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Genetic mechanisms of the resistance of *Escherichia coli* to amino acid antimetabolites. 2. Study of the frequency of induction and properties of glyphosate resistant mutants

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The frequency of the induction by nitrosoguanidine of glyphosate resistant mutants was compared for recipient and donor, as well as for lysogenic and non-lysogenic E. coli cells. It was found that integration of viral genomes and also larger replicons such as F-factor into host chromosome increased the level of glyphosate resistance by the factor ranging from 1.6 to 6. Mutants tolerating 0.2 mM of the inhibitor were obtained one order of magnitude more frequently than mutants tolerating 1 mM of this inhibitor. One half of the mutants of every group were resistant not only to the analogue of glycine,but also to the analogue of lysine. An attempt to clone an insertion from a gene library of one of the mutants was attempted but failed. Study on the nature of this gene is in progress.

Introduction. Antimetabolites are extensively used as drugs and pesticides and are widely studied as «lead» compounds in «drug design» programs [1] (for rev. see [2, 3]). However in practice cellular drug resistance developing in different taxonomic groups makes the use of such inhibitors less efficient. What is genetic and molecular basis of such a resistance? To investigate this the well-studied mechanisms of glyphosate inhibition may be used as a good experimental model.

The broad-spectrum non-selective herbicide glyphosate N-[phosphonomethyl]-glycine specifically inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyses the sixth step in the shikimate biosynthetic pathway the occurence of which is restricted to bacteria, fungi and plants [4]. Mutational changes rendering this enzyme insensitive to the inhibitor, as well as target site overproduction due to gene amplification (or specific promoter changes) have been shown to lead to the glyphosate resistance (for rev. see [5]). However these events as well as reduced inhibitor uptake, increase in its degradation are rare events [6—8], whereas the probability of glyphosate-resistant (gly^r) colonies appearance is 2 orders of magnitude higher in *E. coli* [6]. We have recently shown that most of the gly^r clones are single gene mutants which are mapped at 4 different loci of the *E. coli* chromosome map [9]. We wondered if integration of different replicons into the host chromosome have an effect on the probability of the appropriate gene mutability. To answer this question the induction probability of gly^r mutants in recipient and donor as well as lysogenic and non-lysogenic *E. coli* cells was measured.Also the nature of the mutants obtained was studied.

Materials and Methods. *Chemicals.* N-methyl-N-nitro-nitrosoguanidine (NG) and S-2-amino-ethyl-L-cysteine were supplied by «Sigma» (Germany). As a source of glyphosate the commercial herbicide Roundup(R) with the isopropylamine salt of glyphosate as an active ingredient was used.

Bacterial Strains and Growth Media. The *E. coli* strains employed and their genotypes are listed in Table 1. As basal salt media, M9 and LB medium were used [10].

Mutagenesis and Genetic Methods. For mutagenesis, nitrosoguanidine treatment was employed as

Table 1
Strains used

N	Sex	Strain	Relevant genotype	Prophage	Source
1	F^-	AB_{1157}	*thr-1, thi-1, lacy-1, mtl-1, xyl15, galK2, proA2, argE3, str31, tsx33, sup37, leu6, ara, his4C*	None	Dr. V. Lantzov, Inst. of Nucl. Res. (Russia)
2	F^-	AB_{1157}	The same	λ^{imm434}	Lysogenized in this work
3	Hfr	C	Prototrophic	λ	Dr. V. Lantzov, Inst. of Nucl. Res. (Russia)
4	Hfr	B2625	The same	None	Inst. Industr. Microbe Genet. and Selection (Russia)
5	Hfr P_3	B346	*cys, gal a,* $^-T1^r$, $T3^r$	None	Ibid
6	F^-	DH5α	*supE44, DlacU169(φ80lacZDM15), hsdR17, recA1, endA1, gyrA96, thi1, relA*	None	Ibid
7	F^-	HB-101	*supE44, hsdS20($r_B^-m_B^-$)recA13, ara14, proA2, lacY1, galK2, rpsl20, xyl15, mtl1*	None	Ibid

Table 2
Average number of gly^r mutants per 10^6 of mutagenized E. coli cells appearing after NG treatment*

Strain	Relevant genotype	Number of survivors at increasing doses of glyphosate**			
		Control	Experiment		
			1 dose	1.5 dose	2.5 dose
AB_{1157}	Recipient nonlysogenic	0	300	150	25
AB_{1157}	Recipient lysogenic	0	400	300	40
Hfr C	Donor nonlysogenic	0	400	200	56
Hfr C	Donor lysogenic	0	1000	500	150
Hfr P_3	Donor	0	400	200	40

*Not less than 3 experiments; **because Roundup (300 g/l of isopropylammonium salt of glyphosate) was used, 1 dose was determined as the minimal quantity required to suppress growth of all untreated cells in control.

described by Miller [10]. Gly^r mutants obtained in primary selection experiments were studied by a passage on increasing concentration of glyphosate alone or a mixture of glyphosate and AEC (200 μg/ml).

Construction of gly^r mutant genomic DNA library and complementation. DNA isolated from this mutant was partially digested with *Sau3A* and fractionated on a 0.8 % agarose gel. Fragments smaller than 3 kb (λ DNA *HindIII* markers) were recovered from the gel using the glassmilk procedure (Bio101, Inc). Analogously molecules of *BamH1* digested, dephosphorylated Bluescript SK^+ vector (from «Stratagene», USA) were recovered from the gel. The fragments from the two sources were ligated in a ratio of 1:1 (total 40 ng of DNA used) at 4 °C overnight [11] and were used to transform cells of competent *E. coli* strain DH5α (from Bethesda Res. Lab). Diluted aliquots of the transformed cells were plated on LB agar containing ampicillin at 50 μg/ml and X-gal and IPTG thus allowing to determine the percent of plasmids having DNA inserts [11].

The remainder of the transformants were inoculated into 3 ml of LB supplemented with ampicillin and incubated overnight at 37 °C. A plasmid library was generated by harvesting the plasmids by alkaline lysis [11]. The library obtained was used to transform both DH5α and HB-101 cells made competent following the method of Hanahan [12]. The transformants were plated on M9 medium containing different concentrations of glyphosate and all auxotrophic additions required.

Results and Discussion. The frequency of the induction of gly^r mutants by NG for different *E. coli* cells represented by recipient — donor and lysogenic-nonlysogenic cells are shown in Table 2.

Table 3
Number of mutants tolerating increasing doses of glyphosate and AEC

Strain	Glyphosate dose		Glyphosate dose + AEC (200 μg/ml)	
	1 dose	2.5 dose	1 dose	2.5 dose
Hfr $C\lambda^r$	50	Not studied	25	Not studied
Hfr $C\lambda^s$	50	Not studied	35	Not studied
Hfr P_3	47	12	18	7

From the results shown in Table 2 it can be concluded that integration of different replicons into the host chromosome slightly increased the probability of a gly^r mutation. The increase was more pronounced in the strain with both F-factor and λ replicons integrated not far from each other (about 10 min on the *E. coli* chromosome map). As was shown earlier, the mutations obtained in this strain map at 4 different loci [9].

On the basis of their tolerance to increasing doses of the inhibitor, mutations obtained could be divided into 2 groups, i. e. those tolerating low doses and those tolerating higher doses. The frequency of the first group is one order of magnitude higher than the other. The frequency of mutants resistant to both glyphosate and AEC is evident from Table 3.

The results in Table 3 demonstrate that almost half of the mutants tolerating both low and higher doses of glyphosate also tolerated a toxic analogue lysine and thus were multiple-resistant.

Thus, gly^r mutants appearing after NG treatment constitute 4 different groups tolerating different doses

Fig. 1. Partia digestion of gly^r mutant genomic DNA with *Sau3A* restriction endonuclease lanes: *1* — λDNA *Hind III* markers; *2—4* — dynamics of a mutant DNA (7 μg) hydrolysis for 20, 40 and 60 min appropriately with 4 units of the enzyme

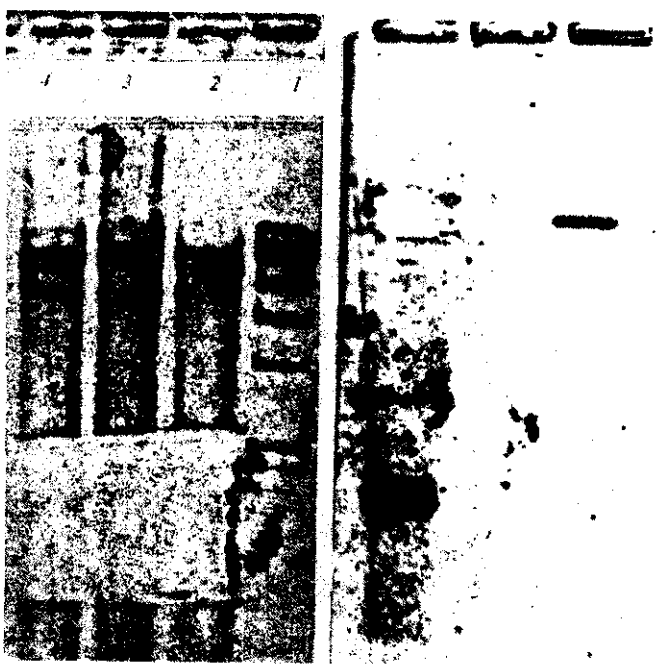

Fig. 2. Demonstration of the size of DNA fragments used for gene library construction lanes: *1* — λDNA *HindIII* markers; *2—4* — gly^r mutant genomic DNA (10 μg) hydrolysed with *Sau3A*
Fig. 3. Purification of Bluescript SK^r vector DNA using glassmilk procedure (Bio101, Inc.)

of glyphosate and cross-resistant to AEC. How many genes are involved in this phenotype and what is their nature?

Because *mdr* genes in eukaryotes are responsible for multidrugresistance based on drug efflux out of the cell (for rev. see [13]), and as such a gene should be dominant over its wild type allele the cloning of multiresistant gly^r mutant DNA was tried. This mutant DNA was partially digested with *Sau3A* (Fig. 1), fragments of ≈2 kb recovered from the gel (Fig. 2) and ligated to the vector (Fig. 3) (see Materials and Methods). Thus, approximately 80 % of the transformants produced due to the ligation product were of white color on the indicator plates and the number of inserts obtained was about 10 000. Using this library, DH5α and HB-101 cells were transformed and after washing with M9 medium plated on selective medium containing 1 dose of glyphosate. A couple of white colonies were obtained in both cases but none of them were highly efficient in retransformation experiments and consequently the gene was not cloned. If this gene codes for a recessive trait another approach should be used [9] and this work is now in progress.

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О. Й. Черепенко

Генетичні механізми стійкості клітин *Escherichia coli* до амінокислотних антиметаболітів. 2. Вивчення частоти індукції та властивостей гліфосатстійких мутантів

Резюме

Вивчено частоту індукції гліфосатстійких мутантів у реципієнт-донорських, а також лізогенних-нелізогенних штамах клітин E. coli. Показано, що інтеграція вірусного, а також більшого F-реплікона підвищує частоту індукції гліфосатстійких мутантів від 1,6 до 6 разів. Мутанти, стійки до 0,2 мМ гліфосату, виникають у 10 разів частіше мутантів, стійких до 1 мМ цього аналога гліцину. Половина мутантів кожної групи стійка не лише до аналога гліцину, але й до аналога лізину. Клонувати ген гліфосатстійкості не вдалося. Досліджується домінантно-рецесивна природа цього гена.

Е. И. Черепенко

Генетические механизмы устойчивости клеток *Escherichia coli* к аминокислотным антиметаболитам. 2. Изучение частоты индукции и свойства глифосатустойчивых мутантов

Резюме

Изучена частота индукции глифосатустойчивых мутантов в реципиент-донорских, а также лизогенных-нелизогенных штаммах. Показано, что интеграция вирусного и более крупного F-репликона увеличивает частоту индукции gly^r-мутантов от 1,6 до 6 раз. Мутанты, устойчивые к 0,2 мМ глифосата, возникают в 10 раз чаще таковых, устойчивых к 1 мМ этого аналога глицина. Половина мутантов каждой группы устойчива не только к аналогу глицина, но и к аналогу лизина. Клонировать ген глифосатустойчивости не удалось. Изучается доминантно-рецессивная природа этого гена.

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