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EXPERIMENTAL ARTICLES

The Role of Antioxidant Systems in the Response of *Escherichia coli* to Acetamidophenol and Some Antibiotics

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Abstract—The role of glutathione and other antioxidant systems in the response of *Escherichia coli* to acetamidophenol (paracetamol), rifampicin, and chloramphenicol was studied. The exposure of aerobically growing *E. coli* cells to acetamidophenol diminished the intracellular level of glutathione by 40% and the reduced-to-oxidized glutathione ratio in the cells by 50%, while it enhanced the expression of the antioxidant genes *soxS* and *sodA* by 2.7 and 1.8 times, respectively. Glutathione-deficient cells were more susceptible to acetamidophenol than were normal cells. All this suggests that acetamidophenol induces a mild oxidative stress in *E. coli* cells. The oxidative stress induced by rifampicin was still less pronounced, whereas chloramphenicol-treated *E. coli* cells exhibited no signs of oxidative stress at all.

Key words: glutathione, acetamidophenol, oxidative stress, *Escherichia coli.*

In nature, bacteria are frequently subject to various stresses, including oxidative. Bacterial cells constitutively synthesize antioxidant enzymes and possess various adaptive response mechanisms, due to which cells that have received low doses of oxidants become tolerant to high doses. The transcriptional factors SoxRS and OxyR, encoded by the regulatory genes *soxRS* and *oxyR*, respectively, are known to be the key regulators of the adaptive response of *Escherichia coli* cells to oxidative stress. SoxRS and OxyR induce a coordinate expression of gene groups (regulons) in response to such exogenous reactive oxygen species (ROSs) as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), respectively [1, 2]. ROSs are also normal byproducts of the aerobic respiratory chain [3, 4]. The disturbance of normal metabolism by various extreme factors may augment the concentration of ROSs to levels that cause oxidative stress.

Little is known about the role of antioxidant systems in the response of the normal microflora of human and animal intestines to drugs, many of which can cause oxidative stress indirectly by inhibiting the activity of antioxidant systems or by activating ROS-producing metabolic pathways.

Our earlier studies showed that the exposure of aerobically growing *E. coli* cells to the membranotropic antibiotic gramicidin, which is not oxidant, gives rise to metabolic changes typical of oxidative stress, such as the decline in the intracellular level of reduced glutathione (GSH) and in the reduced-to-oxidized glutathione ratio (GSH : GSSG); the increase in the expression of the antioxidant genes *sodA, soxS*, and *gor*; and the enhancement of the gramicidin susceptibility of the mutants deficient in the synthesis of GSH and the superoxide dismutases SodA and SodB [5].

The aim of this work was to study the role of antioxidant systems in the response of *E. coli* to acetamidophenol, which, together with its derivatives paracetamol, panadol, etc., is extensively used in medical practice as a potent antipyretic analgesic but may cause oxidative damage to hepatocytes at high concentrations [6]. Relevant studies were also performed with rifampicin and chloramphenicol, as information about the possible involvement of antioxidant systems in bacterial resistance to these antibiotics is scarce.

MATERIALS AND METHODS

The *Escherichia coli* strains used in this work are listed in Table 1. The strains were grown overnight in mineral M9 medium [7] supplemented with 0.4% glucose, 0.2% casamino acids, and 10 μg/ml thiamine. Cells were precipitated by centrifugation, resuspended in fresh medium (100 ml), and cultivated at 37°C in 250-ml flasks on a shaker (150 rpm). Growth was monitored by measuring culture turbidity at 670 nm. The antibiotics and acetamidophenol were added in the midexponential growth phase, when the culture density was 0.3–0.4 g dry wt. cells/l.

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Table 1. The *E. coli* strains used in this study

Strain	Relevant genotype	Source
AB1157	*thr-1 leuB6 thi-1 arg-E3 his-4 proA2 tsx-33*	
	supE44 lacY1 galK2 ara-14 xyl-5 mtl-1 rpsL	CGSC*
JTG10	AB1157 *gshA* (*gshA20::Tn10 km*$^{-}$)	B. Demple
NM23	As AB1157, but with pKT1033 (*katG::lacZ*)	Laboratory collection
NM31	As AB1157, but with pRSkatE16 (*katE::lacZ*)	Laboratory collection
NM122	As AB1157, but with pGOR-12 (*gor::lacZ*)	Laboratory collection
QC772	GC4468 *sodA49* (*sodA::lacZ*)	D. Touati
TN521	Δ(*lac*)*U169 rpsL* ΔsoxRS *soxR*$^{+}$ *soxS::lacZ*	B. Demple
BGF611	As AB1157, but *katG17::Tn10*	B. Demple)
GC 4468	F^{-} Δ(*lac*) *U169 rpsL soxRS*$^{+}$ *sup*(*Am*)	B. Demple
QC 909	As GC 4468, but (*sodA sodB*) *E. coli*	B. Demple
AE 1318	*araD139* Δ(*argF-lac*)*169 flhD5301* Δ(*his-grd*)*295 relA1 rpsL150 deoC1*	A. Eisenstark
AE1319	As 1318, but (*gor*$^{-1}$*::Muc*)	A. Eisenstark
SH646	F^{-} *ggt-2 recA56 rpsL sr1300::Tn10*	H. Suzuki
SH641	As SH646, but *ggt*	H. Suzuki
Plasmids		
	pRK4936/pKT1033 c *katG::lacZ*	K. Tao
	pRS*katE16* c *katE::lacZ*	A. Eisenstark
	pGOR-12 c *gor::lacZ*	A. Eisenstark

* CGSC is the *Escherichia coli* Genetic Stock Center.

The intracellular level of reduced and oxidized glutathione (GSH and GSSG, respectively) was determined spectrophotometrically [8] as has been described earlier [9]. Intracellular K^{+} was measured using a flame photometer. Samples for this analysis were prepared by rapid filtration through membrane filters [10]. Protein concentration was determined by the Lowry method with bovine serum albumin as the standard. The expression of the relevant genes was studied by measuring the β-galactosidase activity of strains harboring the promoter fusions of these genes with the structural gene of β-galactosidase [7].

All the experiments were performed at least in triplicate. The results presented in this paper are the mean values ± the standard deviation. The statistical significance of the data was evaluated by using Student's *t*-test for significance level $P < 0.05$.

Agar, casamino acids, thiamine, deoxycholate, glutathione reductase, mercaptoethanol, DTNB, EDTA, NADPH, GSH, GSSG, NEM, rifampicin, chloramphenicol, and acetamidophenol were purchased from Sigma (United States). All other reagents used were of analytical grade.

RESULTS

The effect of acetamidophenol. This compound at concentrations higher than 5 μg/ml inhibited the biomass yield and the growth rate of *E. coli* AB1157 in a dose-dependent manner. At a concentration of 1 μg/ml, however, acetamidophenol slightly enhanced bacterial growth at 37°C under both aerobic and anaerobic conditions, while only under anaerobic conditions at 42°C (Figs. 1a, 1b). At 42°C, the inhibitory effect of acetamidophenol on *E. coli* cells under aerobic conditions was stronger than under anaerobic conditions, suggesting that acetamidophenol may cause oxidative stress.

To prove this suggestion, we studied the expression of antioxidant genes in aerobically growing *E. coli* cells by measuring β-galactosidase activity in the strains harboring the promoter fusions of the *katG, katE, sodA, gor*, and *soxS* genes (which code for the antioxidant system components) with the *lacZ* gene operon. These experiments were carried out at an acetamidophenol concentration equal to 7.5 μg/ml, which partially inhibited bacterial growth and, hence, biosynthetic processes (Figs. 1a, 1b).

The *sodA* gene codes for the superoxide dismutase SodA, which plays a key role in protecting *E. coli* cells from superoxide-induced stress. The exposure of *E. coli* QC772 cells, which bear the *sodA::lacZ* fusion, to acetamidophenol enhanced the β-galactosidase activity of this strain by 1.8 times. It is known that the *sodA* gene is under the control of several regulatory genes, including the two-gene system *soxRS* of bacterial response to superoxide-induced stress [2]. In this

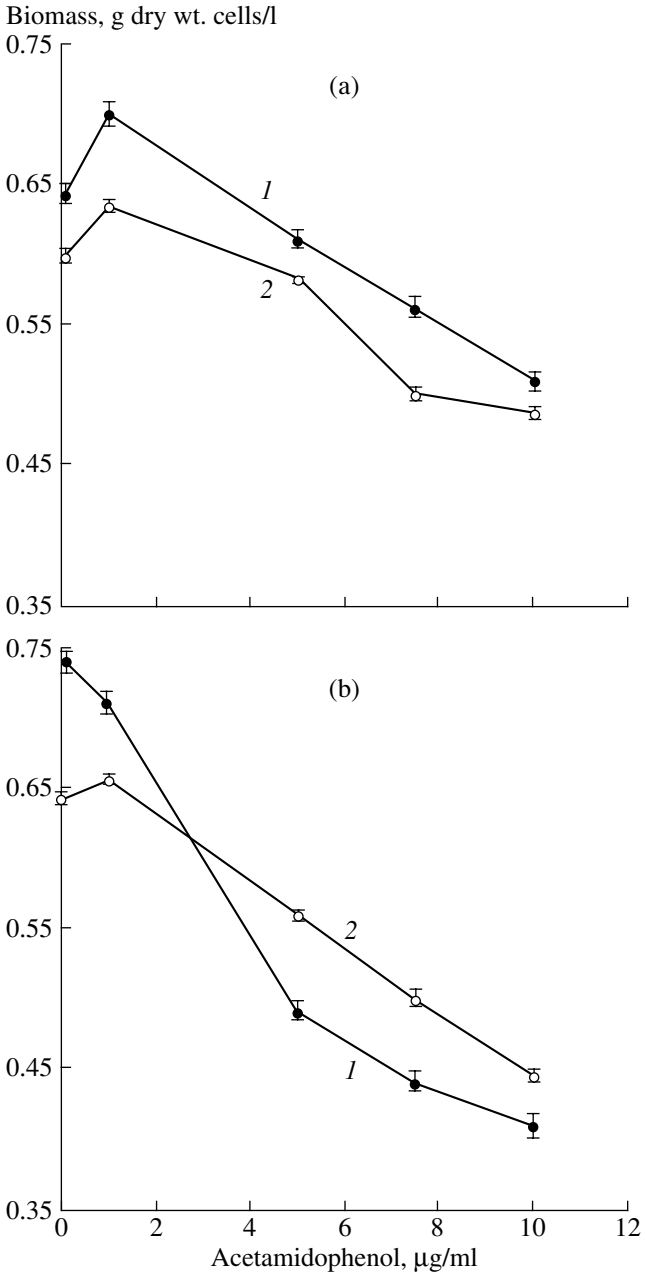

Fig. 1. The effect of acetamidophenol on the biomass of *E. coli* AB1157 grown (*1*) aerobically and (*2*) anaerobically at (a) 37°C and (b) 42°C.

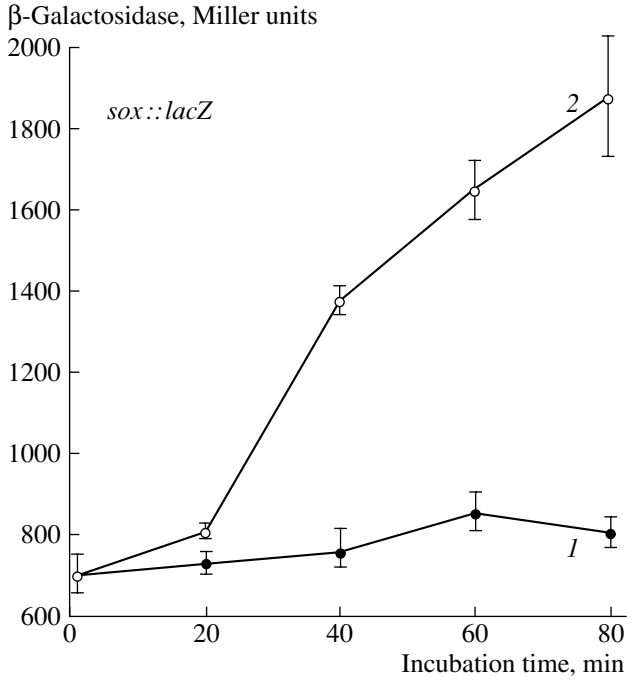

Fig. 2. The effect of acetamidophenol on expression of the *soxS::lacZ* fusion in *E. coli* TN521: (*1*) control cells, (*2*) cells treated with 7.5 μg/ml acetamidophenol.

regard, of interest is the fact that acetamidophenol augmented 2.7-fold the β-galactosidase activity of *E. coli* TN521 cells, which carry the *soxS::lacZ* fusion (Fig. 2).

Hydrogen peroxide in *E. coli* cells is detoxified by two catalases, HPI and HPII, which are encoded by the *katG* and *katE* genes, respectively. In the presence of acetamidophenol, the activity of β-galactosidase in the growing *E. coli* NM23 and NM33 cells, which bear the *katG::lacZ* and *katE::lacZ* fusions, decreased by 44 and 31%, respectively. Therefore, acetamidophenol not only failed to activate, but even inhibited, the expression of the *katG* and *katE* genes.

In *E. coli* cells, oxidized glutathione is reduced by NADPH-dependent glutathione oxidoreductase (GOR) encoded by the *gor* gene. The exposure of *E. coli* MB1417 cells, which bear the *gor::lacZ* fusion, to acetamidophenol did not cause statistically significant changes in the activity of β-galactosidase.

To evaluate the contribution of antioxidant systems in the response of *E. coli* cells to acetamidophenol, we measured the specific growth rate of the strains that have mutations in the genes controlling the particular components of these systems and found that only the glutathione deficiency mutation of the *gshA* gene enhanced cell susceptibility to acetamidophenol. Namely, in the presence of 7.5 μg/ml acetamidophenol, the specific growth rate of the strain *E. coli* JTG10 bearing such a mutation was 18% lower than in the case of the parent strain. Under the same conditions (i.e., in the presence of acetamidophenol), the strain *E. coli* QC909 (*sodAsodB*), deficient in the superoxide dismutases A and B, and the strains NM23 and NM33, deficient in the hydroperoxidases HPI and HPII, showed the same specific growth rates as their parent strains.

It is known that γ-glutamyltranspeptidase (GGT) is involved in glutathione metabolism and in the response of animal cells to oxidative stress, playing either a positive or negative role in relation to the type of cells and environmental conditions. The role of this enzyme in the response of bacteria to oxidative stress is unknown.

Table 2. The effect of antibiotics and acetamidophenol on the status of intracellular reduced glutathione (GSH_{in}) in *E. coli* cells

Incubation time, min	Control	Acetamidophenol, 7.5 μg/ml	Rifampicin, 10 μg/ml	Chloramphenicol, 38 μg/ml
0	19.2 ± 0.81 (240 ± 10)	16.7 ± 1.7 (278 ± 16)	19.0 ± 1.1 (317 ± 18)	18.0 ± 0.59 (257 ± 9)
30	20.4 ± 1.2 (340 ± 20)	10.5 ± 1.9 (210 ± 12)	21.0 ± 1.9 (1050 ± 76)	21.1 ± 0.82 (264 ± 10)
60	20.5 ± 0.8 (342 ± 14)	10.0 ± 0.9 (167 ± 7)	24.2 ± 1.7 (1210 ± 113)	21.7 ± 0.4 (310 ± 5)

Note: The data presented in the table show the intracellular concentration of reduced glutathione in μmol/g dry wt. cells. The data in parentheses show the GSH_{in} : $GSSG_{in}$ ratios.

In our experiments, GGT-deficient *E. coli* SH646(*ggt*) cells exposed to acetamidophenol (7.5 μg/ml) did not differ in growth rate from cells of the parent strain.

According to earlier observations [9], the exposure of *E. coli* cells to the superoxide anion-generating compound menadione elevates the levels of oxidized glutathione inside and outside cells and, respectively, decreases the GSH : GSSG ratio on both sides of the cytoplasmic membrane.

It was of interest to compare the effects of menadione and acetamidophenol on the status of glutathione in *E. coli*. Experiments showed that the exposure of *E. coli* cells to acetamidophenol (7.5 μg/ml) lowered the intracellular level of reduced glutathione by 40% and did not influence the intracellular level of oxidized glutathione, so that the GSH_{in} : $GSSG_{in}$ ratio decreased by approximately 50% (Table 2). In this case, the extracellular level of reduced glutathione increased by 29%, whereas the extracellular level of oxidized glutathione decreased by two times. As a result, the GSH_{out} : $GSSG_{out}$ ratio increased from 12 to 36. Thus, acetamidophenol acts to augment the concentration of GSH in the medium and to diminish its concentration in the cytoplasm of *E. coli* cells, suggesting that an amount of intracellular reduced glutathione is released into the medium.

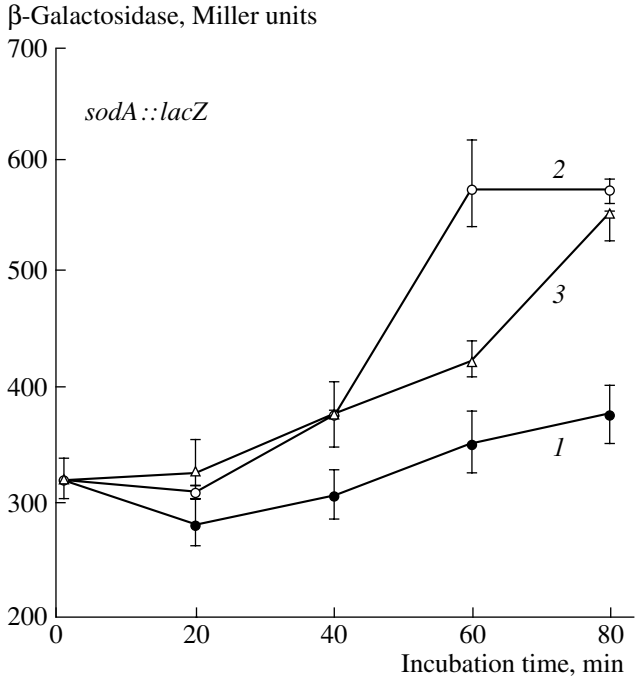

Fig. 3. The effect of acetamidophenol on the expression of the *sodA::lacZ* fusion in *E. coli* QC772: (*1*) control cells, (*2*) cells treated with 0.09 μg/ml rifampicin, (*3*) cells treated with 1 μg/ml rifampicin.

It is known that the intracellular pool of potassium is controlled by the redox state of *E. coli* cells and that glutathione may be involved in this process [10, 11]. It was of interest to study the effect of acetamidophenol on the intracellular level of potassium. Experiments showed that the treatment of *E. coli* cells with acetamidophenol (7.5 μg/ml) stimulated the excretion of potassium from the cells, so that the intracellular concentration of potassium decreased by 32% one hour after acetamidophenol had been added to the medium.

The effect of rifampicin. This antibiotic inhibited the growth of *E. coli* AB1157 cells under both aerobic and anaerobic conditions in a dose-dependent manner, so that bacterial growth was completely suppressed at a rifampicin concentration of 20 μg/ml. Aerobic *E. coli* cells were more susceptible to rifampicin than were anaerobic cells at both 37 and 42°C.

Within a wide range of concentrations (0.03–10 μg/ml), rifampicin stimulated the expression of the *sodA* gene coding for superoxide dismutase A, as is evident from the effect of this antibiotic on the activity of β-galactosidase in the aerobically growing *E. coli* QC772 cells harboring the *sodA::lacZ* fusion. Figure 3 exemplifies the induction of this fusion by rifampicin at concentrations of 0.09 and 1 μg/ml, due to which the activity of β-galactosidase increased by approximately 50%. It should be noted that the *sodA::lacZ* fusion could be induced even by rifampicin concentrations that are not inhibitory to cell growth.

The expression of another fusion, *gor::lacZ*, was enhanced with 0.03 μg/ml rifampicin by 17%. At the same time, this antibiotic did not influence the expression of the *katG, katE*, and *soxS* genes in aerobically growing *E. coli* cells.

At a concentration of 10 μg/ml, rifampicin inhibited the aerobic growth of hydroperoxidase HP1 deficient

cells of *E. coli* BGF611 and glutathione deficient cells of *E. coli* JTG10 by 30 and 21%, respectively. Other mutations did not exert any noticeable influence on the growth of *E. coli* cells in the presence of rifampicin.

The exposure of growing *E. coli* AB1157 cells to rifampicin (10 μg/ml) lowered the intracellular level of oxidized glutathione by three times and elevated the intracellular level of reduced glutathione by 18%, so that the GSH_{in} : $GSSG_{in}$ ratio considerably increased (Table 2). In this case, the extracellular level of reduced glutathione approximately halved, whereas the extracellular level of oxidized glutathione did not change. As a result, the GSH_{out} : $GSSG_{out}$ ratio declined by 1.5–2 times. Thus, rifampicin acts to increase the concentration of GSH in *E. coli* cells and to diminish its concentration in the medium, suggesting that an amount of extracellular reduced glutathione is transported from the medium to the cells.

The exposure of aerobically growing *E. coli* cells to rifampicin (0.06 μg/ml) diminished the intracellular potassium concentration by 24%.

The effect of chloramphenicol. This antibiotic inhibited the growth of *E. coli* cells under both aerobic and anaerobic conditions in a dose-dependent manner, so that bacterial growth was completely suppressed at a chloramphenicol concentration of 38 μg/ml. Aerobic *E. coli* cells were more susceptible to chloramphenicol than were anaerobic cells, but the difference in cell susceptibility was small.

At a concentration of 7.5 μg/ml, chloramphenicol enhanced by 1.5-fold the β-galactosidase activity of aerobically growing *E. coli* QC772 cells harboring the *sodA::lacZ* fusion, but suppressed it at higher concentrations. The expression of another fusion, *soxS::lacZ*, was not significantly influenced by this antibiotic.

According to earlier observations, the catalase activity and the expression of the *katG* and *katE* genes coding for catalases HP1 and HPII tended to increase in the course of the aerobic growth of *E. coli* [12, 13]. Experiments performed in the present study showed that chloramphenicol at low concentrations did not influence the expression of these genes, while slightly suppressing it at a concentration of 38 μg/ml.

The expression of the *gor::lacZ* fusion did not change in the presence of chloramphenicol.

The chloramphenicol resistance of *E. coli* cells was most affected by the loss of the activity of two superoxide dismutases, A and B. It should be noted that the growing SOD-deficient cells of *E. coli* QC909 (*sodAsodB*) were less susceptible to chloramphenicol than were the cells of the parent strain GC4468 ($sodA^+sodB^+$).

The mutations of *gshA*, *katG*, and *ggt* did not notably influence the chloramphenicol resistance of *E. coli* cells.

The exposure of aerobically growing *E. coli* AB1157 cells to chloramphenicol did not considerably change the status of intra- and extracellular glutathione (Table 2) and the intracellular level of potassium.

The combined effect of acetamidophenol and antibiotics. *E. coli* AB1157 cells aerobically growing at 37°C were first treated with various acetamidophenol concentrations and then (after 30 min) by one of the antibiotics. The experiments showed that acetamidophenol (7.5 μg/ml) and rifampicin (10 μg/ml), added separately, inhibited the biomass yield of *E. coli* by 13 and 15%, respectively, compared to 38% upon the combined treatment with these compounds. Similarly, acetamidophenol (7.5 μg/ml) and chloramphenicol (38 μg/ml), added separately, inhibited the growth rate of *E. coli* cells by 17 and 23%, respectively, compared to 32% upon the combined treatment with these compounds. If acetamidophenol was added at a low concentration (1 μg/ml), the subsequent addition of rifampicin or chloramphenicol did not affect bacterial growth at all. These data suggest that the effects of acetamidophenol and the antibiotics are additive, provided that the concentration of acetamidophenol is sufficiently high.

DISCUSSION

It is known that the expression of some genes coding for components of the antioxidant defense of bacterial cells is enhanced in response to elevated concentrations of oxidants, such as hydrogen peroxide or superoxide anion, in the medium [1, 2]. Such an enhanced expression of the antioxidant genes is considered to be the major sign of oxidative stress. In our experiments, the exposure of aerobically growing *E. coli* cells to acetamidophenol substantially enhanced the expression of two antioxidant genes, *soxS* and *sodA*. The latter gene is known to be under the control of several factors, including the two-gene system *soxRS*, which is mainly induced by superoxide anion [2, 14]. The concurrent increase in the expression of the *soxS* and *sodA* genes suggests that the activation of *sodA* is due to an elevated level of ROS. At the same time, *sodA* can also be activated by the transcriptional factor MarA, which plays an important part in bacterial resistance to antibiotics. Of interest is the fact that acetamidophenol may induce the transcription of the *marRAB* operon [15].

The cytotoxic effect of high concentrations of acetamidophenol on hepatocytes may be due to the ability of this drug to inflict oxidative damage [6]. Evidence for this comes from the decreased level of intracellular glutathione, which is a major sign of oxidative stress in eukaryotic cells. The results obtained in this study also indicate that acetamidophenol may induce oxidative stress, as is evident from the concurrent increase in the expression of the *soxS* and *sodA* genes and the decline in the intracellular level of glutathione and the GSH_{in} : $GSSG_{in}$ ratio. The presence of glutathione in eukaryotic and prokaryotic cells augments their resistance to acetamidophenol.

Table 3. The generalized effect of acetamidophenol and antibiotic on *E. coli* cells

Compound	GSH_{in}	GSH_{out}	Gene expression					Mutant susceptibility				K^+_{in}
			sodA	*soxS*	*katG*	*katE*	*gor*	*gsh*$^-$	*sodAB*$^-$	*katG*$^-$	*ggt*$^-$	
Acetamidophenol	–	+	+	+	–	–	0	+	0	0	0	–
Rifampicin	+	–	+	0	0	0	+	+	0	+	0	–
Chloramphenicol	0	0	+	0	–	–	0	0	–	0	0	0

Note: "+," "–", and "0" stand, respectively, for an increase, decrease, and no changes in a given parameter (level of glutathione and potassium, gene expression).

The effect of acetamidophenol on *E. coli* cells is manifold. The data available in the literature show that the exposure of prokaryotic and eukaryotic cells to elevated temperatures under aerobic conditions may increase the level of ROS [16, 17]. The higher susceptibility of aerobic *E. coli* cells to acetamidophenol at 42°C may serve as indirect evidence that this compound induces oxidative stress.

Rifampicin can also induce oxidative stress, though rather mildly. This follows from the high rifampicin susceptibility of *E. coli* mutants deficient in the synthesis of hydroperoxidase HPI and glutathione reductase. The enhanced expression of the *sodA::lacZ* fusion in response to rifampicin may not be certainly related to an increased level of ROS and, hence, oxidative stress, since the *sodA* gene is under the control of several factors, including the transcriptional activator MarA. It should be noted in this regard that, unlike acetamidophenol, rifampicin does not activate *soxS*. The high rifampicin susceptibility of aerobic *E. coli* cells growing at 42°C can be considered indirect evidence for oxidative stress. In view of the fact that the glutathione-deficient *E. coli* cells are less resistant to rifampicin than are cells of the parent strain, the elevated level of intracellular glutathione and the GSH_{in} : $GSSG_{in}$ ratio against the background of the diminished level of extracellular glutathione can be treated as an indication of the involvement of glutathione in the adaptive response of *E. coli* cells to the rifampicin-induced stress.

Unlike acetamidophenol and rifampicin, chloramphenicol did not induce cell responses typical of oxidative stress.

Table 3 summarizes the main experimental results obtained in this study. Acetamidophenol influenced the maximum number of cell parameters (8 of the 11 investigated), four of which can be assigned to oxidative stress and the functions of antioxidant systems. For comparison, rifampicin and chloramphenicol influenced, respectively, 6 and 4 parameters out of the 11. The only cell parameter that changed in a similar fashion (specifically, increased) under the action of all three compounds investigated was the expression of the *sodA* gene.

It is known that glutathione can regulate the intracellular level of potassium by controlling K^+ channels. The data presented in Table 3 show that there is no profound correlation between the status of intracellular glutathione and the potassium level in the *E. coli* cells exposed to acetamidophenol or the antibiotics.

Acetamidophenol, as a component of the therapeutic drugs paracetamol, panadol, etc., is fairly well studied with respect to its action on animal and human cells. Although the effect of this compound on the normal microflora of animal and human intestines has not been adequately investigated, it is well known that uncontrolled application of antibiotics may suppress the normal enteric microflora and cause disbacterioses. Moreover, the results obtained in this study show that acetamidophenol may enhance the detrimental effect of antibiotics on *E. coli*, which is an important component of the enteric microflora of animals and humans. It should be noted that this study is only an attempt to understand the combined effect of acetamidophenol and antibiotics. Further studies should provide better insight into this problem.

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