Available online at www.sciencedirect.com
SCIENCE DIRECT
Mutation Research 595 (2006) 184–190
Fundamental and Molecular Mechanisms of Mutagenesis
www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Characterization of *cycA* mutants of *Escherichia coli* An assay for measuring in vivo mutation rates

Tamás Fehér, Botond Cseh, Kinga Umenhoffer, Ildikó Karcagi, György Pósfai *

Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences, Temesvári krt. 62, Szeged H-6726, Hungary

Received 9 August 2005; received in revised form 7 November 2005; accepted 18 November 2005
Available online 22 December 2005

Abstract

Quantitative assessment of the spontaneous or induced genomic mutation rate, a fundamental evolutionary parameter, usually requires the use of well-characterized mutant selection systems. Although there is a great number of genetic selection schemes available in *Escherichia coli*, the selection of D-cycloserine resistant mutants is shown here to be particularly useful to yield a general view of mutation rates and spectra. The combination of a well-defined experimental protocol with the Ma-Sandri-Sarkar maximum likelihood method of fluctuation analysis results in reproducible data, adequate for statistical comparisons. The straightforward procedure is based on a simple phenotype–genotype relationship, and detects mutations in the single-copy, chromosomal *cycA* gene, involved in the uptake of D-cycloserine. In contrast to the widely used rifampicin resistance assay, the procedure selects mutations which are neutral in respect of cell growth. No specific genetic background is needed, and practically the entire mutation spectrum (base substitutions, frameshifts, deletions, insertions) can simultaneously be measured. A systematic analysis of *cycA* mutations revealed a spontaneous mutation rate of 6.54×10^{-8} in *E. coli* K-12 MG1655. The mutation spectrum was dominated by point mutations (base substitutions, frameshifts), spread over the entire gene. IS insertions, caused by IS1, IS2, IS3, IS4, IS5 and IS150, represented 24% of the mutations.

Keywords: *Escherichia coli*; Mutation rate; D-Cycloserine resistance

1. Introduction

The genomic mutation rate is a fundamental evolutionary parameter of any population. Cells maintain a balance between faithful replication and mutation-generating mechanisms. While most mutations are deleterious, genetic diversity on which selection can work is a necessity for long-term survival in a changing environment. Genetic adaptation was shown to be an uneven process. Certain environmental stresses can result in increased mutation rates creating higher genetic diversity [1,2], although the long-term selective advantage of such temporary mutagenesis in some genetic systems has been brought into question [3].

Mutations in bacteria occur typically at a rate of $<10^{-6}$/gene/replication [4]. Analysis of such rare events usually requires the use of a screen, preferably a positive selection system which filters out the mutants from a much larger population. Ideally, the mutation detection system selects mutations which (i) can be of any type, (ii) are neutral in respect of cell growth, (iii) do not require a specific genetic background, (iv) have a simple phenotype–genotype relationship and (v) can be easily and reproducibly counted.

* Corresponding author. Tel.: +36 62 599 778; fax: +36 62 433 506.
E-mail address: posfaigy@brc.hu (G. Pósfai).

0027-5107/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrfmmm.2005.11.004

The work horse of molecular biology, *Escherichia coli*, is a prominent subject of mutation research. A wealth of information on mutations occurring under various conditions was derived from studies performed with this bacterium, including recent observations on the increase of starvation-induced point mutation rates [5], or detection of beneficial insertion element-mediated rearrangements in long-term-cultured cells [6].

A great number of mutation detection systems have been used in *E. coli* for mutagenesis studies [7], however, the use of them is frequently limited by various factors. Narrow range of mutation types detected, interference with cell metabolism/growth, need for specific genetic background, multiple mutation target genes, multicopy phage- or plasmid-coded components are the causes of limitations associated with the mutation selection systems. In the course of our work studying insertion element (IS)-mediated mutagenesis, we developed an assay, based on the selection of *cycA* mutants, which proved to be particularly useful for the analysis of mutation rates and spectra. We provide a characterization of this system here.

The *cycA* gene of *E. coli* codes for a permease locating in the outer membrane and transporting D-alanine, D-serine, and glycine into the cell [8–11]. D-Cycloserine, an antibiotic interfering with cell wall synthesis [12], is also transported by CycA [13,14]. Mutations in CycA render the cell resistant to D-cycloserine. Since D-cycloserine has multiple targets (D-alanyl-D-alanine ligases A and B, and alanine racemase) [15–18], the simultaneous modification of which can practically be excluded, we hypothesized that resistance under the conditions of the assay is exclusively due to mutations of the transporter, CycA. We show here that *cycA* mutants resistant to D-cycloserine can carry any type of mutations (point mutations, insertions, deletions), and these mutations tend to be neutral for growth in minimal medium. Results obtained by the test are reproducible, and the assay is easy to perform. It is based on a simple phenotype–genotype relationship, there is no requirement for a specific genetic background, and mutations are detected in a single-copy, chromosomally located gene.

2. Materials and methods

2.1. Strains, plasmids and media

Mutation rate experiments were done using K-12 MG1655 [19]. MDS42, a deletional derivative of MG1655, will be described in detail elsewhere. Plasmids were prepared from DH5α host. To clone *cycA* of MG1655, a 1877-bp genomic region encompassing *cycA* was amplified using flanking primers cycA1R (5′-cggaattcatggtagatcaggtaaaagt) and cycA2R (5′-accccaagcttgcgccatccagcatgata). The amplified fragment was cloned into the *Eco*RI–*Hin*dIII sites of pBADαβγ [20], downstream of the inducible promoter. Expression of *cycA* was induced by adding 0.1% arabinose to the medium. In the experiments involving D-cycloserine, the minimal medium (MT) described by Hall [21] was used. The medium was supplemented by 0.2% glucose or glycerol carbon source. Antibiotics were used at the following end-concentrations: ampicillin (Ap) 50 μg/ml, rifampicin (Rp) 100 μg/ml, D-cycloserine (Cyc) 0.04 mM. Stock solution of Cyc (4 mg/ml) was prepared freshly before use.

2.2. Fluctuation assay and statistical analysis

To detect the rate and spectrum of spontaneous mutations, cells resistant to D-cycloserine were selected. In a fluctuation assay, 20 tubes of 1 ml glucose-MT were inoculated from an overnight starter with 10^4 cells each, and cultures were grown with agitation to saturation at 37 °C. Fifty microliters of aliquots from each tube were then spread on minimal plates containing D-cycloserine (0.04 mM) and incubated for 30 h at 37 °C. The estimated number of mutations per tube (*m*) was calculated from the number of colonies using the Ma-Sandri-Sarkar maximum likelihood (MSS-MLL) method [22] (see Supplementary Material at http://www.szbk.u-szeged.hu/~posfaigy/supplement_cyc.htm for details). Eq. (41) given by Stewart et al. [23] was used to extrapolate the obtained *m* value—valid for 50 μl to 1ml. The total number of cells in a tube was determined by spreading dilutions from four tubes onto nonselective plates. Dividing the number of mutations per tube by the average total number of cells in a tube gives the mutational rate (mutation/cell/generation).

2.3. IS-specific primers

In case of insertion mutants, the identity of some ISs was determined by PCR using combinations of oppositely oriented IS-specific primers (IS1: 5′-tcgctgtcgttctca, 5′-aagccactggagcac; IS2: 5′-tcgcaggcataccatcaa, 5′-cagacgggttaacggca; IS5: 5′-gacagttcggcttcgtga, 5′-gctcgatgacttccacca; IS150: 5′-acgtgccgagatgatcct, 5′-cagacctatatgcctcgt) and primers flanking *cycA* (cycA1: 5′-ctgatgccggtaggttct, cycA2: 5′-gcgccatccagcatgata).

3. Results

3.1. Selection of D-cycloserine resistant mutants

For selection of D-cycloserine mutants, the inhibitory effect of the antibiotic was studied in the 0.01–0.06 mM range on glucose/minimal plates. Earlier reports defined 0.01 mM as the minimal concentration of D-cycloserine

needed to completely inhibit growth of *E. coli* K-12 on solid medium [9]. We found that 0.02 mM inhibited growth on agar plates. However, for complete elimination of the background of slow-growing non-resistant cells, use of 0.04 mM was necessary. For routine assays, cells were grown to saturation (approx. 10^9 cells/ml), and 50 μl of the dense culture was spread on glucose/minimal plates supplemented with 0.04 mM D-cycloserine. Since D-cycloserine slowly looses its bactericidal activity in agar plates [24], the plates were prepared immediately before use. Antibiotic resistant colonies emerging on the plates were uniform in size and appearance.

3.2. Resistant mutants are sensitized by expression of wild-type CycA

Complementation of D-cycloserine mutants by the wild-type (wt) *cycA* gene indicates that the mutations causing resistance are located in *cycA*. Wild type *cycA* was cloned in an arabinose-inducible expression vector, resulting in pBADcycA. Twelve randomly chosen D-cycloserine resistant mutants of MG1655 were transformed either by pBADcycA or the insert-less vector pBAD. Transformants were then spread on glycerol/minimal plates with or without D-cycloserine and/or arabinose inducer. Results indicated that expression of the wt gene sensitized the resistant mutants to the antibiotic.

In a another experiment, another set of 12 randomly chosen D-cycloserine resistant mutants were separately grown in liquid cultures, and arabinose was added in early logarithmic phase. Expression of wt *cycA* resulted in growth inhibition in the presence of D-cycloserine (Fig. 1).

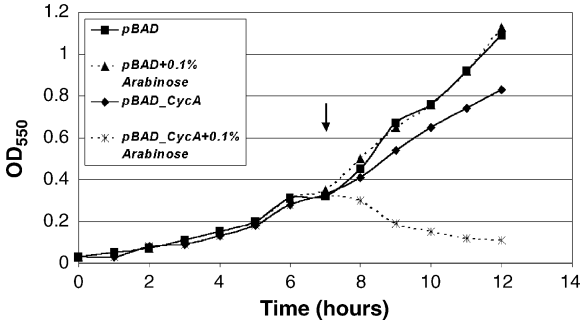

Fig. 1. Effect of wt *cycA* expression on the growth of D-cycloserine resistant mutant MG1655 cells in liquid culture. Plasmids carried by the cells are indicated. Cultures were supplemented with 0.04 mM D-cycloserine, and 0.1% arabinose inducer was added at OD = 0.3 (indicated by a vertical arrow), where relevant. All 12 randomly chosen mutants yielded the same results. Only one typical experiment is shown.

3.3. Mutations in cycA are neutral for cell growth

We hypothesized that in the absence of the transport of substrate amino acids (e.g., in minimal medium), mutations of *cycA* are neutral in respect to growth rate. Starter cultures of wt MG1655 or MDS42 and their randomly chosen D-cycloserine resistant mutants were mixed at a ratio of 50:50, and grown without antibiotic selection for 45–65 generations in minimal medium by repeated inoculum transfer from cultures reaching saturation into fresh medium. For comparison, mixed cultures of wt MG1655 or MDS42 and their randomly chosen rifampicin resistant mutants were grown in an identical way. After every ~10–20 doublings, the ratio of the antibiotic-resistant and the wt cells was determined. Five to five competition experiments were done using independent, randomly chosen *cycA* or *rpoB* mutants (Fig. 2). Competition experiments can show wide fluctuations, but the observed tendency was clear. The ratio of D-cycloserine resistant cells remained largely constant in the mixed cultures, indicating that the *cycA* mutations did not cause growth defect or growth advantage in minimal medium. In contrast, rifampicin resistant mutants displayed variable results in similar competition experiments. While some of them were gradually overgrown by wt cells in the mixed culture, others outcompeted the wt cells.

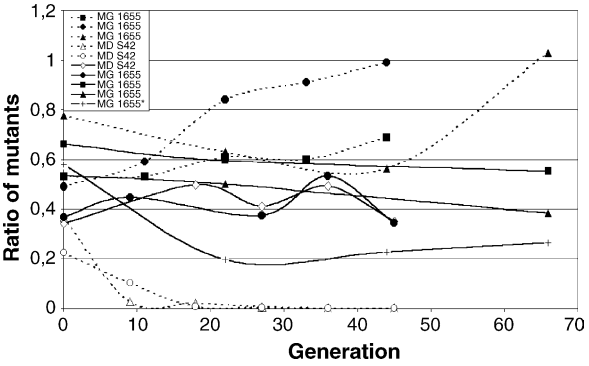

Fig. 2. Competition of wt cells vs. D-cycloserine resistant mutants (solid lines) and wt cells vs. rifampicin resistant mutants (dashed lines) in liquid cultures under non-selective conditions. Each line represents an independent experiment involving a different, randomly picked mutant of MG1655 or MDS42, as indicated. Based on PCR analysis, one of the *cycA* mutants carried an IS150 insertion (indicated by an asterisk), the others carried point mutations. Starter cultures of the competing cells were mixed initially at a ratio of 50:50. At intervals corresponding to ~10–20 doublings, 100 μl inoculums were transferred into 100 ml fresh MT minimal medium. At various intervals, the ratio of mutant cells was determined by plating dilutions onto selective and non-selective plates.

3.4. Measuring the spontaneous mutation rate of *cycA*

To characterize the spontaneous mutation rate of growing cells, D-cycloserine resistant mutants of MG1655 were counted in 20-tube fluctuation assays as described in Section 2. Briefly, parallel cultures were grown to the end of logarithmic phase in minimal medium, and D-cycloserine resistant mutants were selected on antibiotic-containing plates. Aliquots from four tubes per assay were spread on nonselective plates to determine the total cell count. The mutation rate (mutation/cell/generation) was obtained by statistical calculations as described in Section 2 and in Supplementary Material. The Ma-Sandri-Sarkar maximum likelihood method was chosen for this task, due to its capability of dealing with all ranges of the *m* (mutation per tube) value, and of yielding normally transformable results [25]. Reproducibility of the assay is demonstrated by showing the results of six fluctuation assays in Fig. 3. The average spontaneous mutation rate of the *cycA* gene in *E. coli* MG1655 was 6.54×10^{-8}.

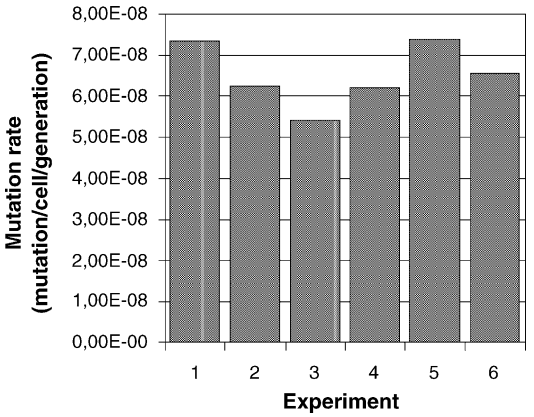

Fig. 3. Demonstration of the reproducibility of *cycA* mutation rate measurements. The results of six independent fluctuation assays are shown.

3.5. Analysis of the spectrum and location of cycA mutations

In order to investigate the type and distribution of *cycA* mutations resulting in D-cycloserine resistance, the gene was analysed by PCR amplification. As a first screen, a 1877-bp genomic segment encompassing the entire gene and the upstream intergenic region was amplified from mutant cells using the primer pair cycA1/cycA2. The amplified fragment was run on agarose gel and compared to a fragment generated on the wt template. Identical size indicated a point mutation affecting only one or a few nucleotides (base substitutions, frameshifts, small deletions and duplications), a decrease in size or failure of amplification reflected a deletion, and an increase in size revealed an IS insertion. In order to avoid a bias caused by the jackpot effect in a growing culture, $\leq$5 mutants per parallel culture of the fluctuation assays were analysed by PCR. Of the 128 mutants investigated, 95 (74.2%) carried mutations that did not result in a change detectable by agarose gel electrophoresis, 2 (1.6%) carried deletions, and 31 (24.2%) carried IS insertions (Fig. 4).

Twenty mutants that did not carry insertions or deletions detectable by agarose gel electrophoresis were further analysed by sequencing. In one case multiple mutations were found in *cycA*. Among the single mutations (Fig. 5), four transitions, five transversions, four (−1) frameshifts, two (+1) frameshifts, one small dele-

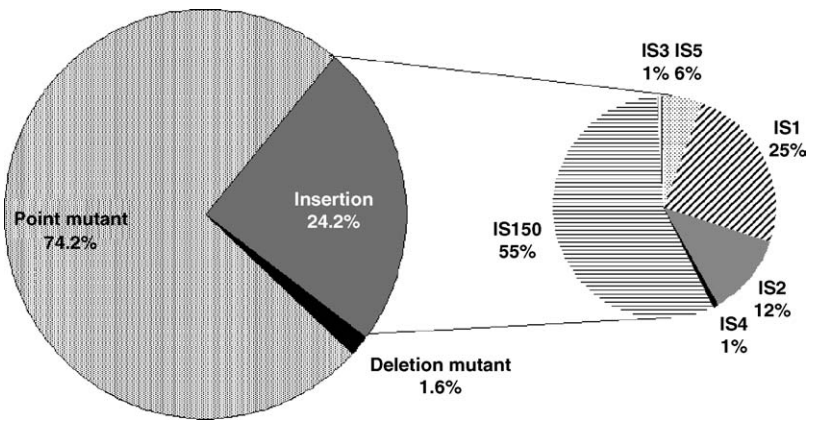

Fig. 4. Spectrum of *cycA* mutations, and the relative share of IS types among insertional mutants, based on PCR analysis of 128 and 118 independently obtained mutants, respectively.

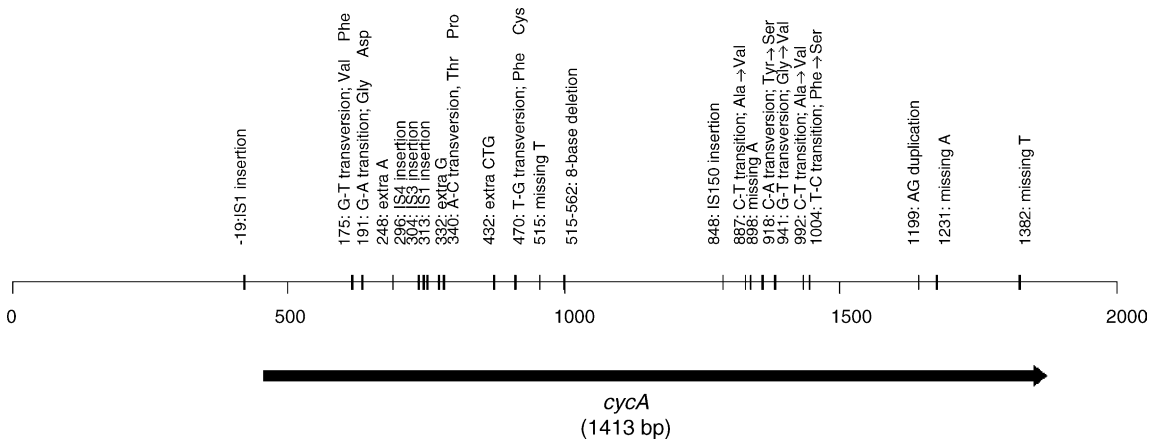

Fig. 5. The location and identity of single mutations in *cycA* of D-cycloserine resistant cells. A sample of twenty randomly chosen *cycA* mutants harboring small mutations and five mutants harboring IS insertions were analysed. Mutation 780:A → C was observed in two independent mutants. Position 1 corresponds to the first nucleotide of the start codon of *cycA*.

tion, one 3-bp and one 2-bp duplication were found. One of the transversions was found in two independent mutants. All base substitutions represented missense mutations.

The identity of IS insertions was determined by PCR using IS-specific primers in combination with the primers flanking the gene. Based on the analysis of 118 independently obtained IS mutants, six different types of ISs (IS1, IS2, IS3, IS4, IS5 and IS150) were found, with IS150 being the most frequent (55%) (Fig. 4). The location of five IS insertions was determined by sequencing (Fig. 5).

4. Discussion

A short sampling of the frequently used mutation selection systems in *E. coli* [7] reveals that many of them provide only partial information on the mutational events in the cell. Variants of the widely used assays utilizing the *lac* operon provide only a screen, limiting the quantitative analysis. Among the positive selection systems, the frequently used rifampicin test detects only base substitutions. Moreover, we show here that these mutations can affect the growth rate. The test based on the activation of the cryptic *bgl* operon detects mostly IS transpositions. Assays based on reversal of auxotrophy require a specific genetic background and detect only specific mutations. Analysis of mutants resistant to ciprofloxacin and other fluoroquinolones is complicated by multiple resistance mechanisms. Several selection systems detect mutations in genes carried on multicopy plasmids, further complicating the analysis. We show here that the *cycA* gene provides a better system for the quantitative analysis of the mutational events.

Earlier reports indicated that resistance of *E. coli* cells to 10–100 μm concentration of D-cycloserine was due to mutations mapped to *cycA*, the gene coding for a permease involved in the uptake of D-cycloserine [9,13]. Theoretically, mutations occurring in the direct targets of the antibiotic can also cause resistance. However, since D-cycloserine has multiple targets, simultaneous relevant modification of them has a negligible probability. We provide here further support that mutations responsible for resistance under our experimental conditions occur exclusively in *cycA*. In two tests, 12–12 D-cycloserine resistant mutants could be complemented by wt *cycA*. Moreover, each of 20 randomly chosen D-cycloserine resistant mutants contained mutations in *cycA*, as revealed by sequencing. Narrowing down the location of mutations responsible for a selectable phenotype to a single gene allows a convenient analysis of the mutation spectrum.

We hypothesized that in the absence of the amino acid transport substrates of CycA, mutations responsible for D-cycloserine resistance are neutral in respect of growth rate. Indeed, the ratio of resistant mutants tended to remain constant when grown with wt cells in competition experiments. In contrast, rifampicin resistant mutants showed a more variable competition potential when compared to wt cells. It is plausible that various mutations in *rpoB*, resulting in rifampicin resistance [26], can affect the transcriptional characteristics of the polymerase, leading to either a defect or advantage in growth. Indeed, rifampicin resistant mutants of *Enterococcus faecium* were reported to have a fitness cost

[27]. Moreover, it was shown that rifampicin resistant *E. coli* mutants can have pleiotropic phenotypes [28]. It can be concluded that rifampicin resistant mutants can be either positively or negatively selected in a growing population of cells, and this can introduce unknown variables in mutation rate measurements. The test based on D-cycloserine resistance offers a way to measure mutation rates without the distorting effects of pre-assay selection.

It can be assumed that practically any type of mutation can incapacitate a permease resulting in resistance to a toxic transport substrate. Indeed, transitions, transversions, frameshifts, small and large deletions, as well as insertions were all found among the selected mutants, representing essentially the full spectrum of mutations in *E. coli*. There are 10 different types of ISs resident in MG1655, and a non-exhaustive screening of the *cycA* insertion mutants identified six species of them (IS1, IS2, IS3, IS4, IS5 and IS150). It is noted that loss of function mutations of *cycA* were distributed along nearly the entire length of the gene. This means that the 1413-bp gene provides a large target for selectable mutations, making it easier to obtain sufficiently large number of mutants for quantitation. A further advantage of the *cycA* assay is that no specific host genetic background is needed for selection, and the mutation target is a single-copy, chromosomal gene.

The overall spontaneous mutation rate of *E. coli* cells, measured by various assays, is usually estimated to be 10^{-6} to 10^{-8} mutation/cell/generation. The rate measured by the *cycA* test (6.54×10^{-8}) is in line with these observations. It is interesting to note, however, that the contribution of different types of mutations varies widely, depending on the mutation markers used. Systematic studies of the spectrum of spontaneous mutations have shown native IS elements to be responsible for 8–60% of mutations in a neutral marker [29–31]. In our experiments, IS insertions represented 24% of all mutations. Among the possible reasons for the varying results are differences in the native IS-sets of the particular strains or hotspots for certain ISs in the target gene. We also note that the *cycA* test detects loss of function mutations. While IS insertions, frameshifts and deletions are likely to result in a non-functional gene product, a large number of base substitutions have probably no effect on the gene function and, as physiologically irrelevant, remain undetected. Different mutation markers can have different density of detectable mutations, and this can also lead to varying results. According to our data, *cycA* serves as an excellent tool to measure mutation rates and spectra by providing a large, neutral mutation target with no apparent hotspots for any particular mutation type. Mutants are easily selected, and results are highly reproducible.

Acknowledgements

We are grateful for the expert technical assistance provided by Ágnes Szalkanovics. This work was supported by grant OTKA T043260 to G.P.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mrfmmm.2005.11.004.

References

[1] S.M. Rosenberg, Evolving responsively: adaptive mutation, Nat. Rev. Genet. 2 (2001) 504–515.
[2] L. Loewe, V. Textor, S. Scherer, High deleterious genomic mutation rate in stationary phase of *Escherichia coli*, Science 302 (2003) 1558–1560.
[3] J.R. Roth, E. Kofoid, F.P. Roth, O.G. Berg, J. Seger, D.I. Andersson, Regulating general mutation rates: examination of the hypermutable state model for Cairnsian adaptive mutation, Genetics 163 (2003) 1483–1496.
[4] J.W. Drake, B. Charlesworth, D. Charlesworth, J.F. Crow, Rates of spontaneous mutation, Genetics 148 (1998) 1667–1686.
[5] I. Bjedov, O. Tenaillon, B. Gerard, V. Souza, E. Denamur, M. Radman, F. Taddei, I. Matic, Stress-induced mutagenesis in bacteria, Science 300 (2003) 1404–1409.
[6] D. Schneider, R.E. Lenski, Dynamics of insertion sequence elements during experimental evolution of bacteria, Res. Microbiol. 155 (2004) 319–327.
[7] R.A. LaRossa, Mutant selections linking physiology, inhibitors, and genotypes, in: F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, H.E. Umbarger (Eds.), *Escherichia coli* and Salmonella typhimurium: cellular and molecular biology, vol. 2, American Society for Microbiology, Washington, DC, 1996, pp. 2527–2587.
[8] R.J. Wargel, C.A. Shadur, F.C. Neuhaus, Mechanism of D-cycloserine action: transport systems for D-alanine, D-cycloserine, L-alanine, and glycine, J. Bacteriol. 103 (1970) 778–788.
[9] R.J. Wargel, C.A. Shadur, F.C. Neuhaus, Mechanism of D-cycloserine action: transport mutants for D-alanine, D-cycloserine, and glycine, J. Bacteriol. 105 (1971) 1028–1035.
[10] S.D. Cosloy, D-Serine transport system in *Escherichia coli* K-12, J. Bacteriol. 114 (1973) 679–684.
[11] J.C. Robbins, D.L. Oxender, Transport systems for alanine, serine, and glycine in *Escherichia coli* K-12, J. Bacteriol. 116 (1973) 12–18.
[12] F.C. Neuhaus, D-cycloserine and *O*-carbamyl-D-serine, in: D. Gottlieb, P.D. Shaw (Eds.), Antibiotics: mechanism of action, vol. 1, Springer-Verlag, New York, 1967, pp. 40–83.
[13] R.R. Russell, Mapping of a D-cycloserine resistance locus in *Escherichia coli* K-12, J. Bacteriol. 111 (1972) 622–624.
[14] I. Chopra, P. Ball, Transport of antibiotics into bacteria, Adv. Microb. Physiol. 23 (1982) 183–240.

[15] M.P. Lambert, F.C. Neuhaus, Mechanism of D-cycloserine action: alanine racemase from *Escherichia coli* W, J. Bacteriol. 110 (1972) 978–987.
[16] L.E. Zawadzke, T.D. Bugg, C.T. Walsh, Existence of two D-alanine:D-alanine ligases in *Escherichia coli*: cloning and sequencing of the ddlA gene and purification and characterization of the DdlA and DdlB enzymes, Biochemistry 30 (1991) 1673–1682.
[17] M. Noda, Y. Kawahara, A. Ichikawa, Y. Matoba, H. Matsuo, D.G. Lee, T. Kumagai, M. Sugiyama, Self-protection mechanism in D-cycloserine-producing Streptomyces lavendulae. Gene cloning, characterization, and kinetics of its alanine racemase and D-alanyl-D-alanine ligase, which are target enzymes of D-cycloserine, J. Biol. Chem. 279 (2004) 46143–46152.
[18] T.D. Bugg, C.T. Walsh, Intracellular steps of bacterial cell wall peptidoglycan biosynthesis: enzymology, antibiotics, and antibiotic-resistance, Nat. Prod. Rep. 9 (1992) 199–215.
[19] F.R. Blattner, G. Plunkett III, C.A. Bloch, N.T. Perna, V. Burland, M. Riley, J. Collado-Vides, J.D. Glasner, C.K. Rode, G.F. Mayhew, J. Gregor, N.W. Davis, H.A. Kirkpatrick, M.A. Goeden, D.J. Rose, B. Mau, Y. Shao, The complete genome sequence of *Escherichia coli* K-12, Science 277 (1997) 1453–1474.
[20] J.P. Muyrers, Y. Zhang, G. Testa, A.F. Stewart, Rapid modification of bacterial artificial chromosomes by ET-recombination, Nucleic Acids. Res. 27 (1999) 1555–1557.
[21] B.G. Hall, Activation of the bgl operon by adaptive mutation, Mol. Biol. E vol. 15 (1998) 1–5.
[22] S. Sarkar, W.T. Ma, G.H. Sandri, On fluctuation analysis: a new, simple and efficient method for computing the expected number of mutants, Genetica 85 (1992) 173–179.
[23] F.M. Stewart, D.M. Gordon, B.R. Levin, Fluctuation analysis: the probability distribution of the number of mutants under different conditions, Genetics 124 (1990) 175–185.
[24] R. Curtiss III, L.J. Charamella, C.M. Berg, P.E. Harris, Kinetic and genetic analyses of D-cycloserine inhibition and resistance in *Escherichia coli*, J. Bacteriol. 90 (1965) 1238–1250.
[25] W.A. Rosche, P.L. Foster, Determining mutation rates in bacterial populations, Methods 20 (2000) 4–17.
[26] D.J. Jin, C.A. Gross, Mapping and sequencing of mutations in the *Escherichia coli* rpoB gene that lead to rifampicin resistance, J. Mol. Biol. 202 (1988) 45–58.
[27] V.I. Enne, A.A. Delsol, J.M. Roe, P.M. Bennett, Rifampicin resistance and its fitness cost in *Enterococcus faecium*, J. Antimicrob. Chemother. 53 (2004) 203–207.
[28] D.J. Jin, C.A. Gross, Characterization of the pleiotropic phenotypes of rifampin-resistant rpoB mutants of *Escherichia coli*, J. Bacteriol. 171 (1989) 5229–5231.
[29] R.M. Schaaper, R.L. Dunn, Spontaneous mutation in the *Escherichia coli* lacI gene, Genetics 129 (1991) 317–326.
[30] H. Rodriguez, E.T. Snow, U. Bhat, E.L. Loechler, An *Escherichia coli* plasmid-based, mutational system in which supF mutants are selectable: insertion elements dominate the spontaneous spectra, Mutat. Res. 270 (1992) 219–231.
[31] K. Kitamura, Y. Torii, C. Matsuoka, K. Yamamoto, DNA sequence changes in mutations in the tonB gene on the chromosome of *Escherichia coli* K12: insertion elements dominate the spontaneous spectra, Jpn. J. Genet. 70 (1995) 35–46.