ISSN 0233-7657. Биополимеры и клетка. 1997. Т. 13. № 5

Genetic mechanisms of *Escherichia coli* resistance to target inactivation. Genes governing purine metabolism in enterobacteria: an unexpected sequence found via complementation selection

E. Cherepenko, S. Craig[1]

Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine
150 Zabolotnogo str., Kyiv, 252143, Ukraine

[1]School of Pharmacy, University of North Carolina
Chapel Hill, NC 27510, USA

Using enterobacterial strains having block in 3 different genes required for GMP synthesis, 3 groups of inserts with different restriction patterns were expected. But the fragments cloned represented 5 such groups. One consisted of Salmonella typhimurium DNA fragments with 2 SacI sites available. Sequencing revealed 100 % homology of the cloned insert to the N-term of Y protein of the hemC-hemD linkage group of E. coli chromosome (85 min locus). It is suggested that Y may represent the gpp gene, coding for guanosinepentaphosphatase. It was also shown that a Salmonella DNA fragment resulting from PCR amplification with 20-mer primers complementary to the N- and C-terms of the hpt gene of E. coli did not encode an hypoxanthine phosphoribosyltransferase (HPRTase)and some other gene complemented GMP synthesis block in de novo and salvage pathways in E. coli cells.

Introduction. If a chemical compound selectively inactivates only a certain type of molecules, a target, and if the function of this target is pivotal for cell survival, then an effective control of cell viability could be possible. To discover such compounds as drugs or pesticides an innovative approach has recently been developed known as structure-based drug design (for rev. see [1, 2]). This approach utilizes three-dimensional structural data to design a selective inhibitor to the active site of an enzyme and allosteric sites as well. Thus, if mutations render a target insensitive to an inhibitor to the active site, inhibitors to the allosteric sites could be developed to overcome the problem of drug resistance.

But alongside with this target approach others are needed as well because non-target specific cell resistance may also occur. Such resistance mechanisms include target site overproduction due to gene amplification or promoter changes, reduced inhibitor uptake, induction of efflux processes and inhibited detoxification or sequestration. Moreover, cells can also exploit some other means to survive with a pivotal metabolic target blocked. For example, *Escherichia coli* thermosensitive phenylalanine-tRNA synthetase could be protected at non-permissive temperatures when the level of its cognate tRNA gene expression was elevated (for rev. see [3]). But the enzyme could also be protected, when cells were transformed with multicopy *colE1* plasmids which use RNA1 resembling the structure of $tRNA^{Phe}$ for controlling plasmid DNA replication [4].

May protection of DNA replication targets inactivation with some unknown genes also occur? To answer this question the study of purine metabolism is of special interest. In enterobacteria, there are *de novo* and salvage pathways for GMP synthesis. Both have been very well studied [5, 6]. Different bacterial strains are available which allow the cloning via complementation selection of genes belonging to both pathways. This makes it possible to determine if genes of only these pathways are capable of complementing the block of GMP synthesis, or if some

© E. CHEREPENKO, S. CRAIG, 1997

other genes also exist which help a cell to overcome this block.

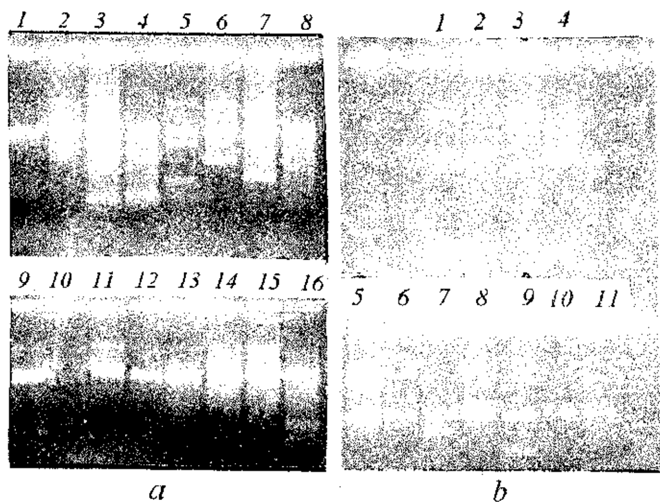
Materials and Methods *Materials.* Guanine was obtained from «Sigma (USA). Bacto-tryptone and vitamin-free Casamino acids were purchased from «Difco» (USA). All other reagents used in this work were from «Sigma» and of the highest purity available.

Bacterial strains and growth media. For complementation experiments *E. coli* SØ609 strain (*F-Dpro-gpt-lac, hpt, purH, J, thi, pup, ara, strA*) [6] unable to grow on selective medium containing guanine was used as a recipient in transformation experiments with the gene library.

Salmonella typhimurium GP36 (*DproAB-gpt, purE66, sug*) [7] with a changed substrate specificity of the *hpt* gene coding for hypoxanthine phosphoribosyltransferase and allowing cells to grow not only on hypoxanthine but on guanine as well. This strain was used for the gene library construction.

Division of S. typhimurium DNA inserts complementing GMP synthesis block in the de novo and salvage pathway into groups on the basis of differences in restriction pattern

Group number	Restriction endonuclease site in the insert cloned	Number of sites available
1	*SacI*	2
2	*EcoRI*	1
3	*EcoRV*	1
	SmaI	1
4	*HindIII*	3
	SalI	1
5	*HindIII*	2
	EcoRV	1
	SalI	1



c

Fig. 1. Examples of restriction patterns of some *Salmonella typhimurium* DNA inserts cloned via complementation of cells with a block of GMP synthesis in *de novo* and salvage pathways in *E. coli*; *a*: 4 clones studied with: lanes *1 — 4*: *EcoRV* (clones 3, 4 contain this site); lanes *5 — 8*: *HindIII* (clones 1—3 contain this site); lanes *9 — 12*: *SalI* (no clone contains this site); lanes *13 — 16*: *SmaI* (clone 4 contains this site); *b*: 11 clones studied with *HindIII* (clones 7, 9, 11 contain this site); *c*: lanes *1* and *9* are Bluescript SK$^+$ vector DNA only, other lanes show top and bottom clones study with *EcoRI, EcoRV, KpnI, PstI, SalI, XbaI, XhoI* appropriately (bottom clone of lane 10 contains *EcoRI* site)

As a basal medium in transformation experiments of SØ609 made competent after Hanahan [8] a defined MOPS medium [9] containing 0.2 % glucose, 0.2 % vitamin-free casaminoacids, 1.5 mM thiamine, 20 μg/ml guanine, 50 μg/ml ampicillin and 40 mM $NaPO_4$-buffer was used.

Generation of a genomic library of S. typhimurium DNA and complementation. High-molecular weight DNA obtained from strain GP36 was partially digested with restriction endonuclease *Sau3A* and fragments obtained were resolved in an agarose gel. Fragments of about 3 kb identified by reference to l *HindIII* size markers were recovered from the gel using the glassmilk procedure (Bio 101 Inc). The fragments thus obtained were ligated in 1:1 ratio with *BamHI* digested, dephosphorylated Bluescript SK$^+$ vector (Statagene) at 4 °C overnight [10]. With this ligation products, maximal efficiency competent DH5a cells (from Bethesda Res. Lab) were transformed giving rise to 80 % of white colonies on indicator LB medium containing X-gal and IPTG [10] a 10000-member gene library was thus constructed with every gene present.

Alternative methods to clone genes capable of complementing a block in GMP synthesis. Numerous clones obtained via complementation selection of *E. coli* SØ609 recipient cells with the GP36 *S. typhimurium* DNA gene library were divided into groups on the basis of different availability of restriction sites in the insertions cloned. The clones from different groups were studied routinely as in [11] in the PCR

reaction with 20-mer primers to the N- and C-terms of *E. coli hpt* gene using the sequence of the gene published in [12]. The sequence of the PCR amplified DNAs of the clones studied were confirmed with ^{35}S-labelled dATP by the dideoxy chain termination method as described previously [13].

Results and Discussion. Using *E. coli* SØ609 competent cells and a GP36 *S. typhimurium* gene library (in both these strains the *gpt* gene is deleted) 3 groups of insertions could be cloned via complementation selection. These groups should represent the *hpt sug* allele of GP36 conferring on the salvage pathway the ability to the recipient to grow on guanine, the *purH, J* gene of *Salmonella* which belongs to the *de novo* pathway and *Salmonella pup* gene making conversion of adenine, adenosine and deoxyadenosine to guanine nucleotides possible [6]. Plating of transformed and washed SØ609 cells onto selective MOPS medium supplemented with 20 μg/ml of guanine and 50 μg/ml of ampicillin produced more than 50 colonies after 2 days of incubation, while the negative control (Bluescript vector alone) produced none. The clones obtained were picked, plasmid DNA minipreps were isolated as in [10] and studied in transformation experiments and with restriction endonucleases. High efficiency of retransformation was shown for every insert cloned.

An example of the restriction patterns of some inserts complementing GMP synthesis block in *E. coli*

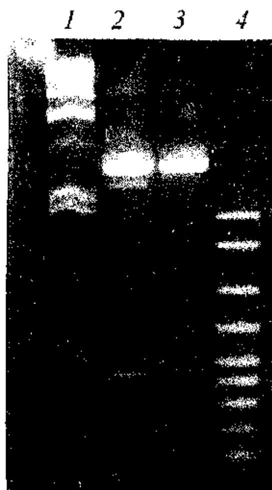


Fig. 2. Study of the *Salmonella typhymurium* DNA insert complementing GMP synthesis block on *de novo* and salvage pathway in *E. coli* cells with *SacI* restriction endonuclease (lanes *1* and *4*: size markers λ *HindIII* and a ladder appropriately; lanes *2* and *3*: concentration of DNA loaded differs by the factor of 2)

CACCTGGTGG AAAAACCAAA
GCCGAAAACC CGCCATCACGT

Fig. 3. A small part of the sequence obtained with the forward primer in sequencing *S. typhimurium* DNA insert containing 2 *SacI* sites and complementing GMP synthesis block on the *de novo* and salvage pathways of *E. coli*

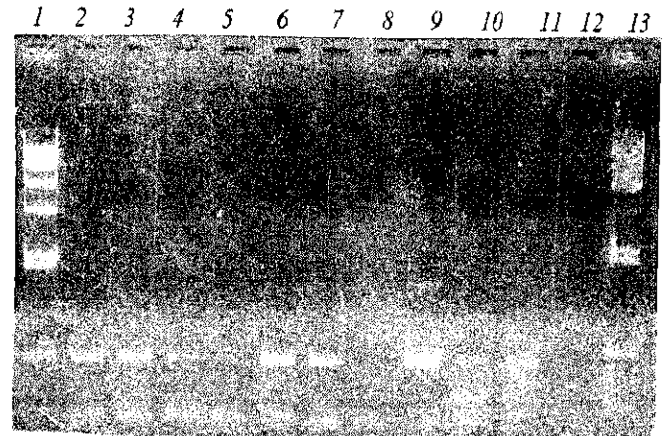


Fig. 4. PCR-amplification with 20-mer primers to the N- and C-terms of *E. coli hpt* gene of *Salmonella typhimurium* DNA inserts complementing GMP synthesis block on *de novo* and salvage pathways in *E. coli*: lanes *1* and *13* are λ *HindIII* size markers; lanes *1*–*8*: different inserts; lane *9*: *E. coli* genomic DNA; lane 10: GP660 *Salmonella* DNA; lane *11*: GP36 *Salmonella* DNA; lane *12*: vector DNA only

SØ609 strain is shown in Fig. 1. As seen from this figure, inserts cloned could be divided into groups on the basis of different restriction sites available. Table demonstrates that all these inserts could be divided into 5 such groups whereas only 3 groups were anticipated with cloning system used.

Because it was known that *pur H, J* of *E. coli* contained an *EcoRI* site [14] and the *hpt* gene of *E. coli* contained both *EcoRV* and *SalI* sites [12], and even though the genes studied belong to different enterobacteria, we paid attention to the fragment containing 2 *SacI* sites (Fig. 2). Double-stranded DNA of this fragment was sequenced using the Sequenase 2.0 kit (U. S. Biochemical Corp. Cleveland, OH) and universal and reverse primers. A small part of this sequence obtained with the forward primer is shown in Fig. 3.

Analysis of the sequence shown in Fig. 3 by Simtran and dfastp programs did not reveal any similarity to genes involved in purine metabolism, but showed strong homology to the hypothetical protein *Y* of *E. coli* [15]. The sequnce coding for this protein was found in 4260 bp *E. coli* DNA fragment mapped at 85 min of the *E. coli* chromosome map and includes three genes (*hemC, hemD, X*) which were suggested to be a part of *Uro* operon. The function of *Y* remains unknown. Because the «2 *SacI*» containing fragment of *Salmonella* DNA is strongly homologous to *Y* and conferred the ability to SØ609 to grow on exogenous guanine, we suggested on the basis of map position that *Y* could be identified as the *gpp* gene coding for guanosinepentaphosphatase. This enzyme is responsible for a conversion of pppGpp into ppGpp which is an important regulator molecule in bacterial cell (for rev. see [16]). In the conditions of overexpression could this enzyme be capable to the resynthesis with exogenous guanine and phosphates providing for DNA synthesis precursor? Because due to this enzyme activity also ppGp compound is formed in cells [16], degradation of this molecule also could supply cells with a precursor needed for DNA replication. This suggestion remains to be verified and the gene identified.

Besides «2 *SacI*» insert we also studied if a fragment containing an *EcoRI* site and fragments containing *EcoRV* and *SalI* sites could be amplified in the PCR reaction with 20-mer primers complementary to the N- and C-terms of the *hpt* gene of *E. coli*. Fig. 4 shows results of this study. The positive PCR amplification with the primers used is observed with *E. coli* DNA containing the wild type *hpt* allele, with *S. typhimurium* GP660 DNA containing also the wild type allele of the gene and with *Salmonella* GP36 DNA containing the *sug* mutation in the *hpt* gene (ability to grow on hypoxanthine and guanine). As seen from Fig. 4, lane *8*, the fragment containing an *EcoRI* could not be amplfied by PCR with primers used.

This shows that there is no cell DNA contamination in the plasmid DNA preparations used. The inserts of *S. typhimurium* DNA containing *EcoRV*, *SalI*, *SmaI* and *HindIII* sites were all positive in the PCR reaction with generic 20-mer primers complementary to the N- and C-terms of *E. coli hpt* gene. At present these fragments are under study, but sequencing of one of the positive fragments (data not shown) demonstrated that it did not encode an HPRTase. If this insert does not include the *pup* gene, then some new gene capable to complement a block in GMP synthesis might be identified.

О. Й. Черепенко, С. Крег

Генетичні механізми стійкості клітин *Escherichia coli* до інактивації мішеней. Гени пуринового метаболізму: клонування за допомогою комплементації невідомої послідовності

Резюме

Використання штамів ентеробактерій, які дозволяють клонувати три різних гени шляхів синтезу GMP, надавало можливість одержання трьох груп рестрикційних фрагментів. Однак було клоновано п'ять груп таких фрагментів. Фрагменти однієї з цих груп вміщували 2 SacI-сайти. Секвенування фрагмента геному Salmonella typhimurium показало 100 % гомологію з N-кінцем гена Y, який належить оперону hemC-hemD E. coli. Зроблено припущення, що цей фрагмент вміщує ген, подібний гену gpp E. coli. Також показано, що фрагмент геному S. typhimurium, який комплементує блок синтезу GMP, ампліфікований у PCR-реакції за допомогою 20-членних праймерів до N- та C-кінців гена hpt E. coli, не кодує HPRT.

Е. И. Черепенко, С. Крэг

Генетические механизмы устойчивости клеток *Escherichia coli* к инактивации мишени. Гены пуринового метаболизма: клонирование с помощью комплементации неизвестной последовательности

Резюме

Использование штаммов энтеробактерий, позволяющих клонировать три различных гена путей синтеза GMP, предполагало получение трех групп рестрикционных фрагментов. Однако были клонированы пять групп таких фрагментов. Фрагменты одной из этих групп содержали 2 SacI-сайта. Секвенирование фрагмента генома Salmonella typhimurium показало 100 % гомологии с N-концом гена Y, принадлежащим оперону hemC-hemD E. coli. Сделано предположение, что этот фрагмент содержит ген, подобный гену gpp E. coli. Также показано, что фрагмент генома S. typhimurium, комплементирующий блок синтеза GMP, амплифицируемый в PCR-реакции с помощью 20-членных праймеров к N- и C-концам гена hpt E. coli, не кодирует HPRT.

REFERENCES

1. *Bugg Ch., Carson W., Montgomery J.* Drugs by design // Sci. Amer.—1993.—**269**.—P. 92—98.
2. *Craig S.* Purine salvage enzymes as targets for the chemotherapeutic treatment of parasitic diseases // Biopolymers and Cell.—1994.—6.—P. 65—71.
3. *Cherepenko E., Matsuka G.* Genes of prokaryotic and eukaryotic aminoacyl-tRNA synthetases // Uspekhi biol. chimii.—1989.—30.—P. 67—105 (in Russian).
4. *Cherepenko E.* ColE1 plasmids can prevent thermoinactivation of phenylalanyl-tRNA synthetase in *E. coli* // Biopolymres and Cell.—1994.—3, N 4.—P. 75—78.
5. *Neuhard J., Nygaard P.* Purines and pyrimidines // *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology / Eds F. Neidhardt, J. Ingraham, K. B. Low et al. // Amer. Soc. Microbiol.—Washigton: D. C., 1987.—Vol. 1.—P. 445—473.
6. *Jochimsen B., Nygaard P., Vestergaard T.* Location on the chromosome of *Escherichia coli* of genes governing purine metabolism // Mol. and Gen. Genet.—1975.—**143**.—P. 85—91.
7. *Benson C. E., Gots J.S.* Genetic modification of substrate

specificity of hypoxanthine phosphoribosyltransferase in *Salmonella typhimurium* // J. Bacteriol.—1975.—**121**.—P. 77—82.

8. *Hanahan D.* Studies in transformation of *Escherichia coli* with plasmids // J. Mol. Biol.—1983.—**166**.—P. 557—580.
9. *Neidhardt F. C., Bloch P. L., Smith D. F.* Culture medium for enterobacteria // J. Bacteriol.—1974.—**119**.—P. 736—747.
10. *Sambrook J., Fritsch E. F., Maniatis T.* Molecular cloning. A laboratory manual.—New York: Cold Spring Harbor Lab., 1989.
11. *Eakin A. E., Bouvier J., Sakanari J. et al.* Amplification and sequencing of genomic DNA fragments encoding cysteine proteases from protozoan parasites // Mol. and Biochem. Parasitol.—1990.—**39**.—P. 1—8.
12. *Fujita N., Mori H., Yura T. et al.* Systematic sequencing of the *Escherichia coli* genome: analysis of the 2.4—4.1 min (110,917 = 193,643 bp) region // Nucl. Acids Res.—1994.—**22**.—P. 1637—1639.
13. *Craig S. P., Mckerow J. H., Newport G. R. et al.* Analysis of cDNA encoding the hypoxanthineguanine phosphoribosyltransferase (HGPRTase) of *Schistosoma mansoni*: a putative target for chemotherapy // Ibid.—1988.—**16**.—P. 7087—7101.
14. *Flannigan K. A., Hennigan S. H., Vogelbacker H. H. et al.* Purine biosynthesis in *Escherichia coli* K12: structure and DNA sequence studies of the pur HD locus // Mol. Microbiol.—1990.—**4**.—P. 381—392.
15. *Alefounder P. R., Abell Ch., Battersby A.* The sequence of *hemC*, *hemD* and two additional *E. coli* genes // Nucl. Acids Res.—1988.—**16**.—P. 9871.
16. *Cashel M., Rudd K. E.* The stringent response // *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology / Eds F. Neidhardt, J.Igraham, K. B. Low et al. // Amer. Soc. Microbiol.—Washington: D. C., 1987.—Vol. 2.—P. 1570—1438.

Received 24.02.97