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EXPERIMENTAL ARTICLES

Lysine Decarboxylase Activity as a Factor of Fluoroquinolone Resistance in *Escherichia coli*

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Abstract—Exposure of *E. coli* cells to sublethal concentrations of fluoroquinolones induced synthesis of lysine decarboxylase LdcC, which was previously considered to be a constitutive enzyme. Under these conditions, a key role in this process is played by RNA polymerase σ^S subunit (RpoS); its quantity increased substantially in the presence of antibiotics. Fluoroquinolones of the second and third generations had a more pronounced effect on *rpoS* expression and LdcC activity than the first-generation antibiotics. A direct correlation was shown between the level of cadaverine, the product of lysine decarboxylase reaction in *E. coli* cells, and their resistance to fluoroquinolones. An increase in endogenous cadaverine reduced effectiveness of the second and third-generation fluoroquinolones, but had no effect on antimicrobial activity of the first-generation antibiotics. This is in good agreement with the hydrophilic properties of antibiotics of different generations and, consequently, with different mechanisms of their penetration into bacterial cells.

Key words: fluoroquinolones, cadaverine, polyamines, antibiotic resistance, lysine decarboxylase, LdcC, *rpoS*.

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Extensive use of antibiotics since their discovery in the early 1950s resulted in the emergence and spread of antibiotic-resistant microorganisms, including those showing evidence of multiple antibiotic resistance [1].

There are at least two mechanisms underlying nonspecific antibiotic resistance: limiting the penetration of antibiotics into bacterial cells and active removal of the drug by means of multidrug efflux pumps [2]. Triggering of these mechanisms in the first moments of contact with an antibiotic is aimed at reducing its concentration in the cell, which prevents rapid death. Later on, this aids in development of a specific high-level antibiotic resistance, which may be achieved either by altering the target of the antibiotic through mutation or enzymatic modification, or by acquiring mechanisms of enzymatic detoxication [3].

Limiting the outer membrane's permeability is common among gram-negative microorganisms as a means of adaptation to many types of stress and is connected with the regulation of the quantity and transport function of porin proteins [4]. Many water-soluble antibiotics are transported into bacterial cells through porin channels; the regulation of porin permeability may therefore contribute to the development of antibiotic resistance.

Recently, it has been shown that an important role in the regulation of porin permeability is played by polyamines, cadaverine in particular [5]. Cadaverine is produced in the lysine decarboxylation reaction. Two lysine decarboxylase isoforms, CadA and LdcC, were found in *E. coli* cells. While the main physiological function of CadA has long since been determined as adaptation to low pH levels [6], the role of the relatively recently described lysine decarboxylase LdcC [7] in microorganisms has remained unknown up to this day. Despite the supposedly constitutive nature of the enzyme, addition of fluoroquinolones in a weakly alkaline medium (pH 7.4) was recently shown to induce significant accumulation of cadaverine in *E. coli* cells [8]. This suggests a possible role of lysine decarboxylase LdcC in *E. coli* adaptation to sublethal concentrations of fluoroquinolones.

The aim of this work was to study alterations of lysine decarboxylase activity in *E. coli* exposed to sublethal concentrations of fluoroquinolones, as well as to determine its isoforms and the role of cadaverine in the development of antibiotic resistance.

MATERIALS AND METHODS

Objects of investigation. *E. coli* strains used in this study, their genotypes and sources are presented in Table 1.

Culturing of microorganisms was carried out in 250-ml flasks, each containing 50 ml of LB medium supplemented with 25 μg/ml of streptomycin, in a thermostatic shaker at 37°C, 100 rpm.

Cell biomass was assayed from optical density (OD_{600}).

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Table 1. *E. coli* strains used in the present work

Strain	Genotype	Reference
HS1600	MC4100DE3 *rpoS13::Tn10*	Schellhorn H.E. [9]
RO91	MC4100 (λRZ5:*rpoS742:lacZ*[hybr])	Hengge-Aronis R. [10]
EH40	GC4468 (λEH40 soxS'::*lacZ* (SoxRS$^+$)	Demple B. [11]

Table 2. Correlation between diameter of growth inhibition zones (mm) and endogenous cadaverine content

Antibiotic	Endogenous cadaverine content/*E. coli* strain	
	26.4 nmol/mg dry weight/RO91	74.1 nmol/mg dry weight/EH40
Levofloxacin	38.3 ± 0.5	34.8 ± 1.9*
Pefloxacin	35.6 ± 1.2	33.4 ± 0.7*
Ciprofloxacin	37.6 ± 1.5	27.3 ± 1*
Nalidixic acid	24.3 ± 1.5	23 ± 1.1

Notes: Average values of ±σ are presented.
* Statistically significant difference from the *E. coli* RO91 culture.

The studied antibiotics (at concentrations given in the Figure captions) were added to the culture at an optical density $OD_{600} = 0.3$.

Activity of β-galactosidase was determined by Miller's method [12].

Enzyme activity. The culture was centrifuged at 2800 rpm, 0°C for 5 min, and then washed twice with physiological saline. Resuspended cells were sonicated (22 kHz, 10 mA, 3 times for 10 s) and the cell debris was precipitated by centrifugation (16000 *g*, 20 min). The incubating mixture for determining the enzyme activity contained 100 mM citrate–phosphate buffer (pH 7.5), 0.04 mM pyridoxal phosphate, 1 mM dithiothreitol, 10 mM L-lysine, and the supernatant containing 100 μg of protein, in the final volume of 0.5 ml [13]. The reaction was triggered by adding the substrate; after 60-min incubation at 37°C, it was stopped by adding perchloric acid to the final concentration of 0.4 M.

Protein determination was performed by the Lowry method [14].

Polyamine determination was performed by the TLC method [15].

Antibiotic resistance determination was performed using the method of twofold serial dilutions on plates and the disc-diffusion test according to the Methodological instructions [16].

Statistical analysis was performed using the standard software package Statistica for Windows 5.0. The average values from a set (not less than three) of uniform experiments are shown in the figures and in Table 2. Vertical lines in the graphs represent the values of the standard error. The significance of differences between the groups was evaluated using the unpaired Student's *t*-test, $p \le 0.05$.

RESULTS AND DISCUSSION

To ensure reliable comparison between the effects of different fluoroquinolones on LdcC activity, we used them in sublethal concentrations, which inhibited the biomass accumulation (OD_{600}) by 40–50 % by the 5th hour of growth. A rise in the cadaverine level, observed in an exponentially growing culture in response to a sublethal concentration of levofloxacin (Fig. 1), in the absence of the major inducing factor for lysine decarboxylase CadA (low pH level), suggested further investigation of lysine decarboxylase LdcC activity.

All tested fluoroquinolones had a pronounced positive effect on the activity of LdcC (Fig. 2), which was earlier regarded as a constitutive form of the enzyme [7].

It was demonstrated that the promotor of the gene encoding the lysine decarboxylase LdcC is specific for the RNA polimerase σ^S subunit and, consequently, the σ^S holoenzyme is involved in the gene's expression [17]. Different fluoroquinolones had an inducing effect on the level of expression of *rpoS* (the gene encoding the specified subunit). Similar to the LdcC activity, it was maximal for the third-generation antibiotics and declined in the second-generation ones (Fig. 3). This points to a direct relationship between the enzyme activity and the σ^S subunit level in cells.

To confirm that the described lysine decarboxylase activity was related to the LdcC form of the enzyme, we studied its dependence upon the presence of the RNA polymerase σ^S subunit by using two isogenous strains, *rpoS*$^+$ (RO91) and *rpoS*$^-$ (HS1600), derivatives of one parental strain (MC4100) (Fig. 4). The RpoS-deficient strain had a fourfold lower lysine decarboxylase activity in the absence of an antibiotic, which did not increase in response to levofloxacin addition. In the cells of *E. coli rpoS*$^+$, the activity of the enzyme

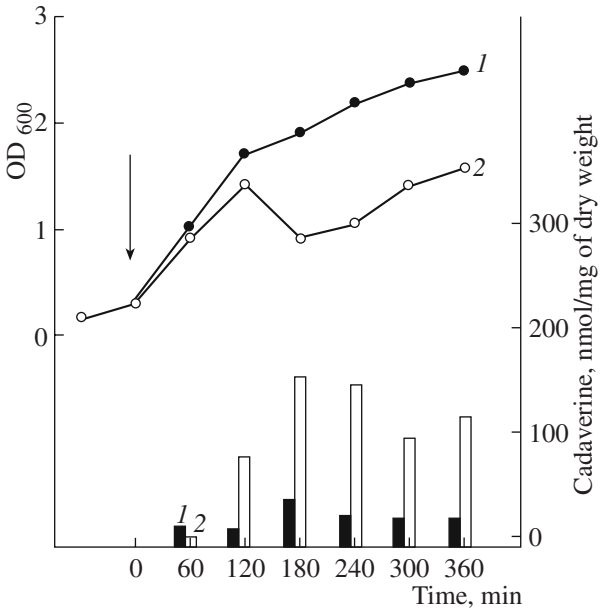

Fig. 1. Changes in the intracellular cadaverine content (columns) and in the optical density of the *E. coli* RO91 culture (curves) in response to a addition of levofloxacin: control culture (*1*); culture exposed to 0.012 μg of levofloxacin (*2*). An arrow indicates the moment when antibiotic was added.

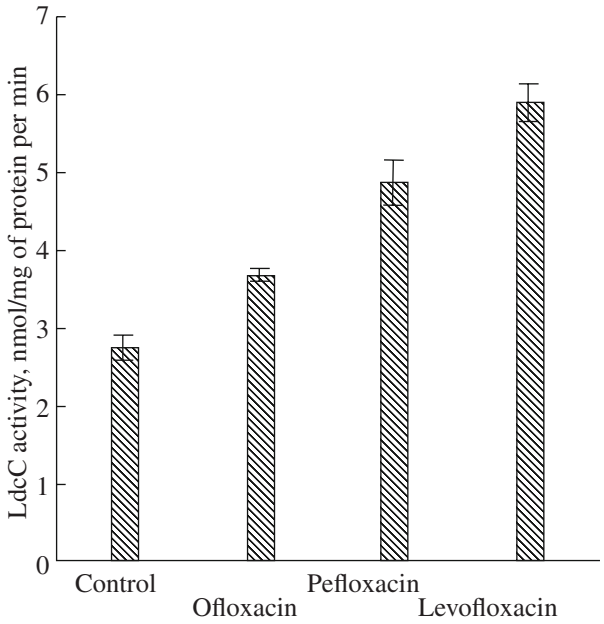

Fig. 2. The level of lysine decarboxylase activity in *E. coli* RO91 cells in the presence of 0.025 μg/ml of ofloxacin, 0.038 μg/ml of pefloxacin, or 0.012 μg/ml of levofloxacin.

increased almost twofold in the presence of the antibiotic, which confirms that the LdcC enzyme form was responsible for the lysine decarboxylase activity.

To this day, the physiological role of this enzyme has remained unknown. We demonstrated that LdcC activity increases significantly in response to sublethal concentrations of fluoroquinolones, which is accompanied by the intracellular accumulation of cadaverine, the product of the reaction catalyzed by LdcC. The adaptive character of this response is obvious, considering the role of cadaverine as a factor decreasing the permeability of porin channels in the outer membrane of gram-negative bacteria [5, 8]. This property of polyamines may influence the resistance of microorganisms to the antibiotics transported through porins.

An exogenous addition of cadaverine to the *E. coli* culture resulted in a twofold increase of the MIC for levofloxacin (from 0.012 to 0.025 μg/ml), while the cadaverine concentration reached 50 mM. A similar concentration of cadaverine had an inhibiting effect upon porin permeability [5, 8]. The effect of exogenously added cadaverine is achieved at a relatively high concentration in the medium; however, it was shown that endogenous cadaverine levels within the cells under inducing conditions are sufficient to produce a similar effect [5]. This is due to cadaverine localization mostly in the periplasm (where its local concentrations may reach comparably high values [5, 18]), as well as to a stronger inhibiting effect of polyamines on porin transport at the outer membrane's periplasmic surface [19]. The reliability of these data is supported by the information on localization of lysine decarboxylase activity in the structures of the cell envelope, which puts the product of the enzymatic reaction close to the place of its action and facilitates creation of high local poliamine concentrations around porin channels [20].

The role of endogenous cadaverine in defining the level of *E. coli* antibiotic resistance was studied with the use of strains that differed in the basic level of this polyamine in the cells. Determination of antibiotic resistance by the disc-diffusion method revealed that the cells of the strain with a higher endogenous level of cadaverine exhibited reliably less distinct growth inhibition zones (Table 2). This indicates a direct correlation between endogenous polyamine concentrations in the cells and the degree of antibiotic resistance.

Along with limitation of porin transport, the defense strategy of microorganisms includes multidrug efflux pumps controlled by the Mar (multiple antibiotic resistance) regulation system [2]. Earlier we demonstrated (by using *E. coli* mar$^-$ mutants and by induction of the *marRAB* operone) that the specific positive effect of polyamines upon *E. coli* resistance to fluoroquinolones was pronounced during functioning of the Mar-dependent multidrug efflux system [8]. This points to the additivity of different mechanisms involved in *E. coli* defense against antibiotics.

Cadaverine effectiveness (Table 2) displays a direct dependence on the generation level of the antibiotics tested: it is maximal with respect to the second and

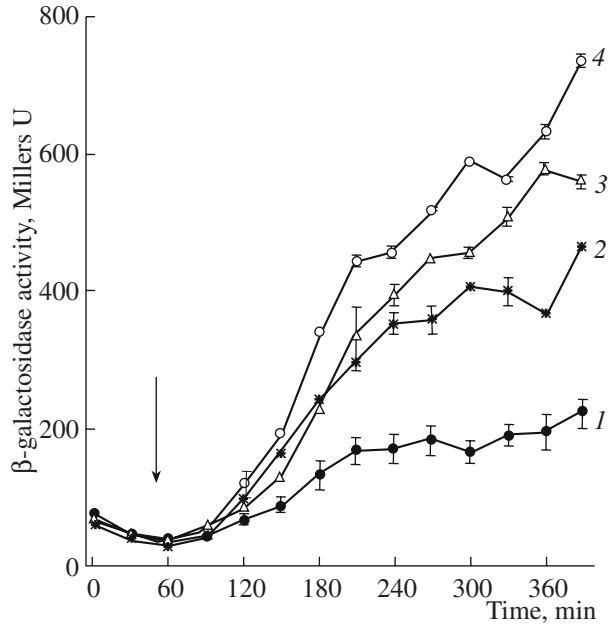

Fig. 3. The change in *rpoS* expression in *E. coli* cells in response to sublethal antibiotic exposure: control antibiotic-free culture (*1*); culture supplemented with 0.025 μg/ml of ofloxacin (*2*); culture supplemented with 0.038 μg/ml of pefloxacin (*3*); culture supplemented with 0.012 μg/ml of levofloxacin (*4*). The arrow indicates the moment when the antibiotic was added.

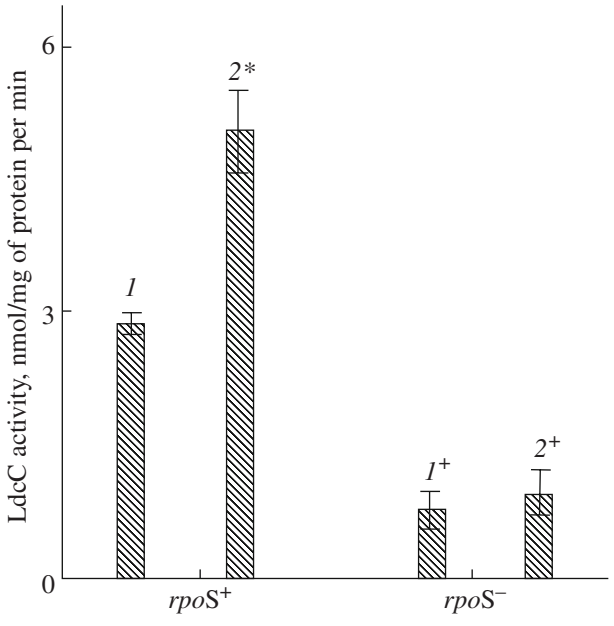

Fig. 4. Lysine decarboxylase activity alterations in response to addition of levofloxacin (0.012 μg/ml) to the cells of the wild-type *rpoS*$^+$ (RO91) *E. coli* strain and the *rpoS*$^-$ (HS1600) mutant: antibiotic-free control culture (*1*); culture supplemented with 0.012 μg/ml of levofloxacin (*2*); * is a statistically significant difference from the antibiotic-free control culture; $^+$ signifies a statistically significant difference from the corresponding parameter in *rpoS*$^+$ strain.

third, but is not statistically significant for the first generation of fluoroquinolones. These data are in agreement with the level of *rpoS* induction (Fig. 3) and the results of investigation of lysine decarboxylase activity (Fig. 2).

Since cadaverine acts upon the outer membrane porin channels, through which hydrophilic compounds are transported, the magnitude of its effect should depend to a certain degree on the hydrophilic properties of the transported substances. It was shown earlier that, the hydrophilic properties of fluoroquinolones increase with their ascending generations. This accounts for a higher affinity to the outer membrane channel proteins OmpF, and thus, promotes the penetration of antibiotics into the cell [21]. More hydrophobic fluoroquinolones of the first generation (nalidixic acid) cross the membrane mostly via a less efficient mechanism of diffusion through the lipid bilayer; this, hypothetically, may be the reason why their antimicrobial effect is essentially independent of cadaverine. By contrast, more hydrophilic fluoroquinolones of the subsequent generations are transported through porin channels [22], which evidently accounts for a more pronounced inverse relationship between their antimicrobial action and cadaverine.

Thus, sublethal concentrations of fluoroquinolones induce expression of the *rpoS* gene coding for the RNA polymerase σ^S subunit, which provides high activity of lysine decarboxylase LdcC, earlier regarded as a constitutive form of the enzyme. The product of the lysine decarboxylase reaction, cadaverine, improves microbial resistance, particularly marked with respect to the second- and third-generation fluoroquinolones.

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