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Original Research

Cystine and Antibiotic Treatment Alters Low Molecular Weight Thiol Levels in *Mycobacterium smegmatis*

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Abstract

Background: Endogenous and exogenous H_2S can influence the virulence of bacteria and their susceptibility to antibiotics and oxidative stress. *Escherichia coli* and *Bacillus subtilis*, when grown in minimal medium with sulfate as the sole sulfur source, produce H_2S when treated with cystine or under stress conditions, including exposure to chloramphenicol and ciprofloxacin. However, it is unknown whether *Mycobacterium smegmatis* is capable of producing sulfide under these conditions and how this production affects cell physiology. **Methods**: Real-time monitoring of dissolved oxygen (dO_2), pH, extracellular K^+, and sulfide was performed directly in culture flasks using selective electrodes. Changes in the level of low molecular weight (LMW) thiols were recorded using spectrophotometric methods and high performance liquid chromatography (HPLC). **Results**: Sudden addition of cystine or chloramphenicol to growing *M. smegmatis* cultures increased the intracellular level of cysteine and induced its homeostasis mechanisms, which include the export of excess cysteine from cells and its incorporation into mycothiol (MSH), along with desulfurization with H_2S formation. Ciprofloxacin also increased intracellular cysteine concentration and sulfide production but did not induce cysteine release. Both antibiotics inhibited growth and respiration, whereas cystine transiently increased respiration and glucose uptake in *M. smegmatis*, in contrast to *E. coli*, which showed a transient inhibition of these processes. **Conclusions**: The mechanisms of cysteine homeostasis under the action of antibiotics in *M. smegmatis* are similar to those in *E. coli* and *B. subtilis*, indicating the universal nature of stress response. The opposing effects of cystine-derived H_2S on physiological parameters in *M. smegmatis* and *E. coli* may be important factors contributing to their susceptibility to antibiotics.

Keywords: chloramphenicol; ciprofloxacin; H_2S; cysteine; mycothiol; *Mycobacterium smegmatis*

1. Introduction

The rapid development of antibiotic resistance of pathogenic bacteria requires searching for adjuvants of existing drugs to improve their therapeutic efficacy and targets to create novel antibiotics. Research into the influence of cysteine and its derivatives on the virulence of bacteria and their susceptibility to antibiotics and oxidative stress is one of the actively developing areas [1]. Endogenous H_2S, formed during L-cysteine degradation, reduced the sensitivity of bacteria to antibiotics and oxidative stress, while mutations and inhibitors of H_2S-producing enzymes increased the efficacy of antimicrobial drugs [2–4]. High intracellular cysteine levels are potentially harmful, since cysteine is capable of autoxidation yielding ROS and can potentiate the Fenton reaction giving rise to toxic hydroxyl radicals by maintaining the Fe^{2+} pool [5,6]. To explain the interplay between L-cysteine metabolism, H_2S production and oxidative stress, a model has been proposed where 3-mercaptopyruvate sulfotransferase (3MST) protects *Escherichia coli* against oxidative stress by catalyzing cysteine degradation to form H_2S, which binds free iron required for the Fenton reaction [3]. However, instead of enhancing antibiotic tolerance, exogenous and endogenous H_2S in *Mycobacterium tuberculosis* and exogenous H_2S in *Acinetobacter baumannii* sensitized them to antibiotics by triggering a pro-oxidative redox imbalance [7–9]. Endogenous H_2S in mycobacteria has been shown to be an effector molecule that maintains bioenergetic homeostasis by stimulating respiration, plays a key role in central metabolism, regulates redox homeostasis, and increases the susceptibility of *M. tuberculosis* to antituberculosis drugs clofazimine and rifampicin through its pro-oxidant function [9]. Host-derived H_2S has also been shown to stimulate *M. tuberculosis* respiration, primarily through cytochrome *bd* oxidase, and regulate genes involved in sulfur and copper metabolism, as well as the Dos regulon [8,10]. The effect of H_2S on antibiotic sensitivity apparently depends on the bacterial species and can be determined by the features of endogenous H_2S production or the nature of its effect on metabolic pathways. We have previously shown that antibiotic exposure of *E. coli* and *Bacillus subtilis* is accompanied by changes in low molecular weight thiol levels and H_2S production [11–14]. It still remains unknown whether mycobacteria are capable of producing H_2S in response to antibiotics.

Addition of cystine to *E. coli* growing in minimal medium with sulfate has been shown to cause cytoplasmic cysteine overload and stimulate cysteine export, its incorporation into glutathione (GSH) and degradation to form H_2S [6,13,15–17]. Multiple bacteria possess cysteine-inducible cysteine-specific efflux pumps for its export [18–20]. However, no such transporters have been detected in *M. tuberculosis* and the ability of mycobacteria to export cysteine into the medium remains unknown, although recently the involvement of the Rv0191 efflux pump in the export of low mlecular weight (LMW) thiols has been suggested [21]. L-cysteine supplementation has been shown to stimulate H_2S production in *Bacillus anthracis, Pseudomonas aeruginosa*, and *Staphylococcus aureus* [2,3]. Various *E. coli* enzymes are known to possess *in vitro* cysteine desulfhydrase activity (TnaA, CysK, CysM, MalY, MetC, 3MST, IscS, CyuA); some of them may specifically cope with cysteine toxicity [2,22–25]. Addition of cysteine to *M. tuberculosis* was also found to increase H_2S production in a cysteine desulfhydrase (Cds1)-dependent manner, suggesting that this pathway could be used to get rid of excess cysteine [9].

Recently, in a series of studies, we have shown that a transient increase in intracellular cysteine concentration and activation of cysteine homeostasis mechanisms occur not only when cysteine/cystine is added to the growth medium, but also as a consequence of abrupt protein synthesis inhibition during various stresses in *E. coli* and *B. subtilis* [11,12,26,27]. These stresses involve nutrient starvation (depletion of glucose, phosphate, or nitrogen) and exposure to antibiotics such as chloramphenicol, tetracycline, and ciprofloxacin. Excess cysteine formed in *E. coli* under these conditions was mainly incorporated into glutathione, and was also exported into the medium and partially degraded to form H_2S. Sulfide release from cells into the medium under stress can be recorded using selective sulfide or platinum electrodes as abrupt changes in the redox potential (Eh jumps). In our earlier studies, Eh jumps suppressed by the thiol reagent N-ethylmaleimide (NEM) occurred upon exposure to various stressors (starvation, elevated temperature, ultraviolet irradiation, treatment with acetate and antibiotics, etc.) on Gram-negative and Gram-positive bacteria, indicating the universal nature of the observed phenomenon [28].

Like other mycobacteria, *Mycobacterium smegmatis* contains no glutathione and uses mycothiol as its major LMW thiol, whose concentration in cells is comparable to that of glutathione in *E. coli* [29–31]. Mycothiol in mycobacteria performs many of the functions of glutathione in Gram-negative bacteria, including the maintenance of the intracellular redox balance, as well as protection against reactive oxygen and nitrogen species, alkylating agents, and antibiotics. Mycothiol (MSH) levels may be influenced by environmental conditions, such as oxidative or osmotic stress or nutrient starvation [30]. In response to the increased need for antioxidant molecules under hostile conditions such as oxidative stress, *M. tuberculosis* actively upregulates CysM-dependent L-cysteine biosynthesis through the transcription factors AosR and SigH, turn enhancing mycothiol and ergothioneine production [21]. The expression of MSH biosynthetic genes was also elevated by acidic conditions [32]. It was shown that MSH turnover occurs in *M. smegmatis* and that MSH can be a cysteine source [30]. Since MSH is autoxidized much more slowly than cysteine, it has been suggested that cysteine incorporation into MSH is a way to conserve cysteine and limit its intracellular concentration. However, no literature data on how the emergence of excess cysteine affects MSH levels are available.

In this study, we examined how cystine supplementation and treatment with chloramphenicol or ciprofloxacin affect changes in cysteine, mycothiol, and H_2S levels in *M. smegmatis*. We also monitored the effects of these treatments on bacterial growth and respiration, which may be important for explaining the effects of H_2S on antibiotic tolerance. *M. smegmatis* is frequently used as a model organism to study *M. tuberculosis* due to its genetic similarity, conserved metabolic pathways (including biosynthesis and turnover of LMW thiols), and lower pathogenicity. However, despite the similarity and widespread use of *M. smegmatis* to investigate the mechanisms of drug resistance in tuberculosis, these bacteria have some differences in gene expression, metabolic pathways, and cell structure. In particular, *M. tuberculosis* was shown to produce significantly more H_2S compared to *M. smegmatis* [9]. Moreover, these bacteria differ significantly in growth rate, which may play an important role in altering thiol levels during antibiotic-mediated growth inhibition. This suggests that some caution is needed when generalizing the results obtained in *M. smegmatis* to *M. tuberculosis*.

2. Materials and Methods

2.1 Bacterial Strain and Growth Conditions

The American Type Culture Collection (ATCC) strain *M. smegmatis* 700084/mc^{2}155 was used in this study. Bacterial cultures were grown in 250 mL flasks at 37 °C with shaking at 150 rpm in 100 mL of minimal M9 medium with glucose (4 g/L) [33]. This medium additionally contained glycerol (4 g/L), Tween 80 (0.15%), $FeCl_3$ (10 µM), $CaCl_2$ (0.2 mM), $MgSO_4$ (2 mM) and 1 mL of trace elements prepared as described previously [34]. Bacteria were pre-grown for 24 hours in the presence of ampicillin (25 µg/mL); part of the culture was then transferred to fresh medium of the same composition. The overnight culture was centrifuged, diluted in 100 mL of fresh medium without antibiotic to OD_{600} 0.25 and cultured as described above. Some experiments on studying H_2S production were carried out in the glycerol-free medium. In this case, the overnight culture after centrifugation was transferred to a medium of the above composition, excluding glycerol.

Cystine (Cys, 30 µM), chloramphenicol (Cam, 25 µg/mL) and ciprofloxacin (CF, 10 and 30 µg/mL) were added when OD_{600} had reached 0.4. The antibiotic concentrations used were selected based on preliminary experiments on their ability to rapidly inhibit bacterial growth and respiration when added to growing cultures. Ciprofloxacin concentrations (10 and 30 µg/mL) were 40–120 times higher than the MIC values (0.25 µg/mL) for *M. smegmatis* mc^2155 reported in the literature [35].

The following equation was used to calculate the specific growth rate (µ): $\mu = \Delta \ln OD_{600}/\Delta t$, where t is the time in hours. Colony-forming units (CFU/mL) were determined by plating serially diluted culture samples onto agar (1.5%) medium of the above composition. CFU were counted after 4 days of growth at 37 °C.

2.2 Real-time Monitoring of Dissolved Oxygen (dO_2), pH, Extracellular K^+ and Sulfide

A Clark InPro 6800 oxygen electrode (Mettler Toledo, Greifensee, Switzerland) and an ESC-10601/7 pH electrode (IT Company, Moscow, Russia) placed in flasks with *M. smegmatis* cultures were used for continuous measurement of dissolved oxygen and pH in the medium, respectively. Data recorded using the dO_2/pH controller of a BioFlo 110 fermenter (New Brunswick Scientific Co., Edison, NJ, USA).

The sulfide content in the culture medium was monitored using an XC-S^{2-}-001 sulfide-specific chalcogenide electrode (Sensor Systems Company, St. Petersburg, Russia) and a cpX-2 computer pH/ion meter (IBI, Pushchino, Russia). The advantages of this electrode are the ability to measure at physiological pH (the electrode operates in the pH range from 6 to 12), no reaction to changes in oxygen content and high sensitivity (sensitivity threshold 5 nM). A standard curve for determining sulfide concentration was generated using known amounts of Na_2S.

Changes in K^+ concentration in the medium were recorded using an ELIS-121K K^+-selective electrode (IT Company, Moscow, Russia) and a cpX-2 computer pH/ion meter (IBI, Pushchino, Russia). When measuring potassium, its content in the growth medium was reduced to 0.2 mM. Synchronous processing of all data from the sensor system was carried out using the RS-232 and Modbus protocols and the Advantech OPC Server v3.0 software package (Advantech Co., Shing-Tien, New Taipei City, Taiwan, China).

2.3 Measurements of Gaseous H_2S and Intracellular and Extracellular Cysteine

Gaseous H_2S was determined based on its specific reaction with lead acetate [$Pb(Ac)_2$]. The lead sulfide formed during the reaction produces a brown spot on $Pb(Ac)_2$-soaked paper strips fixed above the surface of the liquid culture [2]. Sequential replacement of paper strips every 30 min allowed monitoring of the H_2S production kinetics. The color intensity was quantified using ImageJ1.54g software (NIH, Bethesda, MD, USA) after scanning the spots. The detection threshold of this method was 0.1 µM.

L-cysteine was determined using our modification of the Gaitonde method [26]. Briefly, culture samples collected at different time points were concentrated ten-fold and sonicated in 0.1 M Tris-HCl (pH 8.6). After protein removal with 0.5 M perchloric acid followed by treatment with potassium hydroxide to pH 8.6, the resulting supernatant was evaporated on an RV10 rotary evaporator (IKA, Staufen, Germany) and treated with dithiothreitol (10 mM) to reduce cystine to cysteine. To determine extracellular cysteine, culture samples were passed through a membrane filter to remove cells; the resulting filtrates were concentrated by rotary evaporation, the protein was then removed and cystine was reduced to cysteine as described above. Cysteine assay in reduced samples was performed as described previously [26]. Standard curves were prepared using known amounts of cysteine treated in the same way as the culture samples.

2.4 HPLC Determination of Intracellular Low Molecular Weight Thiols

The intracellular LMW thiols, including mycothiol, were determined using the high performance liquid chromatography (HPLC) method with fluorometric detection following the reaction of thiols with the fluorescent dye monobromobimane (mBBr) [36]. *M. smegmatis* culture samples (15 mL) were centrifuged (5 min at 8000 ×g). The resulting pellet was resuspended in 0.5 mL hot (60 °C) 50% acetonitrile containing 20 mM Tris-HCl (pH 8) and 2 mM mBBr, and kept in a water bath at 60 °C for 15 min. Next, 2.5 µL of 5 N methanesulfonic acid was added, the protein was removed by centrifugation (5 min at 10,000 ×g), and the resulting supernatant was used for measurements. A separate thiol-blocked control was prepared by adding 5 mM N-ethylmaleimide to the extraction buffer instead of mBBr, incubating at 60 °C for 5 min, and treating with mBBr and methanesulfonic acid as above. Before being injected into the HPLC column, the sample was diluted 4-fold in 10 mM methanesulfonic acid. HPLC analysis was performed using a Shimadzu chromatograph (Shimadzu corporation, Kyoto, Japan) equipped with an autosampler (Shimadzu, model LC-20AD), a degasser (Shimadzu, SPD-M20A) and a fluorescence detector (Shimadzu, model RF-10AXL). Twenty microliters of the sample were injected into a C18 Phenomenex (Phenomenex Co., Torrance, CA, USA) column (4.6 × 250 mm; particle size, 5 µm). Eluent A was 0.25% acetic acid (pH 3.5), eluent B was methanol. Separation was carried out under the gradient conditions: 0 min — 15% B; 5 min — 15% B; 15 min — 23% B; 45 min — 42% B, followed by a 10-min column wash with eluent B and 10-min re-equilibration with 15% eluent B. The eluent flow rate was 1 mL/min. Fluorescence was detected at

λex 395 nm and λem 475 nm. The LMW thiol content was expressed in relative units (peak area divided by OD600 of the sample).

2.5 Statistical Analysis

All the experiments were performed at least in triplicate, with results presented as means ± standard error (SEM). Statistical analyses were carried out using Statistica 8.0.360 (StatSoft Inc., Tulsa, OK, USA, accessed August 27, 2007). Significant difference was analyzed by Student's *t*-test. For analyses, $p < 0.05$ was defined as thresholds for statistical significance.

3. Results

3.1 Changes in Physiological Parameters of *M. smegmatis* Treated With Cystine, Chloramphenicol and Ciprofloxacin

Compared to *M. tuberculosis*, a slow-growing virulent strain (doubling time, 24 h), *M. smegmatis* is a fast-growing non-virulent strain (doubling time 90 min) [37]. Under our experimental conditions, *M. smegmatis* grew at an average rate of 0.25 ± 0.01 h^{-1} (doubling time ~165 min). Addition of 30 μM cystine had no statistically significant effect on the growth rate (Fig. 1a). In contrast to *E. coli* and *B. subtilis*, where treatment with chloramphenicol (25 μg/mL) or ciprofloxacin (10 μg/mL) caused abrupt growth inhibition [12–14], a statistically significant ($p < 0.05$) decrease in *M. smegmatis* growth rate was observed only after 60 min of exposure to these antibiotics.

Due to the rapid oxygen consumption by cells, the content of dissolved oxygen in control cultures gradually decreased with increasing culture density, despite constant rotation of the flasks (Fig. 1b). The rate of oxygen consumption expressed as a percentage of dO_2 per OD_{600} per minute was 0.36 ± 0.01. The addition of cystine increased the rate of oxygen consumption to 0.67 ± 0.03, i.e., 1.86 times ($p = 0.0004$), for 30 min, after which the rate of oxygen consumption decreased. Treatment with both antibiotics tested stopped the fall in dO_2 within 10 min after their addition, indicating a decrease in respiratory rate (Fig. 1b). After 2 hours of exposure, the dO_2 value was 2.9 ± 0.4% and 7.4 ± 1.2% higher than its level before the addition of ciprofloxacin and chloramphenicol, respectively. The stronger inhibition of oxygen consumption by chloramphenicol compared to ciprofloxacin may be due to the more rapid growth inhibition caused by chloramphenicol.

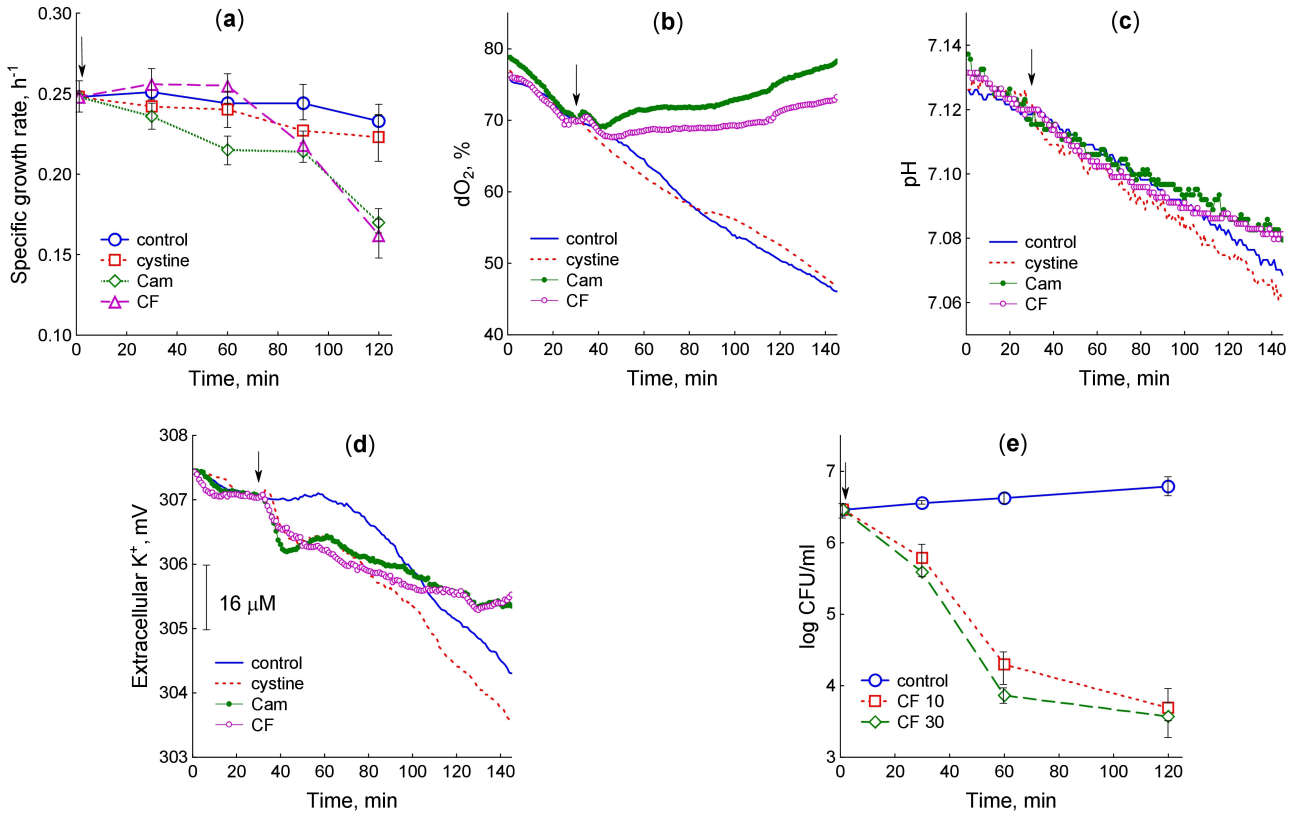

Fig. 1. Changes in physiological parameters of *M. smegmatis* upon treatment with cysteine, chloramphenicol and ciprofloxacin. (a) Specific growth rate. (b) Concentration of dissolved oxygen. (c) pH of the medium. (d) Extracellular K^+ concentration. (e) Ciprofloxacin-induced killing curves. The arrow shows the moment of adding the test compounds. (a,e) The average of the data from several experiments is plotted with the standard error indicated (bars). (b–d) The graphs show typical curves obtained from three independent experiments. Cys, Cystine; Cam, chloramphenicol; CF, ciprofloxacin; dO_2, dissolved oxygen.

Glucose metabolism during *M. smegmatis* growth was accompanied by accumulation of acidic by-products, which reduces pH (Fig. 1c). In this regard, sensitive pH recording can be used as a real-time indicator of glucose consumption. Two hours after the test compounds had been added, the change in pH relative to the baseline (ΔpH) was 0.056 $\pm$ 0.001, 0.069 $\pm$ 0.001, 0.034 $\pm$ 0.003, and 0.04 $\pm$ 0.002 for control, cystine, ciprofloxacin, and chloramphenicol, respectively. The significance of the difference from the control for all the values was $p < 0.01$. The acidification rate of the medium, expressed in units of pH/OD_{600} per min, increased by 1.23 times when exposed to cystine and decreased by 1.3 and 1.54 times when bacteria were treated with chloramphenicol and ciprofloxacin, respectively.

During bacterial growth, the K^+ content in the medium decreased due to its uptake by the cells (Fig. 1d). Treatment of *M. smegmatis* with cystine and antibiotics caused an abrupt decline in K^+ concentration in the medium, indicating an accelerated influx of K^+ ions into the cells. The change in the K^+ sensor potential relative to the initial value 10 min after treatment with cystine and antibiotics (ΔK^+) was 0.11 $\pm$ 0.016 mV (control), 0.73 $\pm$ 0.04 mV (cystine), 0.58 $\pm$ 0.04 mV (CF), and 0.89 $\pm$ 0.04 mV (Cam). After 15-min exposure to chloramphenicol and ciprofloxacin, K^+ uptake by cells was slowed down, while bacteria treated with cystine continued to consume potassium at a rate close to that for control. Overall, all the tested compounds caused significant changes in energy metabolism, and antibiotics inhibited the growth of *M. smegmatis*. The 60-min delay in growth inhibition with ciprofloxacin was apparently not due to a low rate of drug uptake, since other parameters recorded (dO_2 and K^+) responded rapidly to addition of ciprofloxacin.

We also tested the bactericidal effect of ciprofloxacin on *M. smegmatis* (Fig. 1e). The maximum decrease in CFU was observed during the first hour of exposure to the antibiotic, when the bacteria maintained a high growth rate. It indicates that, like in the case of *E. coli*, growing bacteria with high metabolic activity suffer more damage, preventing colony formation during subsequent cultivation on antibiotic-free plates [38]. There was no significant difference in the effects of 10 and 30 µg/mL ciprofloxacin.

3.2 All the Tested Compounds Accelerated Sulfide Production by M. smegmatis Cells

Cysteine or cystine can be transported into *M. smegmatis* and *M. tuberculosis* [39], which may lead to intracellular cysteine overload and activate desulfurization of excess cysteine to form H_2S, as observed in *E. coli* [13,16]. Indeed, genetic and biochemical evidence has been provided that conversion of cysteine to H_2S mediated by Cds1 desulfhydrase functions as a sink for excess cysteine in *M. tuberculosis*, but H_2S production in *M. smegmatis* was barely detectable [9]. Using a highly sensitive method for recording sulfide with a selective electrode, we studied the changes in H_2S production upon exposure of *M. smegmatis* to cystine and antibiotics.

Under our experimental conditions, the addition of 30 µM cystine caused an abrupt drop of 52.6 $\pm$ 5.5 mV in the sulfide electrode potential from the baseline, corresponding to the release of 310 $\pm$ 40 nM sulfide (Fig. 2a). Due to its high volatility, H_2S was accumulated in the gas phase above the culture surface, where its emergence was recorded using $Pb(Ac)_2$-soaked paper strips (**Supplementary Fig. 1**). Since growth of *M. smegmatis* in control cultures is not accompanied by H_2S formation, the color of the $Pb(Ac)_2$-soaked paper strips remains virtually unchanged throughout the experiment and is close to the color the strips had before it began (100%). Cystine addition caused H_2S release from cells, which intensified the color of the strips fixed above the culture surface throughout the experiment (90 min) to 227 $\pm$ 29% (not shown). Successive replacement of strips every 30 min allows one to track the kinetics of H_2S release. The maximum rate of H_2S production was observed during the first 30 min of cystine exposure, consistent with the sulfide electrode readings (Fig. 2a). Interestingly, the accumulation of H_2S in the gas phase was strongly influenced by the presence of glycerol in the medium (Fig. 2b; **Supplementary Fig. 1**). In the absence of glycerol, the total accumulation of H_2S over 90 min was 367 $\pm$ 17% (not shown). Kinetic experiments showed that glycerol not only reduced the amount of H_2S formed, but also significantly slowed down its emergence in the gas phase after cystine supplementation (Fig. 2b). Since glycerol is required to maintain the integrity of the *M. smegmatis* cell wall after culturing under minimal growth conditions [40,41], it can be assumed that the observed changes in H_2S production are related, at least partially, to alterations in cell wall permeability.

Chloramphenicol caused the sulfide electrode potential to drop by 4.2 $\pm$ 0.8 mV, corresponding to a release of 10–15 nM sulfide (Fig. 2c). Sulfide leakage in response to Cam addition was reversible and involved a rapid release phase lasting ~15 min and a slower return to baseline values. H_2S in the gas phase was not detected when *M. smegmatis* was exposed to Cam in M9 medium containing glycerol, but appeared when glycerol was excluded (Fig. 2d; **Supplementary Fig. 1**). The addition of ciprofloxacin also caused a drop in the potential of the sulfide sensor; however, unlike in the case of chloramphenicol, the decrease in potential was irreversible (Fig. 2c) and after 2 hours of exposure, the difference with the control level was 6.4 $\pm$ 1.4 mV, corresponding to 10–20 nM sulfide. We detected no H_2S in the gas phase when *M. smegmatis* was treated with ciprofloxacin, regardless of whether glycerol was present in the medium or not (Fig. 2d).

HPLC analysis of monobromobimane derivatives revealed the presence of sulfide in the cell extract (**Supplementary Fig. 2**). After 60-min exposure, cells treated with cystine contained 4 times more sulfide than

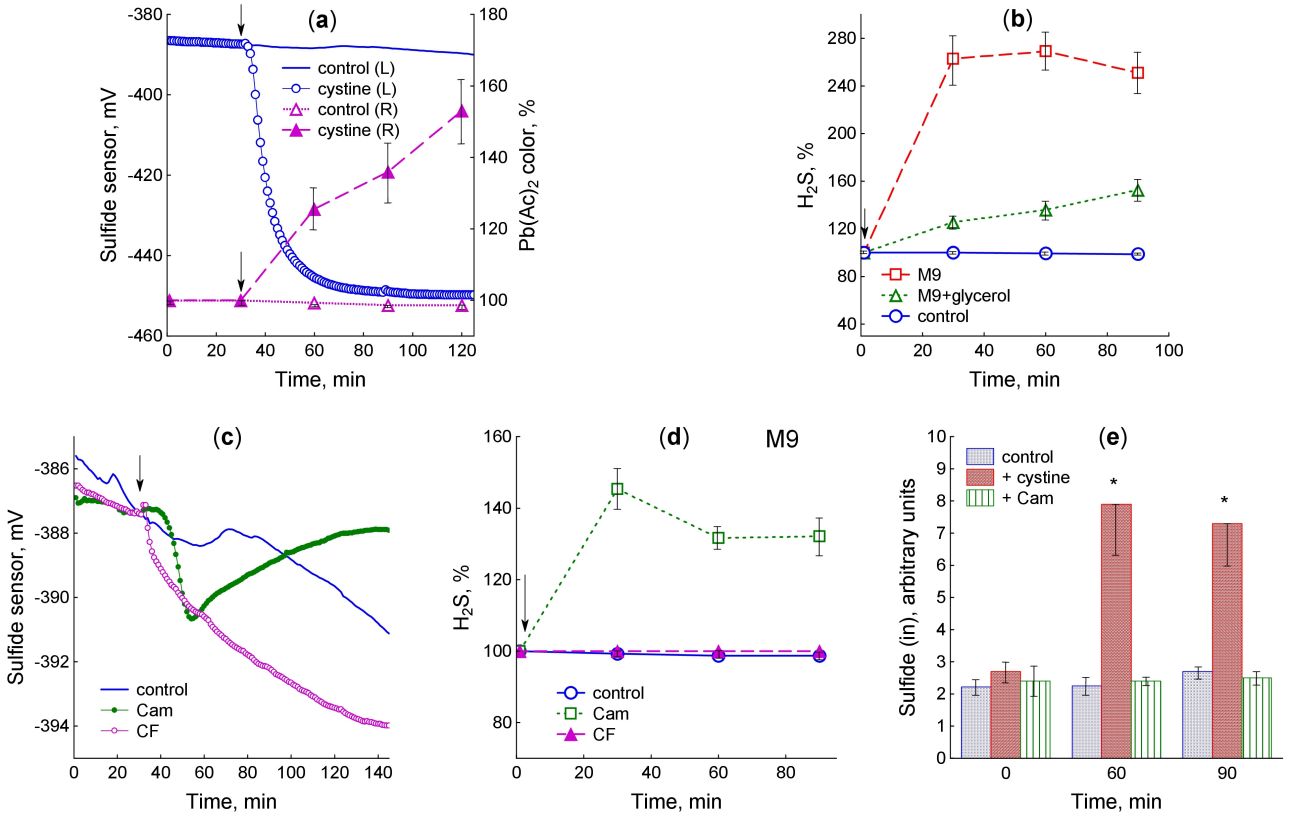

Fig. 2. *M. smegmatis* releases H_2S upon addition of cystine and antibiotics. (a) H_2S production after cystine addition to glycerol-containing medium: left panel (L) – sulfide sensor readings; right panel (R) – color change of $Pb(Ac)_2$-soaked paper strips upon their successive replacement every 30 min. (b) H_2S accumulation kinetics determined using the $Pb(Ac)_2$-soaked paper strips after cystine addition to the medium with and without glycerol. (c) Sulfide sensor readings after addition of chloramphenicol and ciprofloxacin to the glycerol-containing medium. (d) The kinetics of H_2S accumulation determined using the $Pb(Ac)_2$-soaked paper strips after addition of chloramphenicol and ciprofloxacin to the glycerol-free medium. (e) The H_2S levels in extracts of cells grown in glycerol-containing medium determined by high performance liquid chromatography (HPLC). Results of HPLC analysis are expressed as arbitrary units (peak area divided by OD_{600}). The arrow shows the moment of adding the test compounds at OD_{600} 0.4. Mean values and standard errors (vertical bars) are shown. Significant differences compared to control are marked with an asterisk (*) ($p < 0.05$).

controls (Fig. 2e). Treatment with chloramphenicol did not affect the level of intracellular sulfide (Fig. 2e).

The methods used allow the assessment of H_2S accumulation in the liquid medium (sulfide electrode), in the gas phase (lead acetate-soaked paper strips), and in cell extracts (HPLC analysis of mBBr derivatives) and differ in sample preparation and detection threshold (5 nM, 0.1 µM and 2 nM, respectively), which may explain some of the discrepancies in the data obtained by these methods, especially at low levels of H_2S production. In general, the use of three different detection methods revealed H_2S production by *M. smegmatis* cells upon addition of cystine to the medium. H_2S release from cells upon exposure to chloramphenicol and ciprofloxacin was only confidently recorded using a selective sulfide electrode, indicating that the level of H_2S production is weak under these conditions. Nevertheless, in all the cases, sulfide release could be caused by an excess cysteine in the cytoplasm, prompting us to measure its levels in the studied situations.

3.3 Effect of Cystine and Antibiotics on Cysteine and Mycothiol Levels

In the absence of exposures, intracellular cysteine in *M. smegmatis* was maintained at a nearly constant level (0.115 ± 0.008 µM/OD_{600}) throughout the entire culture period. Addition of cystine resulted in a rapid 3-fold increase in intracellular cysteine levels, while chloramphenicol and ciprofloxacin caused a statistically significant increase 1.4-fold ($p = 0.004$) and 1.3-fold ($p = 0.017$), respectively (Fig. 3a). In contrast to cystine, exposure to antibiotics resulted in maximum cysteine levels only after 90 min. HPLC analysis confirmed an increase in intracellular cysteine upon addition of cystine and chloramphenicol (Fig. 3b).

Since excessive cysteine is cytotoxic, multiple bacteria utilize cysteine-specific efflux pumps for its export [18–20]. We measured the concentration of extracellular cysteine during exposure to antibiotics in order to elucidate the involvement of cysteine export in regulation of its in-

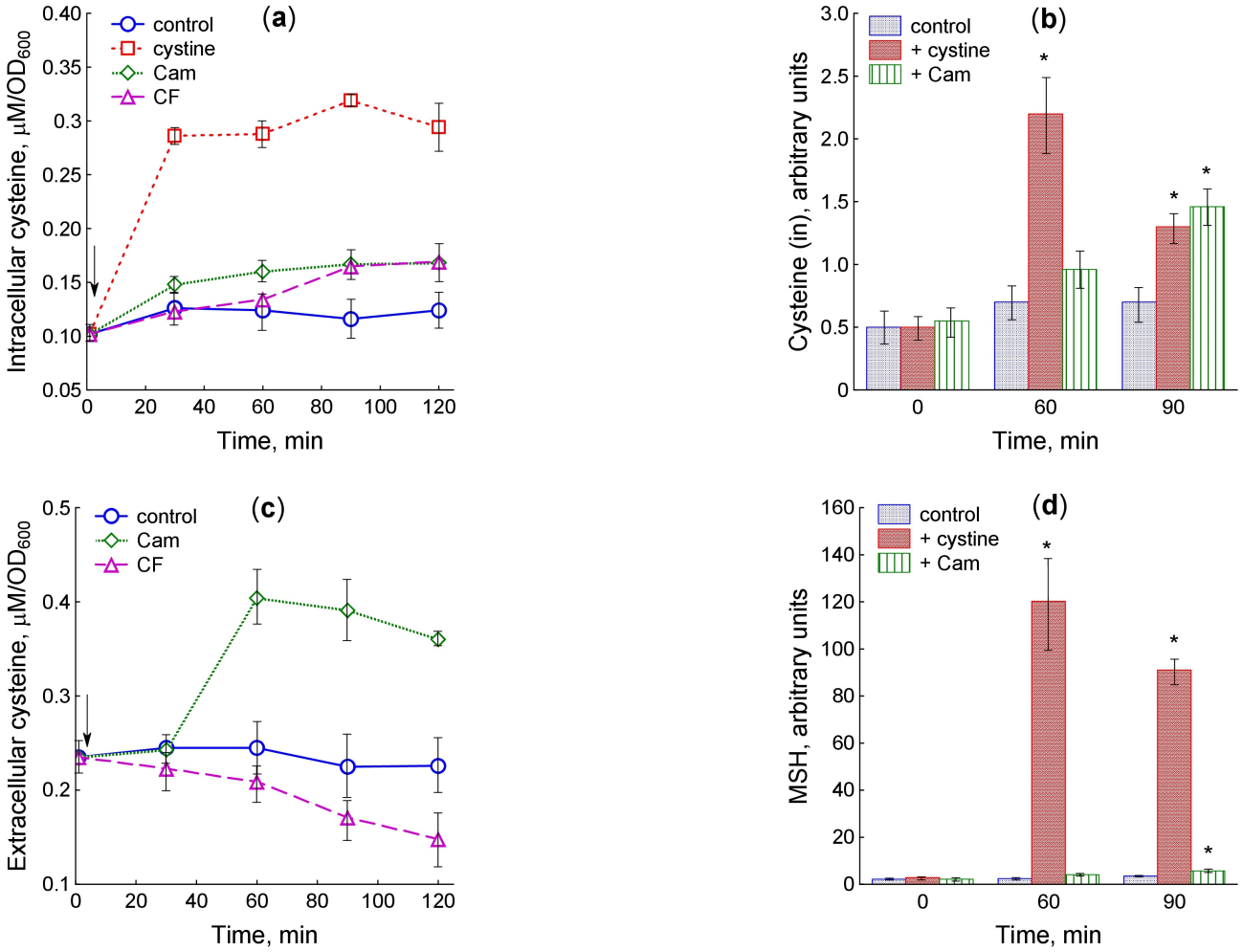

Fig. 3. Changes in cysteine and mycothiol levels upon treatment of *M. smegmatis* with cystine and antibiotics. (a) Intracellular cysteine determined using the Gaitonde's method, (b) intracellular cysteine determined by HPLC, (c) extracellular cysteine determined using the Gaitonde's method, (d) mycothiol (MSH) levels determined by HPLC. The arrow shows the moment of adding the test compounds at OD_{600} 0.4. Results of HPLC analysis are presented in arbitrary units (the peak area divided by OD_{600}). Significant differences compared to control are marked with an asterisk (*) ($p < 0.05$).

tracellular homeostasis under stress. Extracellular cysteine concentration per OD_{600} unit was 2-fold higher than its intracellular level, remaining approximately constant during growth in the untreated culture. Chloramphenicol accelerated cysteine efflux and increased its extracellular concentration by 1.8 times 60 min after the start of exposure (Fig. 3c). In contrast to chloramphenicol, ciprofloxacin gradually reduced the extracellular cysteine levels.

In addition to cysteine and sulfide, HPLC analysis of mBBr derivatives revealed the presence of mycothiol (**Supplementary Fig. 2**). Intracellular mycothiol levels unchanged during growth of *M. smegmatis* in the control culture, but increased significantly after cystine supplementation (Fig. 3d; **Supplementary Fig. 2**). When exposed to chloramphenicol, the mycothiol level statistically significantly increased after 90-min incubation with the antibiotic, coinciding in time with the maximum increase in cysteine content (Fig. 3d; **Supplementary Fig. 2**). Statistical analysis of the data presented in Fig. 2b,d revealed a high correlation between the cysteine and mycothiol levels when *M. smegmatis* was treated with cystine ($r = 0.95$, $p < 0.05$) and chloramphenicol ($r = 0.99$, $p < 0.05$). Overall, treatment of *M. smegmatis* with cystine and chloramphenicol induced excess cysteine in the cytoplasm, which activated homeostasis mechanisms including cysteine export and its incorporation into mycothiol.

4. Discussion

In this work, we have shown that, just like in *E. coli* and *B. subtilis*, the main cause of endogenous H_2S production in *M. smegmatis* is an increase in intracellular cysteine concentration, which may result from over import of exogenous cysteine/cystine or from dramatic inhibition of protein synthesis by chloramphenicol treatment. In both situations, the increase in intracellular cysteine levels in-

duced mechanisms of its homeostasis. In *E. coli*, when protein synthesis is inhibited by antibiotics or as a result of nutrient starvation, most of the excess cysteine is incorporated into glutathione, while the remaining portion is exported into the medium and degraded to form H_2S [11,12,26]. All these mechanisms of cysteine homeostasis are maximally activated during intracellular cysteine overload as a result of excess cystine import when it is suddenly added to a medium with sulfate as the only source of sulfur [6,13,16,17]. During *E. coli* growth in cysteine-containing Luria-Bertani (LB) medium (Miller), H_2S production by cells is carried out without any external influences. Under these conditions, the onset of H_2S release coincides with the slowdown of growth and respiration, which may also indicate the occurrence of excess cysteine at this stage of culture growth [27,38].

In *B. subtilis*, which does not contain GSH, as well as in the glutathione-deprived *gshA* mutant of *E. coli*, the main pathways to restore cysteine homeostasis are its export and degradation to form H_2S, which is more intense and prolonged than in the wt strain of *E. coli* [12–14,26]. It appears that bacillithiol, the major LMW thiol in *B. subtilis* as well as other *Firmicutes* [42], does not function as a cysteine buffer, unlike GSH in *E. coli*.

Mycothiol is the unique protective thiol of *Actinobacteria* [30] and the most abundant LMW thiol in *M. smegmatis* [29]. In this work, we have shown that an increase in intracellular cysteine is accompanied by an increase in mycothiol levels, indicating its role as a cysteine buffer in *M. smegmatis*. We also observed cysteine release from cells and H_2S production upon exposure to chloramphenicol, indicating that *M. smegmatis* possesses all the mechanisms of cysteine homeostasis characteristic of *E. coli*: incorporation of excess cysteine into buffer molecules, release into the medium, and desulfurization.

Measurements showed that *M. smegmatis* growing under normal conditions contained a low cysteine level (0.115 ± 0.008 μM/OD_{600}), which is close to the cysteine level in *E. coli* cells (0.13 ± 0.02 μM/OD_{600}) [12] and corresponds to a concentration of about 0.1 mM. The need to maintain a low concentration of free cysteine in the cytoplasm is related to its ability to generate ROS upon heavy metal-catalyzed autoxidation and to reduce Fe^{3+} to Fe^{2+}, which potentiates the Fenton reaction yielding toxic hydroxyl radicals [5,6]. In GSH, γ-glutamyl and glycine residues reduce the rate of Cu-catalyzed autoxidation from 8- to 26-fold, and in MSH, acetyl and GlcN-Ins residues make Cu-catalyzed autoxidation of MSH about 30-fold slower than cysteine and 7-fold slower than GSH [30]. It makes MSH in *M. smegmatis*, like GSH in *E. coli*, a suitable buffer for safely storing cysteine when its excess occurs. MSH levels in *M. smegmatis* have been shown to range from 16 nmol/10^9 cells (~4.5 mM) in the early exponential phase to 26 nmol/10^9 cells (~7.5 mM) in the late stationary phase [29], being comparable to GSH concentrations in *E. coli*. Unfortunately, using our method, we were unable to determine changes in the other major LMW thiol ergothioneine (ERG) in *M. smegmatis*, whose content is much lower than that of MSH and which is preferentially present as a thione rather than a thiol at physiological pH [37,43]. MSH and ERG were shown to contribute to protection of mycobacteria against reactive oxygen and nitrogen species, alkylating agents and antibiotics, and also play a regulatory role and be involved in adaptation to low pH and other stresses, including through S-thiolation of proteins [31,32].

E. coli can utilize cysteine-specific efflux pumps (EamA, EamB, Bcr) to export cysteine into the medium [18–20]. Thus, constitutive expression of the cysteine exporter EamA in *E. coli* has been shown to prevent H_2S formation by reducing the intracellular concentration of cysteine [26,44]. However, no such transporters have been identified in *M. tuberculosis*. Nevertheless, we observed cysteine release from *M. smegmatis* cells and its accumulation in the medium upon exposure to chloramphenicol, which may indicate that an unidentified cysteine export system is present in this bacterium. In *E. coli*, AlaE was found to be the primary exporter of excessive intracellular cysteine, although this protein was previously identified as an alanine exporter [17]. In contrast to *E. coli*, where ciprofloxacin (10 μg/mL) caused a 3-fold accumulation of cysteine in the medium compared to the control [45], *M. smegmatis* did not increase extracellular cysteine levels under these conditions. Chloramphenicol-induced cysteine release in *M. smegmatis* was also 2.6 times lower than that in *E. coli*. This may be explained by the slower inhibition of growth and hence protein synthesis in *M. smegmatis* compared to *E. coli*, where the higher initial growth rate (0.68 ± 0.01 h^{-1} compared to 0.25 ± 0.01 h^{-1} in *M. smegmatis*) and its rapid decline immediately after antibiotic exposure result in a greater excess of intracellular cysteine. The action of other antibiotics on mycobacteria can also be accompanied by the release of LMW thiols. Accumulation of extracellular thiols has been previously observed when *M. tuberculosis* was treated with bacitracin. Mutants unable to produce extracellular thiols showed increased sensitivity to this antibiotic, supporting their role in detoxification [46].

M. tuberculosis encodes multiple enzymes that may produce H_2S. Cysteine desulfurization involving Cds1 has been shown to be the major, although not the only, source of endogenous H_2S in *M. tuberculosis* [9]. Cds1 is also present in *M. smegmatis* [9], but the involvement of this or other enzymes in H_2S generation upon addition of cystine and chloramphenicol needs to be further studied. *M. smegmatis* has been reported to produce little H_2S [9]. However, our experiments showed that sudden addition of cystine to *M. smegmatis* growing in sulfate medium causes the release of 0.3 μM sulfide, which is 4.5 times lower than that of *E. coli* (1.4 μM) [13], but is well detectable by the methods we used. The difference in H_2S production may be due to variations in the culture medium composition. We found

that excluding glycerol from the medium intensified H_2S generation when cells were treated with cystine and chloramphenicol, which may be due to changes in the cell wall composition and permeability [40,41]. The amount of sulfide released by *M. smegmatis* under the action of chloramphenicol is significantly lower than that of *E. coli* (10–15 nM and 180 nM, respectively). The reason, as in the case of cysteine export, may be a smaller excess of intracellular cysteine that occurs in *M. smegmatis* during rapid inhibition of protein synthesis.

Antibiotic-induced H_2S formation appears to result from disruption of cysteine homeostasis due to protein synthesis inhibition. Under these conditions, growth inhibition in *M. smegmatis* was accompanied by a decrease in the rate of respiration and glucose consumption, as observed previously in *E. coli* [11–13]. However, although both bacteria released H_2S when cystine was added, the physiological response was opposite. Reversible inhibition of growth and respiration was observed in the case of *E. coli* [13], whereas temporary stimulation of respiration and activation of glucose consumption occurred in *M. smegmatis*. These findings can be attributed to the peculiarities of the effect of H_2S on cytochrome oxidases: in *E. coli*, low micromolar concentrations of H_2S inhibit cytochrome *bo* oxidase [16,47], while in *M. tuberculosis,* H_2S stimulates respiration via cytochrome bc_1/aa_3 and, primarily, via cytochrome *bd* [9]. Another evidence of the participation of H_2S in regulation of energy processes, including the membrane potential, in *M. smegmatis* may be the abrupt acceleration of K^+ entry into cells, whose kinetics coincide with sulfide production upon addition of cystine and antibiotics. The ability of H_2S to interact with K^+ ion channels has been previously shown [48]. The special role of exogenous and endogenous H_2S in regulating central metabolism and accelerating respiration and growth in mycobacteria may be among the reasons for the opposite effect of H_2S on antibiotic susceptibility in *M. tuberculosis* and *E. coli* [2,8,9].

We have previously shown that the absence of one of the mechanisms of cysteine homeostasis leads to increased activation of the remaining mechanisms when excess cysteine appears in the cytoplasm. In particular, the lack of glutathione in the *gshA* mutant of *E. coli* causes more intense production of H_2S and cysteine export when cells are treated with ciprofloxacin, whereas the *mstA* mutant, which does not produce H_2S under these conditions, synthesizes more glutathione than the wt strain does [13]. The *gshA* mutant exhibited an increased sensitivity to ciprofloxacin in minimal M9 medium, especially with fractional addition of cystine during cultivation. In the present work, we showed that with increasing concentration of intracellular cysteine in *M. smegmatis*, the level of mycothiol rises, similar to that of GSH in *E. coli*. We speculate that, similar to *E. coli*, a decrease in MSH levels due to mutations or inhibitors of the enzymes involved in its synthesis will intensify H_2S production under stress conditions. Since exogenous and endogenous H_2S have been shown to enhance the killing of *M. tuberculosis* by the anti-TB antibiotics clofazimine and rifampin [8,9], it is conceivable that inhibition of MSH synthesis may increase the susceptibility of mycobacteria to antibiotics via the same mechanism. Mycothiol is essential for *M. tuberculosis* survival and intracellular levels of this thiol are associated with changes in resistance to antibiotics and oxidative stress [39]. *M. smegmatis* devoid of MSH exhibited an increased susceptibility to several antibiotics, such as erythromycin, azithromycin, rifamycin S, penicillin G, and vancomycin [30,49], suggesting that MSH is involved in multiple detoxification mechanisms, one of which may be the maintenance of cysteine homeostasis. Although caution is needed when extrapolating the results obtained with *M. smegmatis* to *M. tuberculosis* and further more in-depth studies are needed, our findings may be promising in searching for ways to improve the efficacy of anti-TB drugs.

5. Conclusions

In this study, we showed that, similar to *E. coli* and *B. subtilis*, addition of cystine and chloramphenicol to growing *M. smegmatis* induces intracellular cysteine accumulation and activates homeostatic mechanisms such as cysteine export, its degradation to H_2S, and incorporation into mycothiol as a cysteine buffer. Ciprofloxacin also increased intracellular cysteine concentration and sulfide production but did not induce cysteine release. Our findings indicate that activation of cysteine homeostasis mechanisms may be part of a universal stress response across bacterial species. The molecular mechanisms underlying these processes in mycobacteria require further investigation, including key gene knockout experiments.

Availability of Data and Materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Conceptualization, GS and OO; Investigation, AT, LS, TK, and VU; Formal analysis, OO, GS, AT, LS, TK, and VU; Methodology, OO, GS, AT, LS, TK, and VU; Data curation, GS and OO; Writing–original draft preparation, GS; Writing–review and editing, OO and GS. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study does not involve human or animal subjects; therefore, according to Russian regulations and policies, ethical committee approval is not necessary.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL44441.

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