadzu UV-1700 spectrophotometer. The change in A_{412} over a 6-min period was used to calculate the total GSH or GSSG after comparing this to standard curves prepared with known quantities of GSH or GSSG. The standard samples of GSH and GSSG were treated as well as samples of the cell suspensions. The amount of GSH in samples was estimated by subtracting GSSG levels from the total GSH value. GSH and GSSG were assayed on the same day as sample preparation.

2.3. Measurement of ATP concentrations

ATP concentration was measured by using a luciferin-luciferase ATP monitoring kit ("Lumtek", Moscow). For ATP extraction, 50 μl of cell suspension was mixed with 450 μl of cell disruption reagent dimethyl sulfoxide (DMSO). Extraction was finished within 0.5–1 min and the ATP concentration was determined as recommended by the manufacturer.

2.4. Alkaline phosphatase activity assays

Cells were grown in MOPS medium containing 4 g/l glucose and 2 mM K_2HPO_4 to OD_{600} = 0.6, washed and incubated in 100 mM MOPS without phosphate for an hour to induce the synthesis of alkaline phosphatase activity (AP) in the *phoR*$^+$ strains, which were not constitutive for Pho regulon. Cell suspension was concentrated 10-fold, treated for 40 min with 5% chloroform and 0.0025% sodium dodecyl sulfate (Michaelis et al. 1986) and AP was determined using AP colorimetric kit with *p*-nitrophenyl phosphate ("Vital Diagnostics", St. Petersburg).

2.5. Measurement of respiration rate

The respiration rate of *E. coli* cells was determined using the polarographic Clark electrode in a chamber with temperature control and continuous agitation. The chamber contained 100 μl of culture (the final density OD_{600} = 0.15), 20 μl of 40% glucose, compound tested, and M9 medium to the total volume of 5 ml. For these experiments, cells were grown in M9 medium to mid-log phase and then washed and resuspended in M9 medium without glucose. When needed, cell culture was preliminary treated with 10 mM EDTA.

2.6. Statistical analysis

Each result is indicated as the mean value of at least three independent experiments ± the standard error of the mean (SEM). Results were analyzed by means of program packet Microsoft Excell and Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA, 2001).

3. Results

3.1. GSH excretion from E. coli cells transferred into fresh medium

Excretion of GSH from *E. coli* cells was investigated using the wild-type bacteria (K12, AN2342, SH646) and mutants of the genes *ggt* (SH641), *gshA* (NM1101), and *cydD* (AN2343) encoding γ-glutamyltranspeptidase (GGT), γ-glutamylcysteine synthetase, and CydD subunit of a heterodimeric transporter CydDC, respectively. As GGT participates in uptake extracellular GSH (in the form of its fragments) into the cytoplasm of *E. coli* cells (Suzuki et al. 1987), the *ggt* mutant could be suitable for investigation of GSH efflux when its influx is diminished. The *gshA* mutant lacking of its own glutathione may be used for study of GSH import in the absence of export. Transporter CydDC mediates transmembrane export of glutathione, and the *cydD1* mutant enables to test the involvement of this transport system in GSH efflux in growing cells (Pittman et al. 2005).

Irrespective of whether glucose was present, a transfer of bacteria *E. coli* K12 (wt) and SH641 (*ggt*) into the fresh aerated medium was accompanied by a rapid excretion of glutathione from the cells (Fig. 1A). The main part of this glutathione was in reduced form, the concentration of oxidized glutathione (GSSG) did not exceed 10%. At 5 min after the addition of cells, the level of GSH in the medium reached 2.5 μmol/g dry wt., whereas the intracellular level of GSH at this time decreased by 2.2 μmol/g dry wt. After this, the rate of GSH efflux in the wild-type culture with glucose strongly decreased, and during all further period of cultivation the concentration of extracellular GSH, counting per unit of biomass, was maintained at a constant level. In the absence of glucose, the initial increase in GSH_{out} in the wild-type culture was followed by its gradual decrease and after 40 min the level of GSH in the medium was close to zero (Fig. 1A). The concentration of GSH_{out} in the growing culture of the *ggt* mutant continued to increase during all period of observation, though with a smaller rate, than in the first rapid phase of efflux. The rate of GSH export in the *ggt* mutant during the second slow phase was 0.036 μmol/g dry wt per min. In medium without glucose these cells did not demonstrate a significant decrease in extracellular glutathione, an observation, which testifies that the main part of GSH returns into the cytoplasm with GGT participation.

We proposed that the second phase of the increase in GSH_{out} in the growing *ggt* mutant could be a result of glutathione export in the absence of its uptake. On the contrary, in the wild-type cells simultaneously with GSH excretion there is a GGT-dependent uptake of GSH. The maintenance of constant concentration of GSH_{out} in the growing wild-type cells can be observed only if the rate of glutathione excretion corresponds to the rate of its uptake. To test this, we have measured a rate of exogenous GSH import in the *gshA* mutant growing in M9 medium with glucose. For this purpose the bacterial culture was grown to the optical density of 0.8 and then different concentrations of GSH were added. The rates of GSH uptake were 0.039, 0.082, 0.112 and 0.262 μmol/g dry wt per min at GSH concentrations 2, 4, 10 and 100 μM, respectively.

The level of intracellular GSH did not exceed 1 μmol/g dry wt when 100 μM of exogenous GSH was added. This result further confirms the main role of GGT, which hydrolyzes glutathione in the periplasm, in the GSH absorption. K_m and V_{max} values for GSH uptake as calculated from experimental data are 13.3 μM

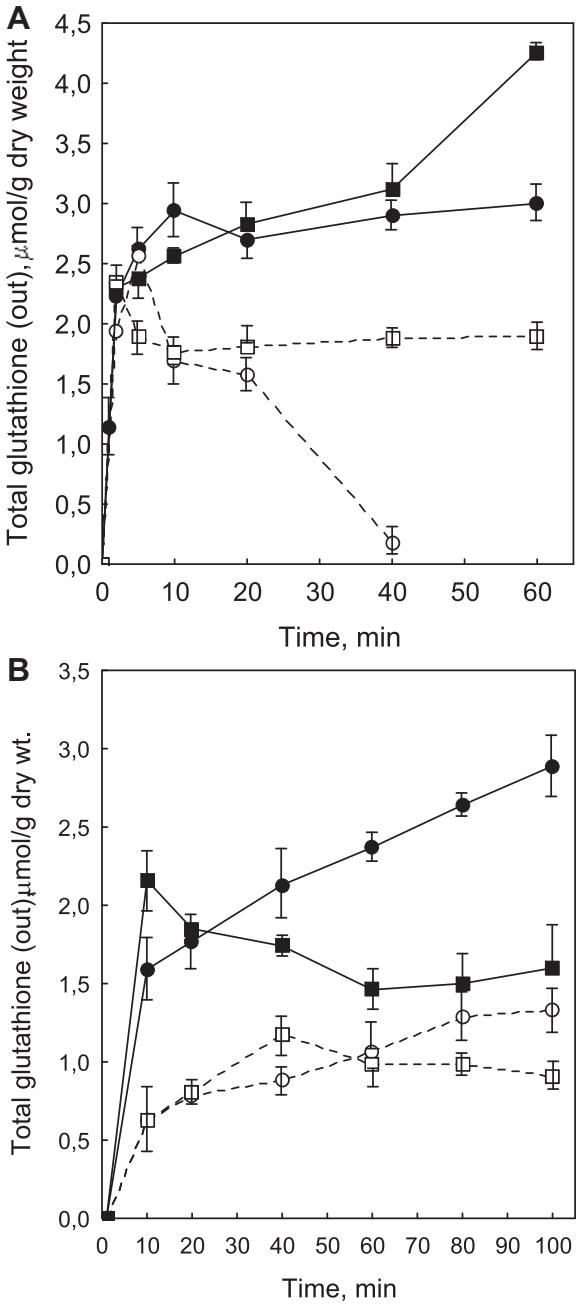

Fig. 1. Glutathione excretion from *E. coli* cells transferred to the fresh medium. A. Strains K12 (wt) (circles) and SH641 (*ggt*) (squares) were grown in M9 medium as described in Section 2 to an optical density of 0.6. After centrifugation and washing, cells from each culture were transferred to 2 flasks with 50 ml M9 medium with glucose (filled symbols) or without glucose (empty symbols) to the initial OD_{600} of 0.5. At various time points, samples for GSH and GSSG determination were removed by membrane filtration. B. Strains AN2342 (wt) (circles) and AN2343 (*cydD*) (squares) were grown in MOPS medium with 2 mM K_2HPO_4 as described in Section 2. After centrifugation and washing, cells were transferred to MOPS medium with 2 mM phosphate (filled symbols) or without phosphate (empty symbols).

and 0.31 μmol/g dry wt per min, respectively. At concentration of GSH_{out} about 1.5 μM observed in our experiments, the rate of glutathione uptake was 0.031 μmol/g dry wt per min that is close to the rate of GSH export in the second phase in growing *ggt* mutant (0.036 μmol/g dry wt per min). Thus, maintenance of a constant concentration of GSH_{out} counting per unit of biomass in growing wild-type culture is probably a result of dynamic balance between processes of glutathione export and its uptake into the cells with GGT participation.

Table 1
Effect of respiratory inhibition on extracellular and intracellular glutathione concentration (μmol/g dry wt) in *E. coli* K12.

	Control	Without agitation	Glucose starvation	Azide
GSH_{out}	3.45 ± 0.1	2.6 ± 0.1	1.9 ± 0.3	1.6 ± 0.1
GSH_{in}	10.3 ± 0.2	11.6 ± 0.2	14.4 ± 0.3	13.3 ± 0.1

The presence of the *cydD1* mutation or sodium orthovanadate addition (2 mM) did not prevent GSH excretion under a transfer of *E. coli* cells into the fresh aerated M9 medium. These data suggest that an alternative system of GSH export in *E. coli* can exist besides ATP-dependent orthovanadate sensitive transporter CydDC. However, when the wild-type and *cydD* mutant cells were transferred into the fresh MOPS medium with 2 mM phosphate (in contrast to 64.2 mM in M9 medium), an essential difference was observed between kinetic characteristics of GSH efflux during the second phase, and the GSH_{out} concentration in the wild-type strain was significantly higher (2.6 ± 0.05 μmol/g dry wt) than that in the mutant (1.5 ± 0.29 μmol/g dry wt) (Fig. 1B, filled symbols). This finding indicates that the CydDC transporter may contribute to the GSH export in the second phase.

When *E. coli* K12 cells grown in aerobic conditions were transferred into the M9 medium previously bubbled with nitrogen, the level of extracellular GSH remained low and reached 0.2 ± 0.08 and 0.42 ± 0.09 μmol/g dry wt. in the absence and in the presence of glucose, respectively. The start of aeration resulted in a rapid 3-fold increase of the GSH_{out} level. Various influences leading to inhibition of respiration (glucose exhaustion, cessation of agitation and treatment with 30 mM of sodium azide) resulted in a significant decrease in the level of GSH_{out} and an increase in glutathione concentration in the cytoplasm (Table 1). The changes in GSH_{out} observed during inhibition of respiration are probably a consequence of arrest of GSH export, while GSH import was continued. Thus, respiration may be an obligatory condition of the second (slow) phase of GSH excretion.

In aerobic cultures, GSH excretion was inhibited also in the presence of sodium sulfide (6.4 mM) and potassium ferrocyanide (0.5 mM), which reduced redox potential (Eh) of the medium measured by platinum electrode by 500 and 90 mV, respectively. Under these conditions, GSH_{out} level was 2-fold lower than that in the control culture.

3.2. Effect of ATP synthesis inhibitors and ionophores on GSH distribution between cells and medium

To decrease ATP synthesis, the inhibitor of membrane F_1F_0 ATP synthase N,N′-dicyclohexylcarbodiimide (DCCD, 2 mM) and sodium arsenate (5 mM) competing with phosphate for ADP binding were used. Treatment of *E. coli* K12 cells with DCCD and arsenate led to a decrease in specific growth rate to 0.5 ± 0.01 and 0.29 ± 0.005 h^{-1}, respectively, compared to 0.65 ± 0.05 h^{-1} in control culture. The ATP level was decreased from 5.88 ± 0.17 μmol/g dry wt in control to 4.47 ± 0.06 and 4.25 ± 0.07 μmol/g dry wt under DCCD and arsenate addition. Both reagents provoked a significant increase in the level of GSH_{out} (Table 2). After DCCD treatment the increase in GSH_{out} (a gain outside was equal 4.9 ± 0.37 μmol/g dry wt) was due to the equivalent decrease in GSH_{in} (a drop inside the cells was 5.3 ± 0.27 μmol/g dry wt). In the case of arsenate, 10.7-fold increase in the GSH_{out} concentration occurred in the absence of essential changes in the level of GSH_{in}. This may suggest that arsenate exposure stimulates not only GSH excretion but also the rate of its synthesis.

In another set of experiments the influence of changes in membrane potential ($\Delta\psi$) or proton concentration gradient (ΔpH) components of the proton motive force ($\Delta\mu H^+$) on intra- and extra-

Table 2
Influence of ATP synthesis inhibitors and acetate on growing *E. coli* K12.

Inhibitor	GSH_{out}	GSH_{in}	Growth rate	ATP	Respiration rate
Arsenate (5 mM)[a]	10.7 ± 0.4	0.9 ± 0.02	0.45	0.72 ± 0.01	n.d.
DCCD (2 mM)[a]	2.9 ± 0.1	0.5 ± 0.03	0.77	0.76 ± 0.08	0.45
CCCP (0.02 mM)[b]	1.9 ± 0.1	0.8 ± 0.03	0	0.54 ± 0.015	0.47
Acetate (50 mM)[c]	1.8 ± 0.1	0.7 ± 0.02	0.5	0.83 ± 0.014	0.33

[a] MOPS with 2 mM of K_2HPO_4.
[b] Low K^+ medium, which was identical to M9 except KH_2PO_4 was replaced by NaH_2PO_4 and 1 mM of KCl was added. Before CCCP addition cells were treated with 10 mM of EDTA.
[c] Under sodium acetate addition, bacteria were grown in M9 medium adjusted to pH 6.5.
Values from treated samples were divided by those from the corresponding control (untreated cells). For comparison, all values were normalized to 1.

cellular GSH concentration was investigated. Use of a protonophore (CCCP) abolishes both the $\Delta\psi$ and the ΔpH. Treatment of *E. coli* cells with sodium acetate is followed by cytoplasmic acidification when ΔpH decreases and $\Delta\psi$ increases (Bakker and Mangerich 1983). The potassium ionophore valinomycin commonly is used to decrease $\Delta\psi$. ΔpH is usually dissipated by using nigericin which carries out electroneutral K^+/H^+ exchange. CCCP addition resulted in complete cessation of growth. The rate of respiration increased by 16% during the first 10 min after the treatment and then decreased by 53%. Addition of sodium acetate (50 mM) caused transient growth cessation, which was followed by the growth resumption at a half initial rate. The rate of respiration decreased by 67% in these conditions. During CCCP and acetate treatment, a decrease in ATP concentration to 3.07 ± 0.09 and 4.69 ± 0.08 μmol/g dry wt was observed as compared to 5.65 ± 0.1 μmol/g dry wt in control. Valinomycin and nigericin inhibited growth by 38 and 68% and increased the rate of respiration by 37 and 34%, respectively.

In contrast to valinomycin and nigericin, which produced a gradual increase in GSH_{out} without essential effect on GSH_{in}, the treatment of cells with CCCP and sodium acetate led to a rapid efflux of a part of intracellular GSH from the cells. GSH excretion was ceased at 10–15 min, then the level of GSH_{out} began to decrease and GSH_{in} gradually increased after minimal level reached at 30 min. Interestingly, we have earlier observed a rapid efflux of GSH from *E. coli* cells under addition of 10 μg/ml of gramicidin S, which abolished $\Delta\mu H^+$ by forming H^+, K^+ and Na^+-channels in phospholipid membrane (Smirnova et al. 2003).

3.3. Influence of external pH on extracellular GSH level

Rapid changes in external pH from 6.8 to 5.5 or from 6.8 to 8.5 caused a transient growth cessation followed by a restoration of growth with slower rates. Both pH shifts led to GSH excretion but the kinetics of these processes was significantly different (Fig. 2A). Under acidic pH shift, after rapid GSH efflux a phase of slower export was observed, and 60 min later GSH_{out} reached a level 3.7-fold higher than that in control. GSH_{in} gradually decreased during all experimental period (data not shown). Under alkaline shift, a short efflux of glutathione with the subsequent stabilization of GSH_{out} at a new level was observed, while GSH_{in} was restored to a base value after an initial decrease.

In other experiment, *E. coli* cells grown at pH 7.0 were transferred in the flasks with M9 medium adjusted to pH 5.5, 6, 7, 7.5, 7.8, 8.5 and after 30 min incubation the level of extracellular GSH was measured. The data obtained revealed the existence of a striking dependence of the extracellular GSH level on the external pH. Decrease or increase in external pH was accompanied by an elevating of GSH concentration in the medium (Fig. 2B). It is remarkable that the lowest GSH_{out} value was observed at external pH 7.5, which is in immediate proximity from intracellular pH 7.6 maintaining in the cytoplasm over a wide range of external pH (Booth 1985).

3.4. Relationship between GSH efflux and availability of inorganic phosphate

Transfer of *E. coli* into the fresh MOPS medium without phosphate inhibited the first phase of GSH efflux at least two fold (Fig. 1B, empty symbols). In contrast, in the medium supplemented with 2 mM phosphate GSH_{out} concentration was supported at a higher level during all experimental period suggesting the significant role of phosphate in GSH export (Fig. 1B, filled symbols). The further experiments have shown that exhaustion of phosphate in the growing wild-type culture led to a decrease in the concentration of extracellular GSH (Fig. 3A). At the same time, a significant inhibition of growth and a twofold decrease in ATP concentration was observed. After 60 min under phosphate starvation, GSH_{out} was recovered to the base level. The increase in GSH_{out} occurred in parallel with the phosphate (Pho) regulon induction, which was monitored by measurement of the alkaline phosphatase (AP) activity (Fig. 3B). The intracellular GSH concentration continued to increase during phosphate starvation and exceeded that in the control by 2-fold after 60 min of phosphate exhaustion (22.8 ± 0.7 μmol/g dry wt in starved culture and 11.2 ± 0.2 μmol/g dry wt in control). Addition of 2 mM K_2HPO_4 in the starved culture resulted in a rapid efflux of a significant portion of the intracellular GSH pool to the medium (Fig. 3A). The intracellular GSH level decreased to 9.9 ± 1.0 μmol/g dry wt in these conditions. Thereafter, GSH efflux ceased and the concentration of extracellular GSH began to decrease in parallel with a decrease in AP activity.

Mutation in the *phoR* gene, coding one of the two proteins of two-component regulatory system (PhoR and PhoB) of the Pho regulon results in constitutive expression of Pho regulon genes in the presence of phosphate (Wanner 1996). In contrast to the wild-type, an increased excretion of GSH was observed under the growth of the *phoR* mutant in the MOPS (with 2 mM phosphate) or in M9 medium and considerable amount of glutathione was accumulated in the growth medium (Fig. 3A). The intracellular concentration of GSH was constant in these conditions (3.24 ± 0.2 μmol/g dry wt) and was maintained at nearly the same level as the extracellular concentration of GSH in the wild-type culture. In the absence of phosphate, the *phoR* mutant ceased to export glutathione and GSH_{out} decreased during further cultivation. The addition of phosphate to the phosphate starved bacteria was accompanied by a rapid efflux of glutathione and its extracellular concentration reached the level accumulated in the culture growing in the presence of phosphate (Fig. 3A).

4. Discussion

In this study we have shown that in exponentially growing aerobic cultures of *E. coli* a continuous cycling of GSH between the cells and growth medium occurs (Fig. 4). As a result of dynamic balance between efflux of GSH and its influx the constant concentration of extracellular GSH counting per biomass unit is maintained. The

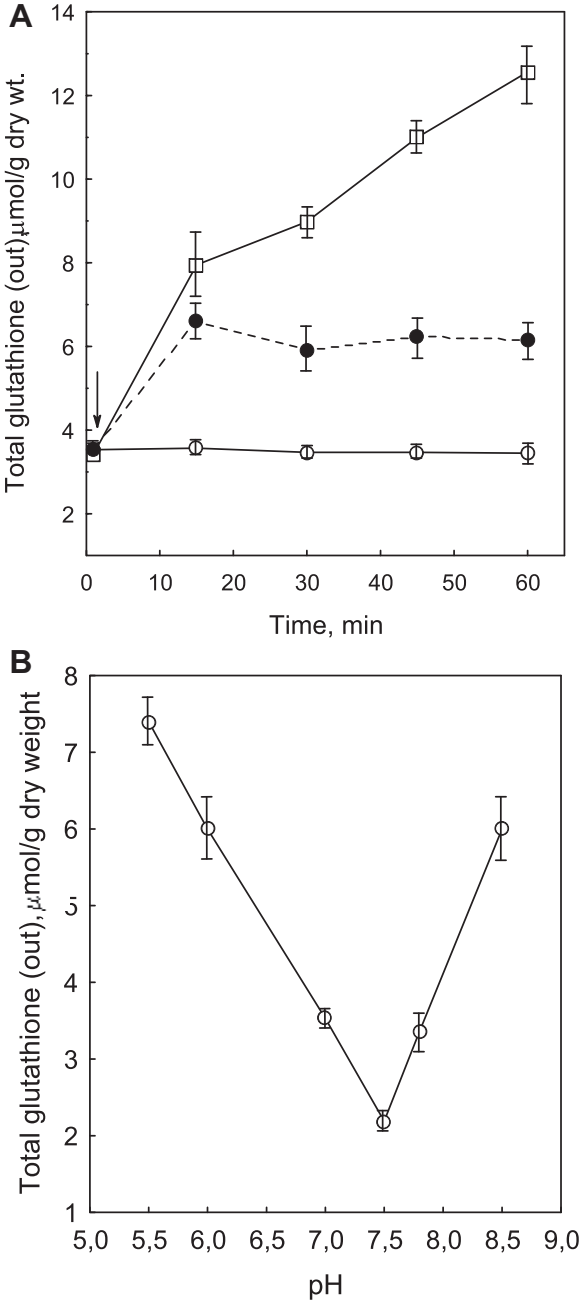

Fig. 2. Dependence of the concentration of extracellular GSH on the medium pH in growing *E. coli*. A. Bacteria were grown in M9 medium to an optical density of 0.6. At the time indicated by the arrow the pH of the external environment was shifted during 2 min from 6.8 to 5.5 (empty squares) using 0.5 M HCl or from 6.8 to 8.5 (filled circles) using 0.5 M KOH. Empty circles show the control culture growing at pH 7.0. Samples were harvested by centrifugation. B. Bacteria were grown in M9 medium to an optical density of 0.8. Then cells were centrifuged and removed into the flasks (the initial OD_{600} = 0.3) containing M9 medium adjusted to pH 5.5, 6.0, 7.0, 7.5, 7.8, and 8.5. Samples were harvested by membrane filtration after 30 exposure to the different pH values. The values of GSH are presented as means ± standard error (SEM) from three or more different experiments.

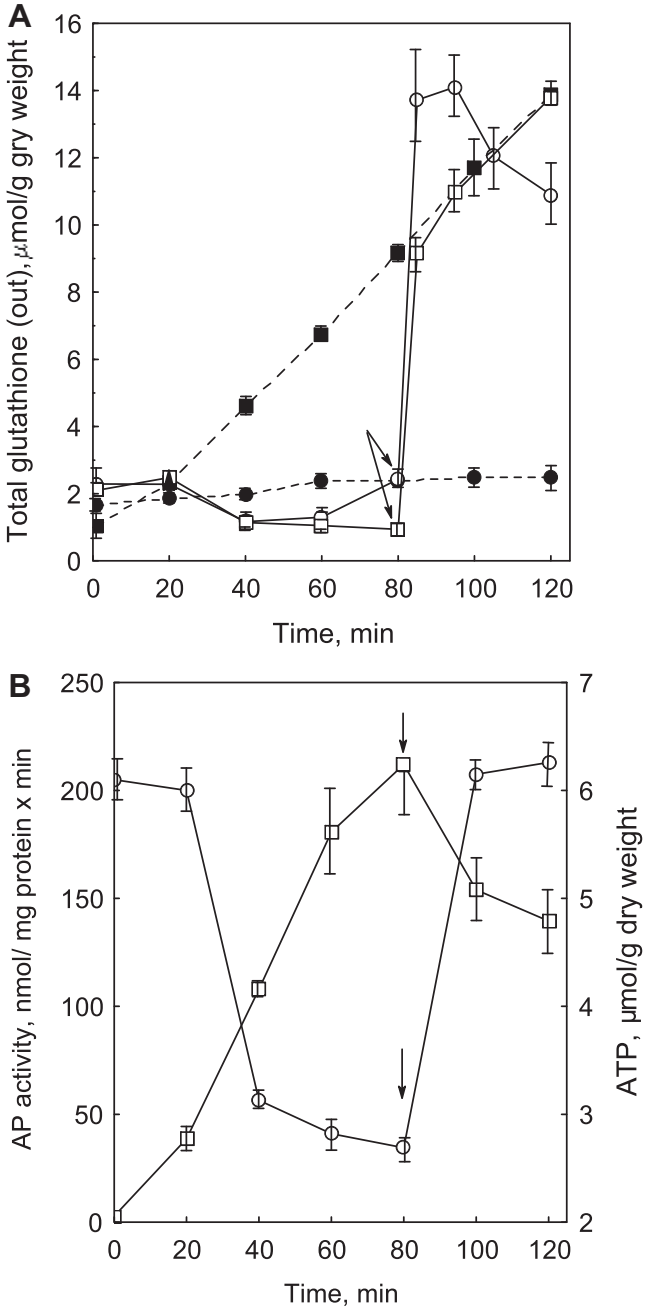

Fig. 3. Effect of phosphate starvation on the GSH export, ATP level and alkaline phosphatase activity in *E. coli* K12. *E. coli* cells were grown in MOPS with 4 mM K_2HPO_4 to an optical density of 0.6. Then cells were centrifuged, removed into the flasks containing the MOPS medium with or without phosphate and grown during 2 h. A. *E. coli* K12 (circles) and RI89 (*phoR*) (squares). Dashed lines show extracellular GSH in the presence of phosphate; solid lines show extracellular GSH in the absence of phosphate and after addition of 2 mM K_2HPO_4 at the time indicated by the arrows. B. The alkaline phosphatase activity (squares) and the level of ATP (circles) in *E. coli* K12. The arrows indicate the time of addition of phosphate.

main system participating in GSH uptake is GGT. It seems likely, that the necessary requirement for extracellular GSH homeostasis is steady state respiration and maintenance of constant $\Delta\psi$ and ΔpH in cells. Exposure to protonophores, ionophores, weak acid and changes in external pH resulted in a transient or constant stimulation of glutathione efflux. The strict dependence of the extracellular GSH level on the external pH value has an especial interest. The participation of protons in GSH excretion may be direct, if transport system uses ΔpH as a driving force, or indirect, if, for example, the activity of transporter is modulated by pH value.

In our opinion, an important finding is also the connection between GSH excretion, ATP synthesis and phosphate metabolism. Tight connection between GSH export and availability of inorganic phosphate was revealed both in the phosphate influence on the steady state level of GSH_{out}, and in the ability of the regulatory protein PhoR to modify GSH efflux. This connection may be mediated

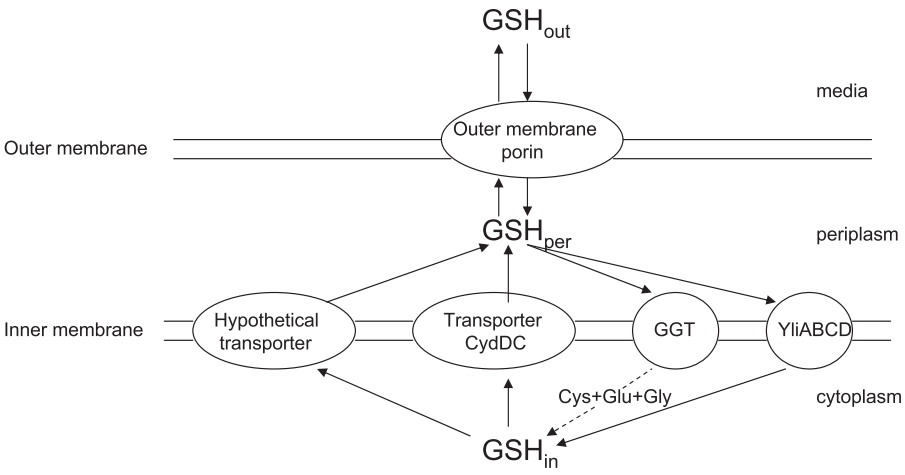

Fig. 4. It is supposed that the CydDC transporter and an unknown additional carrier mediate the export of glutathione to the periplasm. YliABCD and GGT promote uptake of GSH from the medium into the cytoplasm. In the periplasm, glutathione may take part in the antioxidant defense and redox homeostasis. From the periplasm, glutathione may pass to the medium through the outer membrane porins. In growing cells, a dynamic balance between GSH efflux and uptake is changed under different stresses.

by changes in energetic parameters under phosphate deficiency or by direct involvement of phosphate in GSH transport.

In mammalian cells, GSH is synthesized in the cytosol, whereas its degradation by the plasma membrane-bound enzyme GGT occurs exclusively in the extracellular space, and thus export from the cell is required for normal GSH turnover (Meister and Anderson 1983). In yeast *Saccharomyces cerevisiae*, it has been shown that endogenously synthesized reduced glutathione undergoes intra/intercellular cycling during growth to stationary phase. Glutathione efflux is strongly influenced by extracellular pH (Perrone et al. 2005).

What can be the physiological meaning of GSH cycling? Glutathione, probably, may be involved in the regulation of redox reactions in the periplasm as a source of oxidizing or reducing power. This is supported by the findings that exported glutaredoxin 3 can promote disulfide bond formation in the periplasm and requires the glutathione biosynthesis pathway for its function. Authors have also noted, that glutathione may pass from the periplasm to the medium through the outer membrane porins. As a result, extracellular glutathione, probably, is at equilibrium with the periplasmic space (Eser et al. 2009). Additionally, it has been shown that in exponentially growing *E. coli* the adventitious autoxidation of dihydromenaquinone in the cytoplasmic membrane releases a steady flux of superoxide into the periplasm (Korshunov and Imlay 2006). This endogenous superoxide may create oxidative stress in the periplasm and the GSH cycling observed in our experiments can probably have a protective function. The production of reactive oxygen species (ROS) may increase in situations disturbing a normal electron flow in the respiratory chain. The rise in the GSH export into the periplasm in these conditions may contribute to the protection against the increased ROS flux in that compartment.

Potential function of the GSH cycling may be regulation of transmembrane ionic fluxes. In *E. coli* glutathione is a negative regulator of the KefB and KefC potassium channels (Meury and Kepes 1982; Booth et al. 1996). The activation of the KefB and KefC channels elicits potassium efflux and the lost potassium is replaced by protons promoting a lowering of cytoplasmic pH. Recently, it has been shown that the two-component transporter KefFC (KefF is ancillary protein) possesses a monovalent cation/proton antiport activity, which is inhibited by GSH (Fujisawa et al. 2007). Remains unknown, whether these transport systems contribute to the intracellular pH homeostasis and whether the observable GSH changes can significantly influence their activity, but in our experiments the *gshA* mutants revealed enhanced sensitivity to alkaline shift than the parent strain (data not shown). Earlier we have shown, that synchronous changes of intracellular K+-ions and low-molecular thiols were observed not only at pH-shifts, but also at other stresses, especially at the initial stage of the response to stresses (Oktyabrsky and Smirnova 1993; Smirnova and Oktyabrsky 1995).

There is another aspect of the role of GSH export in the defense against oxidative stress. It has been found that when cysteine homeostasis is disrupted, intracellular cysteine acts as an adventitious reductant of free iron in the Fenton reaction and thereby promotes oxidative DNA damage (Park and Imlay 2003). Cysteine reduces ferric iron better than GSH does, so cysteine incorporation in GSH may be one of the mechanisms of the protection against oxidative stress. The transmembrane cycling of GSH observed in exponentially growing *E. coli* culture may be an element of cysteine homeostasis. Various stresses result in inhibition of constructive metabolism and growth, and simultaneously with enhanced ROS production an increase in cysteine pool may occur. In such conditions the binding of potentially toxic cysteine in GSH and GSH excretion from the cells is an important process.

Acknowledgements

This work was supported by grant of Program MCB RAS and by grant of Russian Foundation for Basic Research No 07-04-96013. We thank J. Beckwith, H. Suzuki and R. Poole for the generous gift of strains.

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