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Original article

Extracellular superoxide provokes glutathione efflux from *Escherichia coli* cells

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Abstract

The aim of the study was to elucidate a possible relationship between transmembrane cycling of glutathione and changes in levels of external superoxide. Exposure of growing *Escherichia coli* to exogenous reactive oxygen species (ROS) generated by xanthine and xanthine oxidase (XO) stimulates reversible glutathione (GSH) efflux from the cells that is considerably lowered under phosphate starvation. This GSH efflux is prevented by exogenous SOD, partially inhibited by catalase, and is not dependent on the GSH exporter CydDC. The γ-glutamyl transpeptidase (GGT) deficiency completely prevents a return of GSH to the cytoplasm. In contrast to wild-type *E. coli*, mutants devoid of GGT and glutathione reductase (GOR) show enhanced accumulation of oxidized glutathione in the medium after exposure to xanthine and XO. Under these conditions, *sodC*, *ggt* and especially *gshA* mutants reveal more intensive and prolonged inhibition of growth than wild-type cells. Treatment with XO does not influence *E. coli* viability, but somewhat increases the number of cells with lost membrane potential. In summary, data obtained here indicate that transmembrane cycling of GSH may be involved in *E. coli* protection against extracellular ROS and may promote rapid growth recovery.

Keywords: Glutathione; Superoxide; Hydrogen peroxide; CuZnSOD; Membrane potential; *Escherichia coli*

1. Introduction

All aerobic organisms experience a steady flux of endogenously generated reactive oxygen species (ROS), the result of inadvertent transfer of either a single electron or two consecutive electrons from solvent-exposed redox moieties of flavoenzymes to oxygen, thereby forming superoxide or hydrogen peroxide, respectively [1–3]. The rate of endogenous superoxide production in the cytoplasm has been estimated at around 5 μM/s, and approximately 15 μM/s H_2O_2 was formed in well-fed cells [4]. ROS can damage all kinds of cellular macromolecules, including DNA, lipids and proteins, and can disrupt metabolism by inactivating [4Fe–4S] dehydratases and mononuclear iron enzymes [4–6]. In order to cope with these oxidative burdens, *Escherichia coli* cells have evolved several enzyme-based defense systems that involve superoxide dismutases, alkyl hydroperoxide reductase and catalases [4,7]. Besides two intracellular SODs (MnSOD and FeSOD), *E. coli* contains CuZnSOD (also called SodC) in the periplasm. As the negatively charged superoxide anion cannot easily cross membranes at neutral pH, it has been suggested that the role of this enzyme is to protect bacteria against extracellular sources of superoxide, such as phagocytic cells, and to defend unidentified periplasmic targets against superoxide that leaks from respiratory chain components on the outer aspect of the cytoplasmic membrane [8,9]. It has been shown that the rate of periplasmic superoxide formation (about

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3 μM/s) approximates cytoplasmic superoxide production, and the highest yield of periplasmic superoxide is reached when cells are growing exponentially [9]. However, SodC is positively regulated by the RpoS system and is strongly induced in stationary phase [10]. Thus, the timing of SodC synthesis does not correlate with superoxide formation in the periplasm.

Besides antioxidant enzymes, most Gram-negative and some Gram-positive bacteria contain millimolar concentrations of low-molecular-weight antioxidant glutathione (GSH) [11]. GSH can non-enzymatically react with many ROS, including superoxide and H_2O_2 [12,13]. The levels and redox states of glutathione significantly change under peroxide and superoxide stress in *E. coli* cells [14]; however, mutants devoid of glutathione do not exhibit enhanced sensitivity to oxidative stress in exponentially growing culture [15,16]. Earlier reports have shown that *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 [17,18]. It has been suggested that an important function of this GSH export is to protect the cells against external potentially toxic electrophilic compounds [19]. Previously, we demonstrated that, in exponentially growing *E. coli*, endogenous reduced glutathione undergoes continuous transmembrane cycling between the cells and medium. This transmembrane GSH cycling is carried out only in respiring cells and is disturbed by influences which change the level of $\Delta\mu H^+$ and ATP [20]. A close connection between GSH cycling and respiration that produces a steady flux of superoxide into the periplasm enabled us to speculate that GSH efflux may protect this compartment against oxidative stress in exponentially growing cultures. The aim of this study was to investigate a probable relationship between GSH efflux and external superoxide. We show that external superoxide (and the product of its dismutation H_2O_2), which is generated by xanthine and XO, stimulates GSH efflux from *E. coli* cells. The presence of endogenous and exogenous GSH promotes fast growth resumption of *E. coli* after growth arrest caused by ROS generated by xanthine and XO, suggesting that GSH may participate in defense against extracellular ROS.

2. Materials and methods

2.1. Strains and growth conditions

A parental strain of *E. coli* BW25113 (wt) and single-gene knockout mutants JW2663 (*ΔgshA*), JW1638 (*ΔsodC*), JW3467 (*Δgor*) and JW3412 (*Δggt*) used in this study were from the Keio collection [21]. AN2343 (*cydD*1) was a generous gift from Prof. R. Poole [22]. The strains NM3021 (*katG::lacZ*) and NM3041 (*rpoS::lacZ*) were constructed by transformation of BW25113 with plasmids pKT1033 [23,24] and *pRS 415 KatF5* [25], respectively.

Bacteria were grown in M9 minimal glucose (0.15%) medium [26]. After centrifugation, cells grown overnight were resuspended in 100 ml of fresh medium (OD_{600} of 0.1) and then grown aerobically at 37 °C in 250-ml flasks with shaking at 150 rpm to OD_{600} of 0.5. For the study of exogenous superoxide effects, these cells were centrifuged and resuspended to OD_{600} of 0.25 in 50-ml flasks, each containing 10 ml of prewarmed M9 adjusted to pH 8 with KOH and supplemented with 0.15% glucose, 1 mM EDTA and 0.05 mM xanthine. Then *E. coli* cells were grown with shaking to OD_{600} of 0.5 and various concentrations of XO were added (0.01–0.06 U/ml) to generate superoxide. At 15-min intervals, 1.5-ml aliquots of culture were removed, passed through membrane filters and used for glutathione assays. Effects of exogenous H_2O_2 (10 and 50 μM) on GSH efflux were studied under the same conditions.

For phosphate starvation experiments, bacteria were cultivated in 3-(N-morpholino) propane sulfonic acid (MOPS) minimal medium [27] containing glucose (0.15%) and 0.4 mM K_2HPO_4. Cells grown overnight were centrifuged, resuspended in fresh medium with 0.1 mM K_2HPO_4 and grown at 37 °C with shaking at 150 rpm to OD_{600} of 0.6. For the study of exogenous superoxide effects, these cells were centrifuged, washed and resuspended to OD_{600} of 0.25 in 50-ml flasks, each containing 10 ml of prewarmed MOPS (with or without phosphate) adjusted to pH 8 with KOH and supplemented with 0.15% glucose, 1 mM EDTA and 0.05 mM xanthine. After 2-h incubation at 37 °C with shaking at 150 rpm, 20 μl XO (0.04 U/ml) or H_2O_2 (10 μM and 50 μM) were added to phosphate-starving cells and glutathione was determined at 15-min intervals.

The specific growth rate (μ) was calculated by equation $\mu = \Delta \ln OD_{600}/\Delta t$, where t is the time in hours.

2.2. Measurement of superoxide anion production

The rate of production of superoxide anion, generated in the reaction catalyzed by xanthine oxidase with xanthine as a substrate, was estimated using the ability of superoxide to reduce ferricytochrome *c* [9]; 50 ml flasks, each containing 10 ml of cell-free M9 adjusted to pH 8 with KOH and supplemented with 1 mM EDTA, 0.05 mM xanthine and 40 μM cytochrome *c* with or without catalase (500 U/ml) were incubated at 37 °C with shaking at 150 rpm. At intervals, 1.2 ml samples were withdrawn before and after addition of 20 μl xanthine oxidase and reduced cytochrome *c* was immediately determined as previously described [9].

2.3. Measurement of the H_2O_2 concentration

The H_2O_2 concentration in culture medium was determined before and after XO addition; 2-ml aliquots of culture were removed at intervals, passed through membrane filters and the H_2O_2 concentration in samples was measured by the Amplex Red-horseradish peroxidase detecting system (AR/HRP) [28] using spectrofluorimeter Shimadzu RF-1501 (λ_{ex} 563 nm and λ_{em} 587 nm).

2.4. Study of ROS-mediated exogenous glutathione oxidation in a cell-free system

To study the interaction of ROS generated by xanthine and XO with GSH, a model system without *E. coli* cells was used;

50-ml flasks, each containing 10 ml of M9 adjusted to pH 8, 0.15% glucose, 1 mM EDTA, 0.05 mM xanthine and 2 μM GSH were incubated at 37 °C with shaking at 150 rpm. At intervals, 1.5-ml aliquots were removed for GSH and GSSG assays before and after addition of 20 μl XO (0.04 U/ml) in the presence or absence of catalase (500 U/ml) and SOD (30 U/ml).

2.5. Determination of β-galactosidase activity and glutathione

β-Galactosidase activity was determined as described by Miller [26] in *E. coli* strains NM3021 and NM3041 carrying transcriptional gene fusions *katG::lacZ* and *rpoS(katF)::lacZ*.

Reduced and oxidized glutathione was measured using the DTNB-glutathione reductase recycling method [29] modified as previously described [20].

2.6. Study of cellular viability and membrane potential

For colony-forming studies, samples of control culture and cultures exposed to XO were washed and diluted in 0.9% NaCl and then mixed with molten soft LB-agar (0.8%) at 42 °C and poured onto agar plates containing solid LB-agar (1.5%). Colonies were counted over 24 h incubation at 37 °C.

For the "live-dead" test, culture samples (0.5 ml) were washed and resuspended in 0.5 ml 0.9% NaCl, treated with SYTO 9 (10 μM) and propidium iodide (PI, 5 μM) and held for 15 min in the dark at room temperature. 10 μl drops of this suspension were plotted on slides with 1% agarose, covered with cover glasses and then fluorescent cells were counted using Leica DM2000 microscope with a filter system I3 (excitation 450–490 nm, detection $\geq$ 515 nm, dichroic mirror 510 nm) to detect SYTO 9 fluorescence and filter system N2.1 (excitation 515–560 nm, detection $\geq$ 590 nm, dichroic mirror 580 nm) to detect PI fluorescence.

Uptake of fluorochrome bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] can be related to loss of membrane potential [30]. DiBAC was dissolved in 70% ethanol at 1 mg/ml and further diluted in deionized water to a final concentration of 100 μg/ml. Portions (20 μl) of this solution were added to 180-μl aliquots of *E. coli* culture to give final dye concentration of 10 μg/ml. The mixtures were held for 10 min in the dark at 37 °C and then 10 μl drops were plotted on slides with 1% agarose, and fluorescent cells were counted using a Leica DM2000 microscope with filter system I3. 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.

2.7. Statistical analysis of the data

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 Statistica 6 (StatSoft Inc. 2001).

3. Results

3.1. Effect of exogenous ROS generated by xanthine and xanthine oxidase on the growth rate and GSH efflux from E. coli cells

To investigate the effects of external superoxide on extracellular GSH, *E. coli* cells were exposed to superoxide, which was generated in the reaction catalyzed by xanthine oxidase, with xanthine as a substrate. In this assay, however, the substrate of the reaction is exhausted after a few minutes and the short-living superoxide anion is rapidly converted into the more stable hydrogen peroxide [31]. Under our conditions in cell-free medium, the rapid phase of superoxide-mediated reduction of cytochrome C was completed within 2.5 min; then, this reduced cytochrome C was gradually oxidized by hydrogen peroxide (Fig. 1S). When catalase was added, however, the second phase with a 100-fold lower rate of cytochrome C reduction was visible for at least 40 min. Exposure to ROS (superoxide and H_2O_2) produced by xanthine oxidase and xanthine led to transient growth inhibition and stimulation of the efflux of glutathione from exponentially growing cells of *E. coli* BW25113 (wt). The extent of growth inhibition and of GSH efflux was dependent on the enzyme concentration, that is, on the rate of ROS production (Fig. 1A). It should be noted that these effects were reversible: the specific growth rate was restored within 40 min, while GSH reached its basal level after about 60 min. In order to distinguish between the effects of superoxide and H_2O_2, we repeated experiments in the presence of enzymes able to selectively remove superoxide (superoxide dismutase) or hydrogen peroxide (catalase). Addition of exogenous SOD (60 U/ml) simultaneously with XO prevented GSH efflux (Fig. 1B). Supplementation with SOD 15 min after addition of XO led to a rapid decrease in extracellular GSH to the basal level. Exogenous catalase (500 U/ml) that was added simultaneously with XO did not completely prevent, but substantially diminished, GSH efflux (Fig. 1B). It should be noted that the presence of SOD or catalase simultaneously with XO influenced the extent of growth inhibition and shortened the time of growth recovery (Fig. 2S). SOD and catalase added without XO did not affect GSH efflux or bacterial growth (data not shown). When we checked the effects of H_2O_2 without XO, we found that hydrogen peroxide can also stimulate GSH efflux at concentrations ranging from 10 μM to 1 mM (Fig. 2). Hydrogen peroxide-mediated efflux of glutathione was dependent on the H_2O_2 concentration and was stopped immediately, while the specific growth rate was completely restored after initial inhibition. In summary, these data indicate that both superoxide and H_2O_2 can provoke GSH efflux, which coincides with a period of slow growth.

We had previously shown that export of GSH from *E. coli* cells is completely inhibited under phosphate starvation, and high levels of GSH may be accumulated in the cytoplasm [20]. Here, we demonstrated that an effect of superoxide and H_2O_2 on GSH efflux from phosphate-starved cells was considerably diminished in comparison to growing culture supplemented

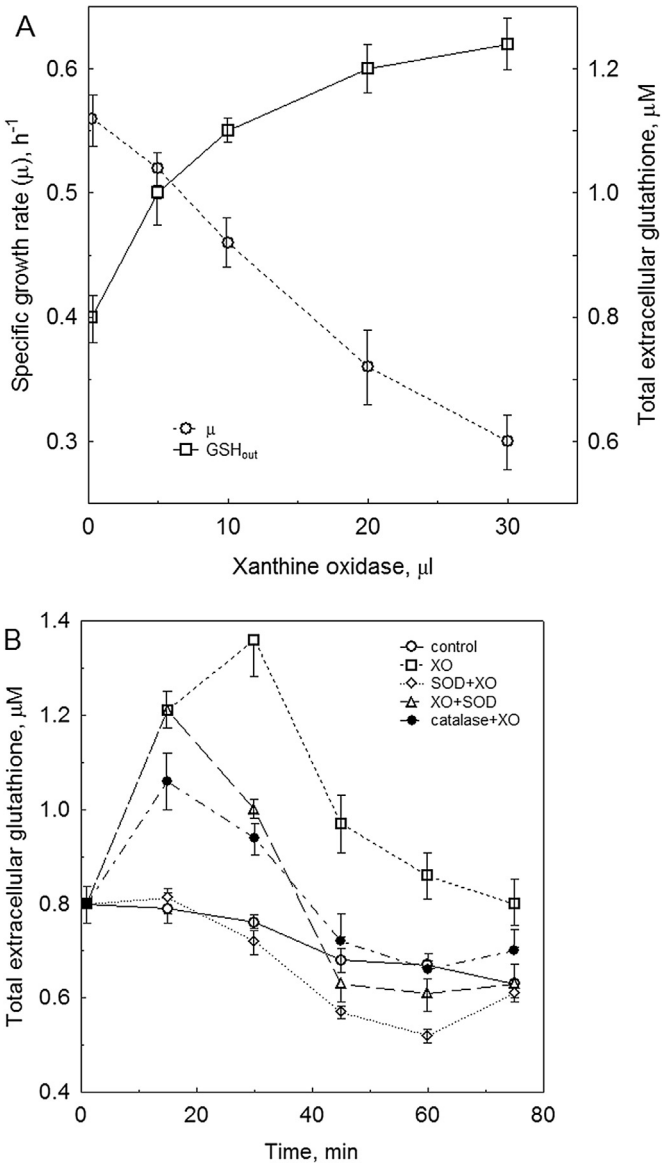

Fig. 1. Exogenous superoxide provokes glutathione efflux from *E. coli* BW25113 (wt) cells. A. GSH efflux and growth inhibition are dependent on the xanthine oxidase concentration, that is, on the rate of superoxide production. Data are presented at 15 min of exposition to XO. B. Exogenous SOD and catalase modulate XO-provoked efflux of GSH. XO (0.04 U/ml) was added at time zero. SOD (60 U/ml) was added just before or 15 min later than XO. Catalase (500 U/ml) was added just before XO. Cells were grown in M9 medium adjusted to pH 8 and supplemented with 0.15% glucose, 1 mM EDTA and 0.05 mM xanthine. Vertical bars in this and other figures represent standard error.

with 2 mM phosphate (Table 1). Under phosphate starvation, GSH efflux in response to XO-generated ROS was only 25% of that in culture growing in the presence of phosphate, despite a 2.5-fold enhanced level of intracellular GSH in starving cells (Table 1). XO-generated efflux of GSH was prevented by exogenous SOD (data not shown). Cells starving for both phosphate and glucose did not release GSH in response to superoxide (data not shown).

The only known transporter that exports GSH from the cytoplasm to the periplasm in *E. coli* is the ATP-binding

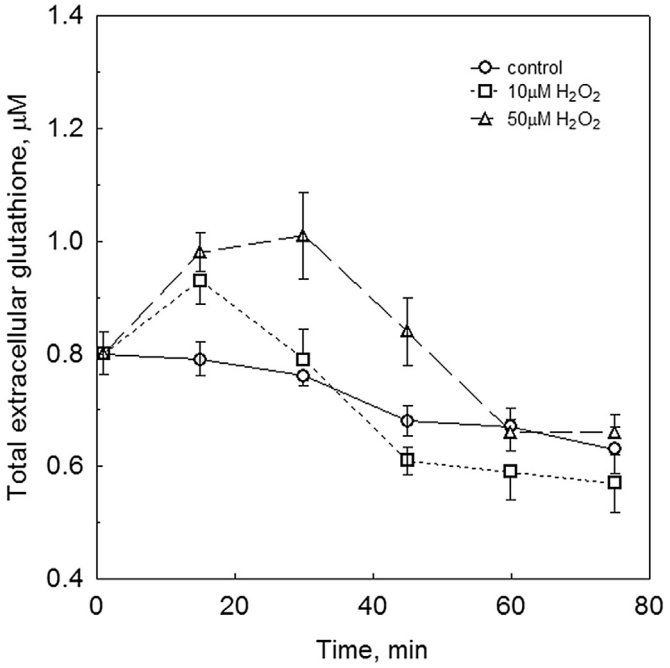

Fig. 2. Changes in glutathione efflux in *E. coli* BW25113 (wt) treated with exogenous hydrogen peroxide. H_2O_2 (10 or 50 μM) was added at time zero. Bacteria were grown in M9 medium adjusted to pH 8 and supplemented with 0.15% glucose, 1 mM EDTA and 0.05 mM xanthine.

cassette-type transporter CydDC [32]. We had previously shown that this transporter does not participate in GSH transmembrane cycling in aerobic exponentially growing cultures [20]. In this study, superoxide-provoked GSH efflux was observed in the *cydD* mutant, indicating that the GSH exporter CydDC is not involved in this process (Fig. 3A). The transport system that exports GSH to the periplasm under these conditions remains unknown. Both *gor* and *ggt* mutants, which were devoid of glutathione reductase (GOR) and γ-glutamyl transpeptidase (GGT), respectively, accumulated more GSH in the medium in response to superoxide than the wild-type cells. A lack of GGT completely prevented a decrease in the level of extracellular GSH, suggesting that this enzyme is involved in a return of glutathione to the cytoplasm after exhaustion of ROS and growth recovery (Fig. 3A).

Mutants devoid of SodC, GGT and GSH (*gsh*A) showed enhanced sensitivity to growth inhibition by ROS generated by xanthine and XO: their specific growth rates decreased to a greater extent and were restored more slowly after exposure to superoxide (Fig. 3B). This effect was especially pronounced in the *gshA* mutant.

3.2. Glutathione oxidation during exposure to xanthine and xanthine oxidase

Both superoxide and H_2O_2 can non-enzymatically react with GSH, producing GSSG [12,13]. Reported values for the second-order rate constants are approximately 200 M^{-1} s^{-1} and 20 M^{-1} s^{-1} for superoxide and hydrogen peroxide, respectively [33]. This is very low compared with values for

Table 1
Total glutathione levels in *E. coli* BW25113 under unlimited growth and phosphate starvation.

Conditions	Total glutathione, μM/OD_{600}	
	Growth	P_i starvation
GSH_{in}, control	9.4 ± 0.6	24.8 ± 0.6
GSH_{out}, control	2.7 ± 0.2 (1.0)	1.46 ± 0.03 (1.0)
GSH_{out}, XO[a]	5.2 ± 0.1 (1.93)	1.81 ± 0.1 (1.24)
GSH_{out}, H_2O_2, 10 μM[b]	3.6 ± 0.1 (1.33)	1.74 ± 0.1 (1.19)
GSH_{out}, H_2O_2, 50 μM[a]	4.1 ± 0.3 (1.52)	1.56 ± 0.09 (1.07)

Indexes in parentheses were calculated as the ratio of GSH_{out} under additions to the control. *E. coli* cells were grown in MOPS medium (pH 8.0; 1 mM EDTA; 50 μM xanthine) with or without 2 mM phosphate to OD_{600} of 0.5 and then 20 μl XO or H_2O_2 were added.

[a] The data are shown at 30 min after XO or 50 μM H_2O_2 addition.

[b] The data are shown at 15 min after 10 μM H_2O_2 addition, when the maximal GSH efflux was reached.

superoxide dismutase and catalase. Thus, the reaction of glutathione with these ROS would not be fast enough for GSH to compete with the enzymes. However, SodC is not induced in the periplasm of exponentially growing *E. coli* [10]. Hydrogen peroxide easily crosses the cytoplasmic membrane and can be scavenged by intracellular enzymes. However, the H_2O_2 concentration in the periplasm may be maintained at the enhanced level over a sufficiently long period. Therefore, periplasmic GSH may contact with both ROS species. In order to determine whether GSH is involved in ROS scavenging in the periplasm, we monitored the level of GSSG in the cultural medium.

Experiments were performed using a cell-free system and cultures of wild-type, *ggt* and *gor* strains. There was no GSH oxidation in the cell-free system without XO during a 40 min shaking period (150 rpm) at 37 °C (Fig. 4A, control). Addition of XO to this system provoked ROS production, and during 40-min incubation, about 60% of GSH (2 μM) contained in the medium was oxidized to GSSG (Fig. 4A). One-third of this oxidation was suppressed by SOD (30 U/ml). Addition of catalase (500 U/ml) led to a decrease in GSSG accumulation by two-third. Thus, both superoxide and hydrogen peroxide were involved in GSH oxidation in the cell-free system, and the contribution of long-lived H_2O_2 was more significant than that of superoxide.

Unlike the cell-free system, XO addition to a culture of wild-type *E. coli* BW25113 growing under the same conditions, but without an exogenous GSH supplement, was not followed by an increase in the GSSG concentration (Fig. 4B). In contrast to the parent, *E. coli* mutants devoid of GGT and GOR showed 1.25-times-higher levels of extracellular GSSG ($GSSG_{out}$) compared to the control after 30 min exposure to xanthine and XO, suggesting acceleration in oxidation of extracellular GSH. It should be mentioned that the basal level of $GSSG_{out}$ in the *gor* mutant was noticeably higher than that in the wild-type and *ggt* cells. The level of $GSSG_{out}$ continuously increased during growth in the *gor* control, but it remained lower than the $GSSG_{out}$ concentration in ROS-treated cells (Fig. 4B). The data suggest that the activity of

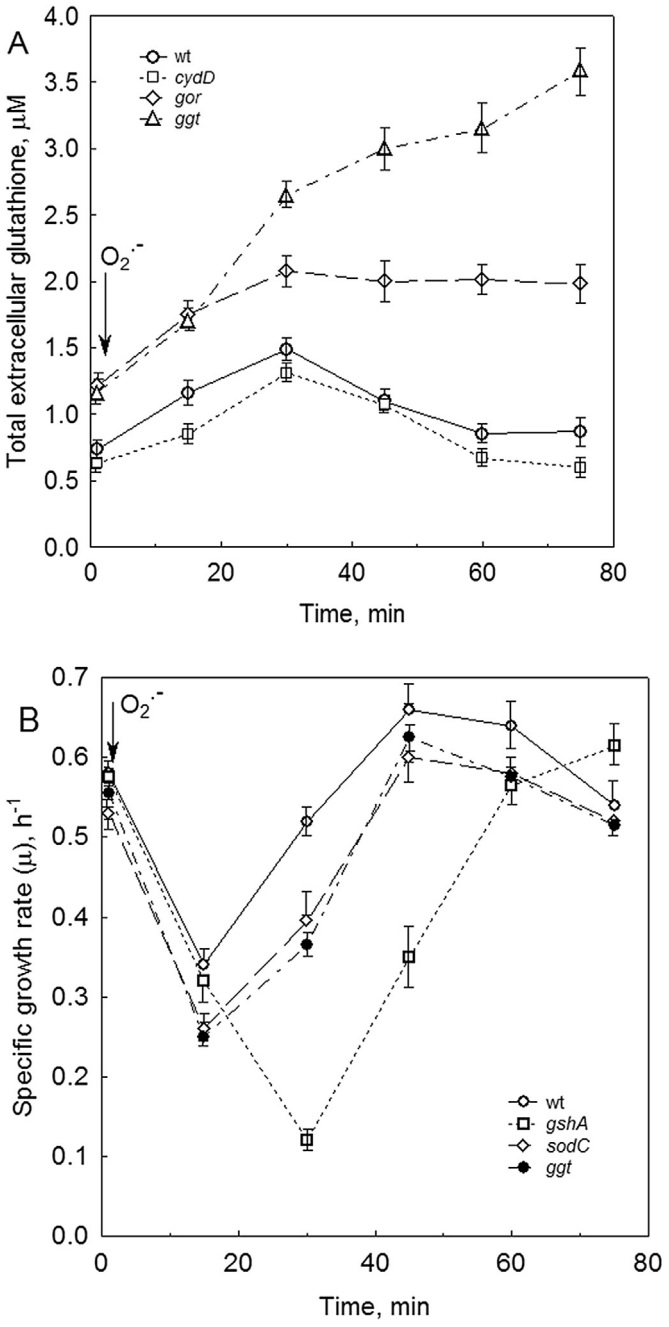

Fig. 3. Mutations in genes involved in GSH turnover influence XO-provoked GSH efflux (A) and growth inhibition (B). *E. coli* BW25113 (wt), JW1638 (Δ*sodC*), JW2663 (Δ*gshA*), JW3467 (*Δgor*), JW3412 (*Δggt*) and AN2343 (*cydD*1) were grown in M9 medium adjusted to pH 8 and supplemented with 0.15% glucose, 1 mM EDTA and 0.05 mM xanthine. XO (0.04 U/ml) was added at time indicated by arrow.

GGT, which is involved in transmembrane GSH cycling, and GOR, which participates in GSSG reduction, may partially explain the absence of the $GSSG_{out}$ increase in the wild-type strain during exposure to ROS. Another reason may be rapid scavenging of H_2O_2 by endogenous enzymes in *E. coli* cells. To test this possibility, we monitored changes in expression of the *katG* and *rpoS* genes and the H_2O_2 concentration in the medium. Treatment of wild-type cells with ROS generated by

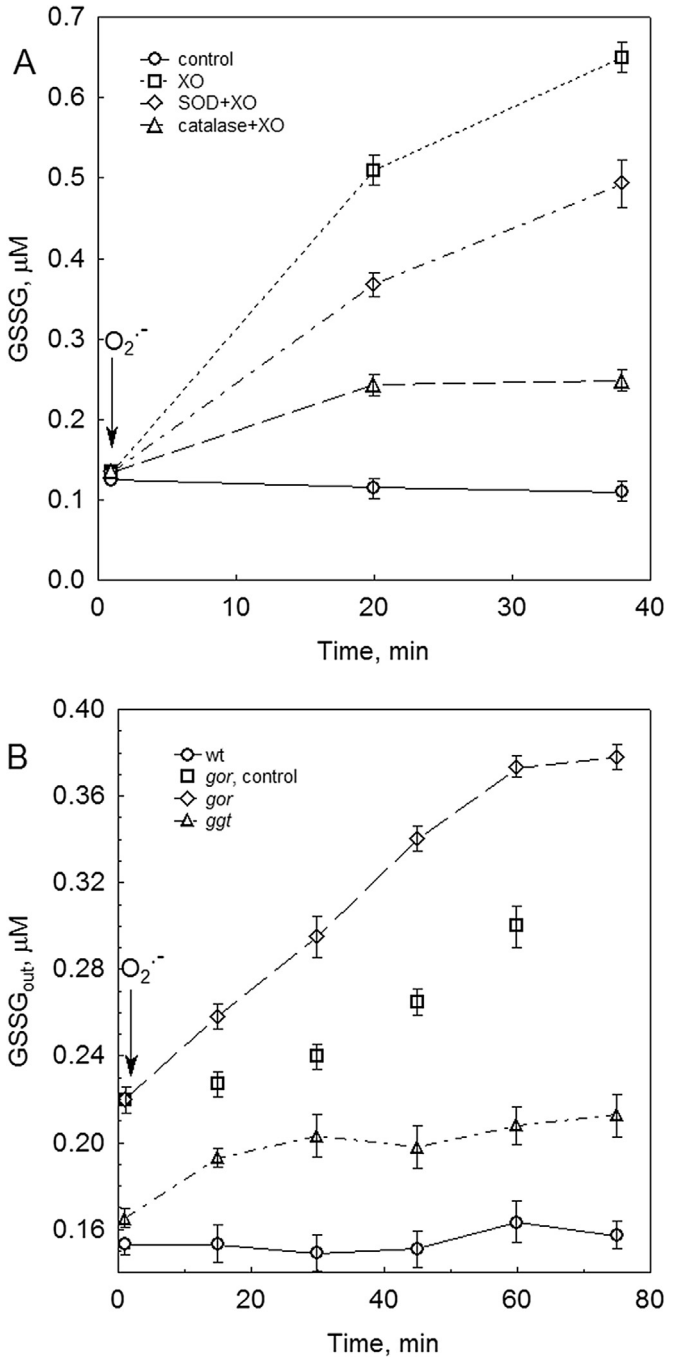

Fig. 4. Accumulation of GSSG in the cell-free system (A) and in the cultural medium of *E. coli* strains (B) under exposure to xanthine and XO. A. SOD (30 U/ml) or catalase (500 U/ml) were added before XO (0.04 U/ml) into cell-free M9 medium adjusted to pH 8 and supplemented with 0.15% glucose, 1 mM EDTA, 0.05 mM xanthine and 2 μM GSH. B. *E. coli* BW25113 (wt), JW3467 (*Δgor*) and JW3412 (*Δggt*) were grown under the same conditions, but without exogenous GSH. XO (0.04 U/ml) was added at time indicated by arrow.

xanthine and XO induced a rapid transient increase in *katG* expression by 1.9 times (Fig. 3A-S). Assays of *rpoS*::*lacZ* expression determined that this regulon was also induced under these conditions (Fig. 3A-S). Therefore, it could be expected that synthesis of OxyR-controlled catalase HPI and GOR and RpoS-regulated catalase HPII are stimulated under exposure to xanthine and XO, leading to rapid H_2O_2 scavenging and a decrease in oxidation of endogenous extracellular GSH. The extracellular H_2O_2 concentration did not exceed 1.3 μM 5 min after XO addition and progressively decreased over a 40 min period to the basal level (Fig. 3B-S).

3.3. Effect of ROS on viability and membrane potential

To determine a possible role for transmembrane GSH cycling in the defense of *E. coli* against exogenous ROS, we monitored changes in viability and membrane potential in the wild-type strain and *gshA* mutant exposed to xanthine and XO. In accordance with previous results [10], ROS generated by xanthine and XO did not kill *E. coli* in log-phase growth, as judged by colony-forming ability and staining with fluorescent dyes SYTO 9 and propidium iodide ("live-dead" test) (Figs. 5 and 4S). *E. coli* starving for phosphate was slightly more sensitive to ROS (number of cells stained by propidium iodide increased by 3%) (Fig. 4S). Comparison of wild-type and *gshA* strains treated with XO showed that the CFU number rises more slowly in the mutant (Fig. 5). This result is in agreement with changes in the specific growth rate (μ) (Fig. 3B) and suggests that the *gshA* mutant is more sensitive to ROS generated by XO than its parent. Growth delay in the *gshA* strain was significantly shortened when the culture was supplemented with exogenous GSH (100 μM) (Fig. 5). The effect of exogenous GSH was less pronounced in the wild-type strain (data not shown). This ability of extracellular GSH to facilitate

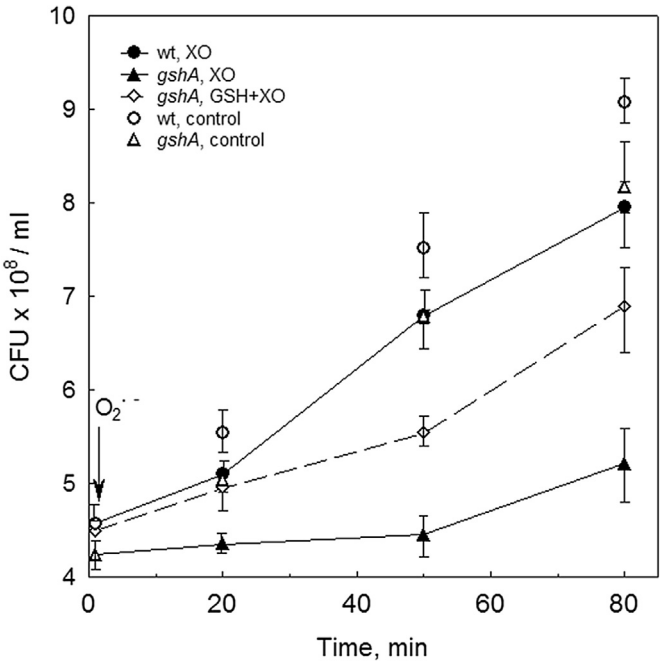

Fig. 5. Influence of endogenous and exogenous GSH on CFU number in *E. coli* BW25113 (wt) and JW2663 (Δ*gshA*) exposed to ROS generated by xanthine and XO. Bacteria were grown in M9 medium adjusted to pH 8 and supplemented with 0.15% glucose, 1 mM EDTA and 0.05 mM xanthine. XO (0.04 U/ml) was added at the moment indicated by the arrow. When needed, GSH (100 μM) was added 15 min before XO.

recovery of fast growth suggests that GSH is involved in adaptation to stress induced by exogenous ROS.

We previously reported that various treatments that change the level of transmembrane electrochemical gradient of protons $\Delta\mu H^+$ ($\Delta\Psi$ and ΔpH) are accompanied by GSH efflux from *E. coli* cells [20,34]. To estimate the influence of ROS on the membrane potential ($\Delta\Psi$) of *E. coli*, we used the membrane-potential-sensitive fluorescent dye $DiBAC_4(3)$ [30]. Negatively charged $DiBAC_4(3)$ cannot penetrate into active cells due to their interior negative charge. Therefore, cells stained by $DiBAC_4(3)$ are considered to be depolarized cells. Only 1% of the wild-type *E. coli* population was permeable to $DiBAC_4(3)$ (i.e. they lost their membrane potential) during exponential growth in M9 or MOPS medium (Fig. 6). Treatment of these cells with the potent artificial protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP, 20 μM) increased the number of cells permeable to $DiBAC_4(3)$ to 54% within 20 min (data not shown). Addition of XO to growing *E. coli* increased the percent of stained wild-type cells to 2.5% (Fig. 6). GSH deficiency did not enhance the effect of ROS on membrane potential. In contrast, the percent of stained cells in the XO-treated *gshA* mutant was slightly lower than that in the parent. Under identical conditions, exposure of *E. coli* cells to hydrogen peroxide also led to slight reversible changes in $\Delta\Psi$ (Fig. 5A-S). During phosphate starvation, a progressive increase in the number of stained cells was observed. Treatment with ROS potentiated the loss of $\Delta\Psi$ under these conditions (Fig. 5B-S). Thus, though exposure of *E. coli* to ROS generated by xanthine and XO does not affect viability, this treatment may somewhat influence membrane potential.

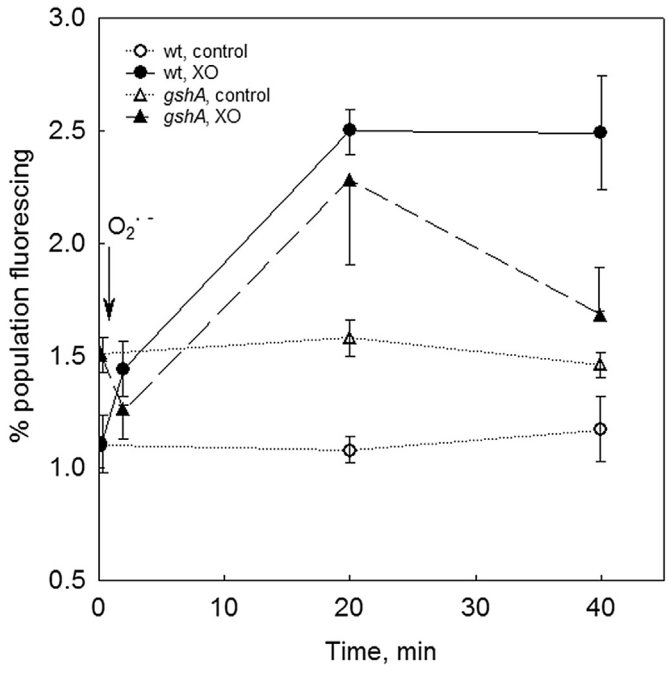

Fig. 6. Percent cells stained by $DiBAC_4(3)$ (loss of membrane potential) under exposure of *E. coli* BW25113 (wt) and JW2663 (Δ*gshA*) to xanthine and XO. Bacteria were grown in M9 medium adjusted to pH 8 and supplemented with 0.15% glucose, 1 mM EDTA and 0.05 mM xanthine. XO (0.04 U/ml) was added at the time indicated by arrow.

4. Discussion

We had previously shown that, in exponentially growing aerobic *E. coli*, the extracellular GSH concentration is a result of a dynamic balance between GSH efflux from the cells and its GGT-mediated return to the cytoplasm [20]. This balance can be disturbed by various environmental factors, which change $\Delta\mu H^+$ and the rate of respiration. GSH efflux completely stops when respiration is inhibited. On the other hand, it was reported earlier that a steady flux of superoxide into the periplasm of exponentially growing aerobic *E. coli* is a result of adventitious auto-oxidation of dihydromenaquinone in the electron transport chain. Superoxide production substantially decreases in *menA* mutants and completely stops in cells starving for glucose [9]. Here we checked a probable connection between fluxes of GSH and superoxide in the *E. coli* periplasm. We demonstrated that exposure of growing *E. coli* cells to exogenous ROS, which are generated by xanthine and XO, stimulates GSH efflux from the cytoplasm. Though both superoxide and low doses of H_2O_2 stimulated GSH efflux in this study, we had previously observed that H_2O_2 concentrations higher than 1 mM may lead to an initial decrease in the level of extracellular GSH [14]. Duration of this delay in the GSH efflux was increased upon an increase in the H_2O_2 concentration and duration of growth arrest. Together with the decrease in the ROS-mediated efflux of GSH under phosphate starvation, these data suggest that either a driving force for glutathione efflux is absent during prolonged growth inhibition, or that some factors, e.g. ppGpp, which are induced under growth arrest, are involved in GSH retention in the cytoplasm.

Since XO-generated ROS oxidized GSH in the cell-free system, it is hypothesized that periplasmic GSH may participate in scavenging of ROS in this compartment. Though we did not observe GSSG accumulation during treatment of the wild-type *E. coli* with xanthine oxidase, GSH oxidation might be masked by rapid turnover and reduction of oxidized glutathione. Interestingly, it was previously shown that, under H_2O_2 stress in *E. coli*, expression of genes of the two transporters that import extracellular glutathione into the cytoplasm, i.e. GGT and ATP-binding cassette transporter YliABCD (GsiABCD), is significantly elevated (12.7-fold and about 5-fold for *ggt* and *yliABCD*, respectively) [35]. GSH may not be the only thiol that provides reducing equivalents to the periplasm in *E. coli*; it has been demonstrated that the inducible L-cysteine/L-cystine shuttle system is required for tolerance of *E. coli* cells to hydrogen peroxide [35].

Under our conditions, the presence of endogenous or exogenous GSH facilitates growth recovery of *E. coli* cells after exposure to ROS generated by xanthine and XO, suggesting that GSH may be involved in defense against oxidative stress. In addition to its participation in ROS scavenging, GSH cycling may have important regulatory functions which help cells to cope with consequences of oxidative stress and to restore rapid growth. Among these GSH-dependent functions, regulation of transmembrane ion fluxes may be the most important. In particular, it is known that GSH is involved in regulation of glutathione-gated potassium efflux systems (KefB and KefC)

that stimulate influx of protons and modulate cytoplasmic pH [36–38]. The activity of the Kef system is subject to complex regulation: S conjugates of GSH with electrophiles, including quinones, activate Kef transporters; in contrast, cytoplasmic GSH and NADH maintain them as inactive; KefF and KefG ancillary proteins, which have quinone oxidoreductase activity, are necessary for full activation. It is also known that, under some conditions, activation of an ion transport "futile" cycle, e.g., the circulation of K^+ catalyzed by (i) the K^+ uniport (which carries out electrophoretic K^+ influx) and (ii) K^+/H^+ antiporter (which facilitates the K^+ efflux in exchange for H^+) can lead to uncoupling ($\Delta\mu H^+$ dissipation) in mitochondria [39]. Uncoupling was postulated to be involved in the cellular defense system preventing formation of superoxide at high $\Delta\mu H^+$. Paradoxically, partial uncoupling stimulates the rate of phosphorylation coupled to respiration [39]. We hypothesize that, in growing *E. coli* cells, periplasmic superoxide and GSH may be involved in a sensory and regulatory mechanism that responds to changes in $\Delta\mu H^+$ under varying growth conditions and corrects transmembrane ion fluxes in accordance with new demands. We previously found that both protonophore CCCP and gramicidin S, which forms ion-permeable channels in phospholipid bilayers, stimulate GSH efflux from *E. coli* cells [20,34]. Here we observed that superoxide and H_2O_2 can provoke GSH efflux and slight $\Delta\Psi$ reduction. Though it is difficult to discriminate between physiologically useful effects and cell damage, it is possible that superoxide-provoked GSH efflux and a slight $\Delta\Psi$ reduction caused by changes in transmembrane ion fluxes, are part of the adaptive response. This type of regulation based on changes in the rate of transmembrane ion fluxes is not suitable for starving cells; therefore, RpoS-regulated SodC is induced to cope with superoxide.

ROS-provoked GSH efflux, shown in this work for the first time, may be important for understanding the mechanisms of bacterial killing by oxidative burst of macrophages. Professional phagocytes generate large amounts of reactive oxygen species, whose production is primed by the single-electron reduction of molecular oxygen to superoxide by the NADPH-dependent NOX2 oxidase complex. This defense mechanism has a key role in control of bacterial infections, as shown by the enhanced susceptibility to infections of individuals with inherited deficiencies of specific NOX2 components [40,41]. However, the mechanisms by which phagocyte-derived ROS kill microbes remain controversial [41,42]. It is clear that phagocytic superoxide damages bacteria in the phagosome and that periplasmic superoxide dismutases can protect bacteria against exogenous superoxide [40,42–44]. Since charged superoxide cannot cross membranes and the role of SodC is genetically separable from cytoplasmic Mn- and Fe-superoxide dismutases, the primary targets of phagocytic superoxide must be located in the periplasm [4,10]. Though superoxide-mediated extracytoplasmic damage is at least as critical as the potential cytoplasmic damage caused by H_2O_2, the targets of exogenous superoxide are as yet unidentified [42]. The efflux of GSH provoked by endogenous superoxide under physiological conditions and involved in regulatory and defense processes in the periplasm of Gram-negative bacteria, might be used by the immune system as a target for exogenous phagocytic superoxide. If this is the case, then the efflux of GSH may sensitize bacterial cells to subsequent treatment with other antimicrobial effectors. In particular, GSH-mediated K^+ efflux under acidic conditions of the phagosome may completely disturb pH homeostasis and make bacterial cells more accessible to proteases. Interestingly, it has been recently shown that glutathione represents a critical signaling molecule that activates the virulence gene expression of an intracellular pathogen *Listeria monocytogenes* [45]. Though further investigations are required to determine molecular mechanisms of superoxide-provoked GSH efflux and its consequences for bacterial cells, this may be a promising direction for understanding new aspects of the antimicrobial action of exogenous superoxide.

Competing interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2015.07.007.

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