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Chapter 8

Bacterial Systems for Testing Spontaneous and Induced Mutations

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/50471

1. Introduction

Changes in genetic material result from introduction of mutations into DNA. Spontaneous mutations can occur because of replication errors or as a consequence of lesions introduced into DNA during normal cell growth. Induced mutations arise after treatment of the organism with an exogenous mutagen being physical or chemical agent increasing the frequency of mutations.

Bacteria are simple and widely used models for examination of mutagenesis and DNA repair processes. The advantages of bacterial systems are their availability, easy cultivation, short time of cell division, and haploidity. Many DNA damaging agents and/or mutator genes cause mutations that are readily and clearly observed in changes of phenotype. Additional observations like (i) analysis of bacterial survival after treatment with mutagenic agents; (ii) microscopic examination of bacterial cells; (iii) examination of plasmid DNAs isolated from mutagen-treated cells for their sensitivity to the specific enzymes that recognize DNA lesions; (iv) induction of the SOS system measured by induction of β-galactosidase in *Escherichia coli*; (v) sporulation of bacteria in *Bacillus subtilis*, all provide a simple and rapid yet highly informative characterization of the examined process. Results of these studies usually constitute a first step of deeper examination of the multiple processes on molecular level.

Two representatives of *Enterobacteriaceae* (Gammaproteobacteria), *E. coli* and *Salmonella typhimurium*, are commonly used in the studies on spontaneous and induced mutagenesis. Other bacterial models like representatives of *Pseudomonadaceae* (Gammaproteobacteria), *Pseudomonas putida* and *Bacillaceae* (Firmicutes), *B. subtilis* have been also used in mutagenesis studies.

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2. Mutation detection systems in *Escherichia coli*

2.1. The *argE3* → Arg$^+$ reversion system in *Escherichia coli* K12

Escherichia coli K12 was isolated from the stool of a convalescent diphtheria patient in USA (Palo Alto, California) in 1922 and deposited in the strain collection of the Department of Bacteriology of Stanford University. Serological studies revealed that after many years of cultivation under laboratory conditions the strain lost the K and O antigens and became incapable of human gut colonization. A lot of mutant derivatives of strain K12 have been obtained in many laboratories around the world. One of them is AB1157 strain with relevant genotype: *thr-1 ara-14 leuB6* Δ*(gpt-proA)62 lacY1 tsx-33 supE44*$_{\text{amber}}$ *galK2 hisG4 rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1* and its derivatives (Bachman, 1987). The *argE3*(ochre), *hisG4*(ochre) and *thr-1*(amber) are nonsense point mutations in genes encoding enzymes involved in arginine, histidine and threonine biosynthesis pathways, respectively. The *supE44* encodes *supE* amber suppressor reading UAG. However, it can only weakly suppress the *thr-1* mutation.

A suppressor mutation is a mutation that counteracts the effects of another mutation. One type of suppressor mutations are mutations that appear in the tRNA encoding genes at the anticodon site. The changed tRNAs are able to recognize a nonsense codon that occur elsewhere in protein-coding genes and incorporate the amino acids specific for them into the polypeptide chain during protein synthesis.

The bacterial test system of mutation detection described here is based on reversion of the auxotrophic *argE3* mutation to prototrophy and subsequent determination of specificity of mutation with the use of a set of bacteriophage T4 amber and ochre mutants. The marker is situated in the chromosome. The *argE* gene encodes acetylornithine deacetylase, one of the enzymes of arginine biosynthesis pathway. The Arg$^+$ phenotype can be restored by (i) any point mutation at *argE3* that changes nonsense UAA codon to any sense nucleotide triplet coding for any amino acid; (ii) an AT→GC transition at *argE3* that changes the UAA nonsense codon to the UAG nonsense codon recognized by *supE44* amber suppressor; and (iii) suppressor mutations enabling reading UAA nonsense codon. The suppressors can be created *de novo* or as the result of a GC→AT transition at *supE44* (formation of *supE* ochre suppressor) (Sargentini & Smith, 1989; Śledziewska-Gójska et al., 1992).

Considering all the theoretical possibilities of the *ochre* suppressor formation in *E. coli* resulting from a single base substitution in tRNA genes it can be seen that such suppressors may arise from tRNA for tyrosine, lysine, glutamine, glutamate, leucine and serine. The following tRNA species that may produce *de novo* an ochre suppressor by a single base substitution in the anticodon site are $tRNA^{Gln}_{UUG}$, $tRNA^{Lys}_{UUU}$, and $tRNA^{Tyr}_{GUA}$. The formed suppressors are, respectively, *supB*, *supL* (*supG*, *supN*) and *supC* (*supO*, *supM*), created as a result of GC→AT (*supB*), AT→TA (*supL*, *supG*, *supN*) or GC→TA (*supC*, *supO*, *supM*) base substitutions in *gln*-tRNA, *lys*-tRNA and *tyr*-tRNA genes, respectively (Table 1). The *supX* suppressor is also found in the Arg$^+$ revertants, but it has not yet been identified. This suppressor can be formed as a result of either GC→TA or AT→TA transversions (Sargentini

& Smith, 1989; Śledziewska-Gójska et al., 1992 and cited therein). Raftery and Yarus (1987) constructed the *gltT*(SuUUA/G) gene encoding $tRNA^{Glu}_{UUA}$ as a result of GC→TA transversion in the *gltT* gene encoding $tRNA^{Glu}_{UUC}$. This construct was expected to explain the mystery of the *supX* suppressor. However, it failed to suppress the *argE3*oc mutation in *E. coli* AB1157 strain (Płachta & Janion, 1992). Moreover, Prival (1996) identified three $tRNA^{Glu}_{UUA}$ suppressors: *supY*, *supW* and *supZ* that arose from the *gltW*, *gltU* and *gltT* genes, respectively. These suppressors were found in late-arising spontaneous Arg^+ revertants.

There are also theoretical possibilities of creating ochre suppressors from $tRNA^{Tyr}_{AUA}$, $tRNA^{Ser}_{UGA}$ and $tRNA^{Leu}_{UAA}$, but these suppressors have not been identified yet (Śledziewska-Gójska et al., 1992). Figure 1 shows two schematic pictures of tRNA suppressors.

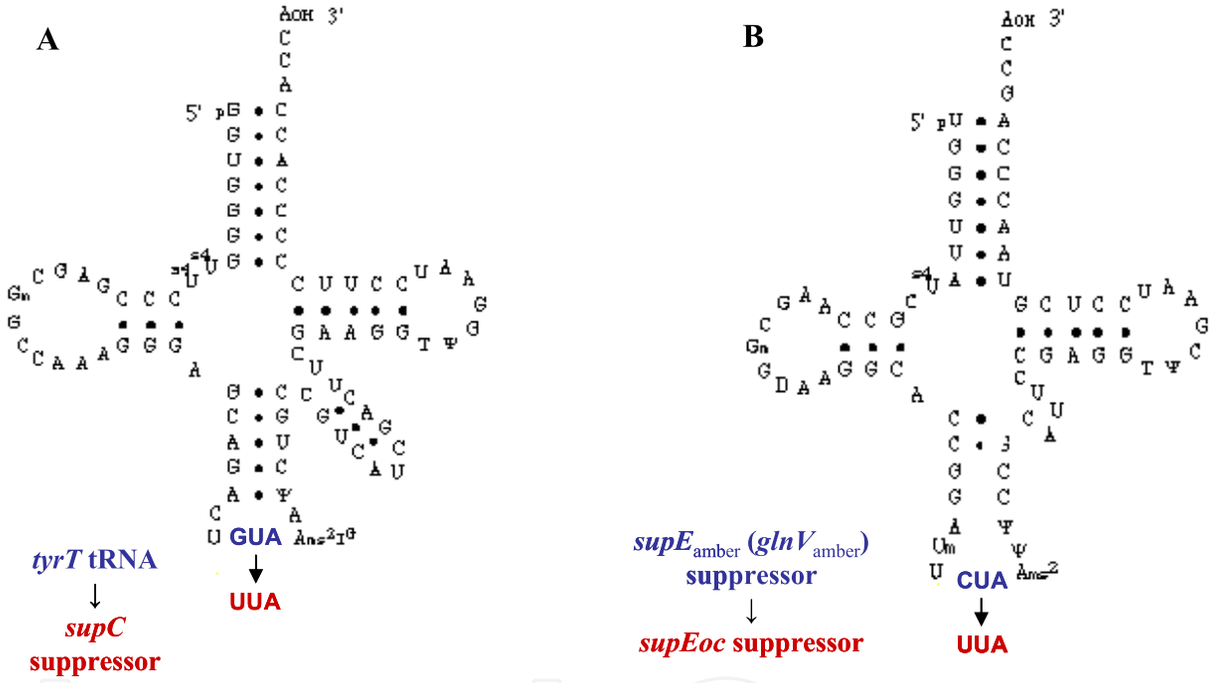


Figure 1. Two examples of tRNA particles. **A.** Tyrosine inserting tRNA into the polypeptide chain (recognizing 5′ UAC 3′ codon in the mRNA) that changes into *sup*C suppressor (reading 5′ UAA 3′) in the result of GC→TA transversion in the anticodon part. **B.** Amber suppressor tRNA arisen from *glnV* gene (encoding tRNA for glutamine) inserting glutamine into the polypeptide chain (recognizing 5′ UAG 3′ codon in the mRNA) that changes into *supE*oc suppressor (reading 5′ UAA 3′) in the result of GC→AT transition in the anticodon part (from *Acta Biochimica Polonica* with permission).

Arg^+ revertants can arise spontaneously or as a result of induced mutagenesis. The first step in the analysis of the Arg^+ revertants is the examination of their requirement for histidine and threonine for growth. Arg^+ revertants have been divided into four phenotypic classes: class I: Arg^+ His^- Thr^-, class II: Arg^+ His^+ Thr^-, class III: Arg^+ His^- Thr^- and class IV: Arg^+ His^+ Thr^+. Because, as mentioned above, *supE44* suppressor to some extent suppresses the *thr-1* mutation, Thr^+ phenotype may be wrongly read, thus the revertants of class I and class II may be incorrectly classified as class III and class IV, respectively. For this reason in practice

only two groups of the Arg+ revertants have been usually considered: a sum of classes I and III, and a sum of classes II and IV (Todd et al., 1979; Śledziewska-Gójska et al., 1992).

The sensitivity of Arg+ revertants to tester T4 phages is the second step in mutational analysis. A set of five T4 phages carrying a defined nonsense mutation includes the following phage mutants: amber B17 and NG19, ochre oc427, ps292 and ps205. Phage multiplication observed as plaque formation on a lawn of tested bacteria indicates that the host bacterium bears a specific suppressor mutation (Kato et al., 1980; Shinoura et al., 1983; Sargentini and Smith, 1989; Śledziewska-Gójska et al., 1992). A schematic procedure for determination of MMS-induced mutagenesis using *argE3* → Arg+ reversion system is shown in Figure 2.

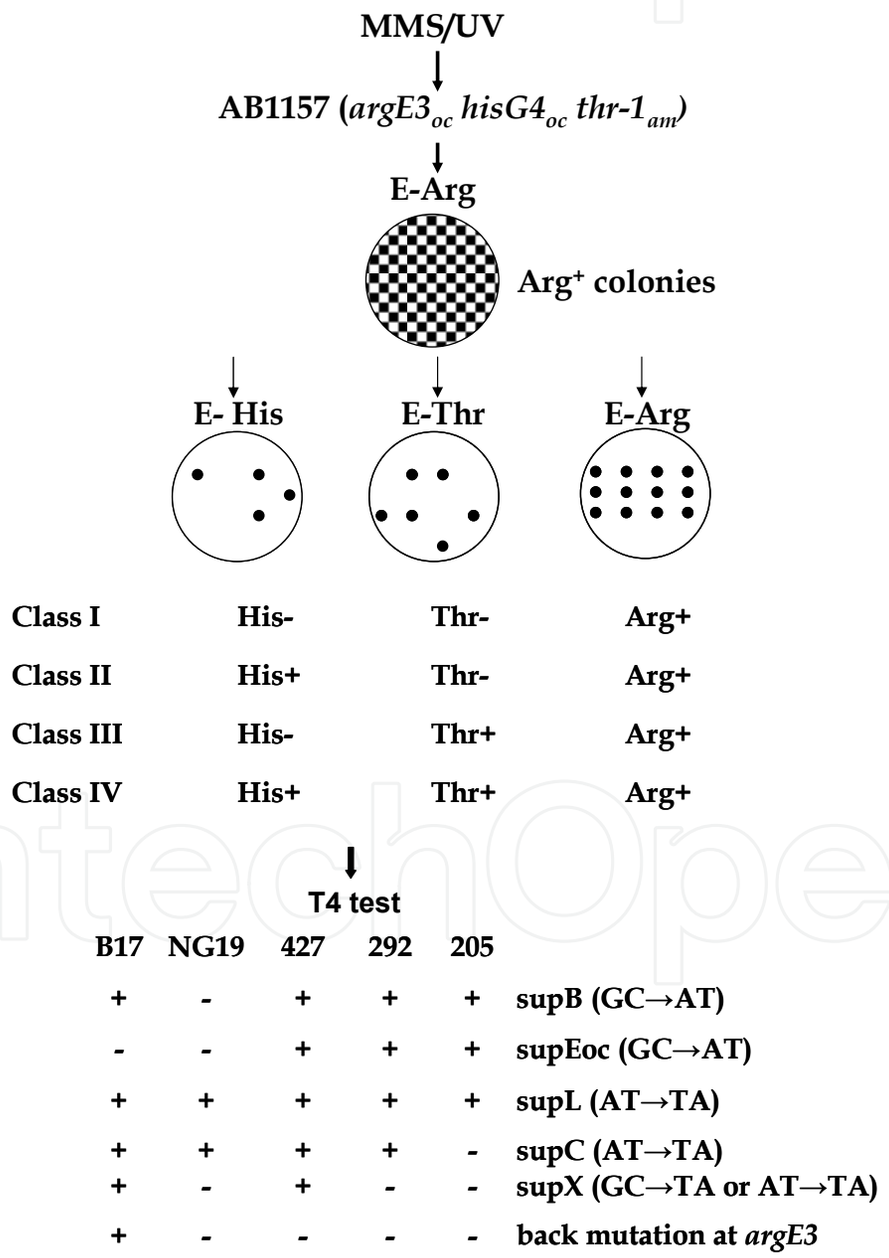


Figure 2. Schematic presentation of MMS-induced mutagenesis assay in *E.coli* AB1157 strain with the use of the *argE3* → Arg+ reversion system (detailed description in the text).

Arg$^+$ revertants of class I are the result of back mutations at the *argE3* site, or *supB* or *supE*oc suppressor formation. Arg$^+$ revertants of class II, III and IV occur as a result of *supL*, *supX* and *supC* suppressor formation, respectively. The details of the above analysis are presented in Table 1 containing species of tRNA producing the indicated suppressor by a single base substitution in the anticodon sites.

Suppressors	T4 phages					Arg$^+$ revertants			Recognized codon	tRNA charged amino acid with	Gene mutation leading to recognition of UAA nonsense codon
	amber		*ochre*			*ochre*		*amber*			
	B17	NG19	oc427	ps 292	ps205	*argE3*	*hisG4*	*thr-1*			
supB	+	-	+	+	+	+	-	-	CAA	Gln	GC → AT
***supC* (*supO*, *supM*)**	+	+	+	+	-	+	+ t^S	+ t^S	UAC	tyr	GC → TA
***supL* (*supG*, *supN*)**	+	-	+	+	-	+	+	-	AAA	Lys	AT → TA
supX	+	-	+	-	-	+	-	+	?	?	GC → TA or AT → TA
***supE*oc**	-	-	+	+	+	+	-	-	UAG	*supE*am (gln)	GC → AT
AB1157 Arg$^-$ *supE*amber	+	-	-	-	-	-	-	-	----------		

Table 1. tRNA suppressors counteracting effects of the *ochre* and *amber* nonsense mutations in T4 phages and Arg$^+$ revertants of *E. coli* AB1157. + t^S means that suppression works better at 30^o C than at 37^o C. (from Acta Biochimica Polonica with permission).

Sargentini and Smith (1989) constructed a set of AB1157 derivatives bearing all the mentioned suppressors: SR2151, SR2155, SR2162, SR2161, SR2154, SR2153 carrying, respectively, *supB*, *supL*, *supN*, *supM*, *supC*, *supE*oc suppressors (Sargentini & Smith, 1989; Śledziewska-Gójska et al., 1992). These strains serve to control respective phage T4 mutations.

Identification of created suppressors allows deducing the specificity of mutation without DNA sequencing. However, such analysis does not indicate the type of mutations in the *argE* gene creating a sense codon from the UAA stop codon. In this case DNA sequencing is required. The proportion of suppressor and back mutations in the *argE* gene depends on the type of mutagenic factor and bacterial background.

There is also a possibility to study the level of *hisG4*→His$^+$ revertants, however, only some of the suppressors may counteract the effect of *hisG4* mutation. The His$^+$ phenotype can be restored by (i) any point mutation at *hisG4* that changes nonsense UAA codon to any sense nucleotide triplet coding for any amino acid, or (ii) only two suppressor mutations enabling reading UAA nonsense codon, namely *supC* (*supO*, *supM*) and *supL* (*supG*, *supN*)

suppressors. In this way many of the arising mutations are lost (Sargentini & Smith, 1989; Śledziewska-Gójska et al., 1992).

2.2. Studies with the use of the *argE3* → Arg$^+$ reversion based system

In the era of intensive development of techniques of molecular biology and genetics studies, information on reversion to prototrophy of the *argE3* mutation still provide new, interesting and valuable information on the mutagenic specificity of different mutagens and mutator genes as well as on the mechanisms of mutagenesis and DNA repair. The applications of the described genetic system are presented below. This system is particularly useful for detection of GC→TA, GC→AT and AT→TA base substitutions and examination of transcription-coupled DNA repair.

2.2.1. Specificity of mutator genes

The system confirms the mutagenic effects of mutator genes such as *mutT*, *mutY* and *fpg* (Wójcik et al., 1996; Wójcik & Janion, unpublished data; Nowosielska & Grzesiuk, 2000) or *dnaQ* (Nowosielska *et al.* 2004a; 2004b).

MutT, MutY and Fpg (MutM), proteins belonging to the GO system, defend bacteria against the mutagenic action of 8-oxoG in DNA. MutT is a pyrophosphatase that hydrolyses 8-oxo-dGTP and prevents its incorporation into DNA. MutY is a DNA glycosylase excising from DNA adenine mispaired with A, 8-oxoG or G. Among others, Fpg excises from DNA 8-oxoG when it pairs with C (or T). The level of spontaneous transversions: AT→CG in *mutT*$^-$ and GC→TA in *mutY*$^-$ and *fpg*$^-$ mutants is, respectively, about 1000 to 10000 and 10 to 100-fold higher than in the wild type strain (Michaels & Miller, 1992). We have analyzed Arg$^+$ revertants arising spontaneously in *mutT*$^-$, *mutY*$^-$ and *fpg*$^-$ derivatives of *E. coli* AB1157 strain. In AB1157 *mutT*$^-$ strain a 1000-fold increase in the *argE3*→Arg$^+$ reversions was observed. All those reversions arose due to back mutations at the *argE3* site (probably as a result of AT→CG transversions). In *mutY*$^-$ and *fpg*$^-$ mutants all of the spontaneous *argE3*→Arg$^+$ reversions were due to GC→TA transversions by *supC* suppressor formation (Wójcik et al., 1996; Wójcik & Janion, unpublished data; Nowosielska & Grzesiuk, 2000).

DNA polymerase III, the main replicative polymerase in *E. coli*, comprises a *dnaQ*-encoded epsilon subunit responsible for proofreading activity. Mutants defective in this subunit chronically express the SOS response and exhibit a mutator phenotype (Echols *et al.,* 1983). Using the *argE3*→Arg$^+$ reversion, the effects of deletions in genes *polB* and *umuDC*, encoding, respectively, the SOS-induced DNA polymerases Pol II and PolV, on the frequency and specificity of spontaneous mutations in the *dnaQ* background were studied. It was clearly shown that deletion of *umuDC* genes significantly decreased the level of spontaneous mutations in *dnaQ* strains (Nowosielska *et al.*, 2004a). The Arg$^+$ revertants in *mutD5* (allele of *dnaQ*) mutant occurred only as a result of tRNA suppressor formation, whereas those in *mutD5 polB* (Pol II deficient) strains arose at 81% by back mutation at the *argE3* ochre site (Nowosielska et al., 2004b).

The SOS response is a bacterial defence system enabling the survival of cells whose DNA has been damaged and replication arrested. The SOS system increases expression of over 40 genes involved in DNA repair, replication, and mutagenesis. The expression of genes of the SOS regulon is tightly regulated. The *umuD* and *umuC* genes encoding the Y-family DNA polymerase V (PolV) are expressed among the last ones. In the process of translesion synthesis (TLS), this low fidelity polymerase, composed of UmuC and two particles of shortened UmuD form, UmuD' ($UmuD'_2C$) bypasses lesions inserting a patch of several nucleotides and allowing resumption of DNA replication by PolIII, the main replicative polymerase in *E.coli* (Janion, 2008). One of the symptoms of the SOS-induction is filamentous growth of bacteria due to expression of the *sulA* gene. The *sulA* gene codes for a protein that blocks cell division by inhibiting assembling of the FtsZ protein into a ring structure leading to filament formation (Bi & Lutkenhaus, 1991). Inhibition of cell division allowed DNA repair processes to be finished before next round of division (Janion et al., 2002; Janion 2008).

It has been shown that BW535 (*nth-1*, Δ*xth*, *nfo-1*), a derivative of AB1157 deficient in base excision repair (BER), chronically induces the SOS system. The *xth, nfo* and *nth* genes encode, respectively, exonuclease III (exo III), endonuclease IV (endo IV) and endonuclease III (endo) III. Exo III and endo IV account for 85% and 5% of the cell's endonuclease activity, respectively (Kow & Wallace, 1985; Cunnigham et al., 1986). Endo III is a DNA glycosylase with a broad substrate specificity which mainly excises oxidized pyrimidines, and also possesses an AP-lyase activity cleaving the sugar-phosphate backbone and generating single- and double-strand breaks in DNA (Dizdaroglu et al., 2000). The triple *nth xth nfo* mutant can not repair AP sites. The chronic induction of the SOS system is due to accumulation of AP sites that left unrepaired in DNA. A mutator phenotype measured by an increased level of spontaneous *umuDC*-dependent *argE3*→Arg^+ reversions was one of the symptoms of the chronic induction of the SOS system in the *nth xth nfo* mutant (Janion et al., 2003).

2.2.2. Specificity of mutagens

The mutagenic specificity of N^4-hydroxycytidine (oh^4Cyd), hydroxylamine (HA), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Śledziewska-Gójska *et al.*, 1992), ethylmethane sulfonate (EMS) (Grzesiuk & Janion, 1993), methylmethane sulfonate (MMS) (Śledziewska-Gójska & Janion, 1989; Grzesiuk & Janion, 1994) and UV light (Wójcik & Janion, 1997; 1999; Fabisiewicz & Janion, 1998) has also been confirmed with the help of the *argE3*→Arg^+ reversion.

Using this system it has been established that HA, a cytosine modifying agen, apart from well known GC→AT transitions may also cause a significant number of GC (or AT)→TA transversions. As much as 30% of the HA-induced Arg^+ revertants were formed by GC (or AT)→TA transversions (Śledziewska-Gójska et al., 1992).

Studies on *E. coli* AB1157 strain and its derivatives revealed that biological effects (survival, mutation induction and mutation specificity) of halogen light irradiation were very similar to those observed after UVC irradiation. The halogen light-induced mutations were

GC→AT transitions (*supB* or *supE* ochre suppressor formation) and back mutations at *argE3* sites resulting from T-C 6-4 photoproducts or T<>T thymine dimers, respectively. The latter damage was observed only in *uvrA* mutants defective in nucleotide excision repair (NER), constituting less than 5% of the total number of Arg^+ revertants (Wójcik & Janion, 1997). These results confirmed previous data showing harmful effects caused by halogen light, such as DNA damage, mutations, genotoxicity and skin cancers in mice due to emission of a broad spectrum of UV light, particularly UVC (De Flora et al., 1990; D'Agostini et al., 1993; D'Agostini & De Flora, 1994).

Analysis of Arg^+ revertants supplied new data on the mechanisms of mutagenesis and processes of DNA repair. The mutagenic properties of DNA damaging agents and the spectra of the induced mutations depend on the bacterial background, *i.e.*, the presence of mutations in genes encoding proteins involved in DNA repair systems.

It is known that EMS, a S_N2-type alkylating agent, is an *umuDC*-independent mutagen and induces GC→AT transitions due to formation of O^6-ethylguanine in DNA. It has been shown that in the AB1157 strain, EMS-induced Arg^+ revertants arise by *supB* and *supE* ochre suppressor formation. However, in *mutS*$^-$, a mismatch repair-deficient strain, the specificity of the EMS-induced *argE3*→Arg^+ reversions was changed and formation of *supL* suppressor by AT→TA transversions was mainly observed. Moreover, these mutations were *umuDC*-dependent. It was suggested that the change in mutation specificity was due to 3meA lesions or creation of apurinic sites. These results also point to different processes of DNA repair in *mutS*$^+$ and *mutS*$^-$ strains (Grzesiuk & Janion, 1993).

MMS, another S_N2-type alkylating agent, predominantly methylates nitrogen atoms in purines. This methylating agent creates the following adducts in double stranded DNA: 7-methylguanine (7meG), 3-methyladenine (3meA), 1-methyladenine (1meA), 7-methyladenine (7meA), 3-methylguanine (3meG), O^6-methylguanine (O^6meG), 3-methylcytosine (3meC), and methylphosphotriesters. In ssDNA, MMS induces the same lesions but in different proportions. In ssDNA, the participation of 1meA and 3meC increases significantly since the ring nitrogens at these positions are not protected by the complementary DNA strand (Wyatt & Pittman, 2006; Sedgwick *et al.*, 2007). Analysis of Arg^+ revertants in *E. coli* AB1157 strain without any additional mutations revealed that 70-80% of those revertants arose by AT→TA transversions in a *umuDC*-dependent process, whereas the rest occurred in a *umuDC*-independent manner either by GC→AT transitions (formation of *supB* or *supE* ochre suppressors) or by back mutations at *argE3* site. The latter ones were detected in less than 5% of the Arg^+ revertants. AT→TA transversions are thought to be the result of 3meA, abasic sites and 1meA, whereas GC→AT transitions come from O^6-meG and 3meC residues in DNA and from depurination of 7meG (Grzesiuk & Janion, 1994; Nieminuszczy *et al.*, 2006a; 2009; Wrzesinski et al., 2010).

The spectrum of the MMS-induced *argE3*→Arg^+ reversions changes in various strains deficient in DNA repair systems. In the *mutS*$^-$ mutant Arg^+ revertants arose mainly by GC→AT transitions (*supB* and *supE* ochre suppressor formation) or back mutations at *argE3* site. The latter group constituted a few percent of the total number of the Arg^+

revertants (Grzesiuk & Janion, 1998). In the *dnaQ49* derivative of the AB1157 strain about half of the MMS-induced Arg$^+$ revertants occurred by AT→TA transversions (*supL* suppressor formation). In a double *dnaQ*$^-$ *umuDC*$^-$ mutant about 90% of the revertants possessed *supB* or *supE* ochre suppressors due to GC→AT transitions (Grzesiuk and Janion, 1996).

2.2.3. Detection of mutations resulting from lesions in ssDNA

Examination of MMS-induced mutagenesis in AB1157*alkB*$^-$ derivatives indicates that the *argE3*→Arg$^+$ reversion system also enables detection of mutations arising from lesions in ssDNA (Nieminuszczy et al., 2006a; 2009; Sikora et al., 2010; Wrzesiński et al., 2010). AlkB is an α-ketoglutarate-, O_2- and Fe(II)-dependent dioxygenase that oxidatively demethylates 1meA and 3meC in ds- and ssDNA and in RNA. However, ssDNA is repaired much more effectively than dsDNA (Trewick et al., 2002; Falnes et al., 2002). It has been shown that in *alkB*$^-$ mutants the level of MMS-induced mutagenesis depends on the test system used, and is several orders of magnitude higher when measured in the *argE3*→Arg$^+$ reversion test system in *E. coli* AB1157 in comparison to *lacZ*→Lac$^+$ reversion studied in CC101-CC106 strains (Nieminuszczy et al., 2006a; 2006b; 2009; Kataoka et al., 1983; Dinglay et al., 2000). The CC101-CC106 tester strains are described in more detail in Chapter 2.4. Briefly, the *lacZ*→Lac$^+$ reversion occurs only by a back mutation at one point in the structural gene encoding the β-galactosidase that if not expressed is primarily in dsDNA form.

The *argE3*→Arg$^+$ reversion-based system has showed that in AB1157 *alkB*$^-$ strain 95-98% of the induced mutations are *umuDC* (Pol V)-dependent AT→TA transversions (*supL* suppressor formation) and GC→AT transitions (*supB* or *supE* ochre suppressor formation). Back mutations in the *argE3* site constitute only about 2-5% of all types of Arg$^+$ revertants (Nieminuszczy et al., 2006a). Genes encoding tRNA are heavily transcribed and exist mostly as ssDNA in cells. It facilitates methylation of A/C to 1meA/3meC. That is why we assume that in AB1157 *alkB*$^-$ strain the targets undergoing mutations leading do Arg$^+$ revertants are predominantly located in ssDNA. Reversion to Arg$^+$ occurs mostly by formation of a variety of *sup*tRNA ochre suppressors. The number of targets undergoing mutations and differences in the reactivity of MMS to form 1meA/3meC lesions in ss- *vs*. dsDNA are the main reasons of the great discrepancy in the frequencies of MMS-induced *argE3*→Arg$^+$ and *lacZ*→Lac$^+$ revertants observed (Nieminuszczy et al., 2009).

An extremely high level of the MMS-induced *argE3*→Arg$^+$ reversions has been observed in *E. coli* AB1157 *nfo xth alkB* strain defected in the repair of AP sites caused by invalid base excision repair system (BER) and deficiency in AlkB dioxygenase. This phenomenon can be explained by local relaxation of dsDNA structure due to the presence of AP sites in AB1157 *nfo*$^-$ *xth*$^-$ strain. We assume that under these conditions more single stranded DNA appears in the bacterial chromosome that facilitating methylation by MMS and resulting in "error catastrophy" in the triple AB1157 *nfo xth alkB* mutant (Sikora et al., 2010). Analysis of MMS-induced Arg$^+$ revertants in *alkB*$^-$ and *alkB*$^-$BER$^-$ strains clearly points to a mutagenic activity of 1meA and 3meC (Nieminuszczy et al., 2006a; Sikora et al., 2010).

2.2.4. Determination of transcription-coupled DNA repair

The *argE3*→Arg$^+$ reversion system in *E. coli* AB1157 also enables studies on preferential removal of lesions from the transcribed DNA strand. This type of DNA repair, called transcription-coupled repair (TCR), requires Mfd protein that removes transcription elongation complexes stalled at non-coding lesions in DNA and recruits to these sites proteins involved in nucleotide excision repair (NER). TCR occurs under conditions of temporary inhibition of protein synthesis and results in a decrease in the frequency of induced mutations (Selby & Sancar, 1993; Savery, 2007). This phenomenon is called mutation frequency decline (MFD) and was discovered for UV-irradiated bacteria by Evelin Witkin (for review see Witkin, 1994). The MFD phenomenon has been studied by the Janion and Grzesiuk's group on UV (or halogen light)- and MMS-induced Arg$^+$ revertants in the AB1157 strain transiently incubated under non-growth conditions (amino acid starvation) after treatment with a mutagen (Grzesiuk & Janion, 1994; 1996; 1998; Wójcik & Janion, 1997; Fabisiewicz & Janion, 1998; Wrzesiński et al., 2010).

Table 2 shows all the mutagenic targets for UV- and MMS-induced DNA damage. Potential targets for UV-modifications (T-C and T-T sequences for creation of 6-4 photoproducts and pyrimidine dimers, respectively) are underlined. Potential targets (single bases) for MMS-induced modifications are shadowed. UV- or halogen light-induced Arg$^+$ revertants occur mainly as a result of a GC→AT transition forming the *supB* and *supE* ochre suppressors, respectively, at the transcribed DNA strand of the *glnU* and the coding DNA strand of the *glnV* amber (*supE44* amber) gene. In *mfd*$^-$ strains the formation of *supB* predominated over *supE* ochre suppressors and their number, in contrast to the *mfd*$^+$ strain, did not decrease during amino acid starvation. The MFD effect observed in *mfd*$^+$ strains is a reflection of repair of premutagenic lesions in the transcribed strand of the *glnU* gene leading to *supB* suppressor formation (Wójcik & Janion, 1997; Fabisiewicz & Janion, 1998).

Studies on TCR involvement in the repair of MMS-induced lesions have included (i) an analysis of Arg$^+$ revertants, and (ii) examination of plasmid DNA isolated from MMS-treated and transiently starved bacteria for their sensitivity to the Fpg and Nth endonucleases. The decrease in the level of MMS-induced mutations during transient starvation was accompanied by repair of abasic sites in plasmid DNA. As it is shown in Table 2, potential targets for MMS damage are located on both the transcribed and coding DNA strands of *glnU*, *glnV* amber and *argE* genes and only on the transcribed strand of *lys*-tRNA genes. Lesions resulting from methylation of the transcribed DNA strand are subject to MFD repair. Previous studies on the MFD phenomenon after MMS treatment of the AB1157 strain and its derivatives focused on the preferential repair of transcribed-strand lesions of genes coding for *lys*-tRNA; this repair was manifested by a decrease in the number of *supL* suppressors (Grzesiuk & Janion, 1994; 1998). Recent studies revealed a significantly slower and completely absent MFD effect in, respectively, AB1157*mfd* and double *alkB mfd* mutants. It has been assumed that the former effect is the result of action of other DNA repair systems and the latter is a reflection of an accumulation of damage to DNA and induction of SOS response. These results again have confirmed the strong mutagenic effects of 1meA/3meC lesions (Wrzesiński *et al.*, 2010).

Interestingly, in a *dnaQ* mutant no TCR was observed indicating that in this mutant the processes of DNA repair are different, probably due to chronic induction of SOS response and the presence of Pol V and Pol IV DNA repair polymerases induced within SOS regulon (Grzesiuk & Janion, 1996).

DNA		→	tRNA
glnU gene ↓ *supB* suppressor	5′----TT ***TTG***AT----3′ 3′--- AA***AA***CTA----5′ ↓ 5′----TT ***TTA***AT----3′ 3′----AA***AA***TTA----5′	→	5'--UUG--3' ↓ 5'--UUA--3' gln-tRNA$_{CAA}$ - tRNA anticodon for glutamine reading 5'CAA3' codon in mRNA ↓ gln-tRNA$_{UAA}$ - tRNA anticodon reading nonsense *ochre* triplet 5'UAA3' in mRNA
glnV$_{am}$ (*supE44*$_{am}$) suppressor ↓ *supE*$_{OC}$ suppressor	5′----T***CTA***----3′ 3′----A***GAT***----5′ ↓ 5′----T***TTA***----3′ 3′----A***AAT***----5′	→	5'--CUA--3' ↓ 5'--UAA--3' gln-tRNA$_{UAG}$ - tRNA anticodon reading nonsense *amber* triplet 5'UAG3' in mRNA ↓ gln-tRNA$_{UAA}$ - tRNA anticodon reading nonsense *ochre* triplet 5'UAA3' in mRNA
lys-tRNA genes ↓ *supL* suppressor	5′---***T TT***-----3′ 3′--***AAA***----5′ ↓ 5′---***TTA***----3′ 3′--- ***AAT***----5′	→	5'--UUU--3' ↓ 5'--UAA--3' lys-tRNA$_{AAA}$ - tRNA anticodon for lysine reading 5'AAA3' codon in mRNA ↓ lys-tRNA$_{UAA}$ - tRNA anticodon reading nonsense *ochre* triplet 5'UAA3' in mRNA
argE3 mutation in *argE* gene ↓ mutations leading to any sense nucleotide triplet or UAG nonsense codon recognized by *supE44* amber suppressor	5′----TTTAAAT----3′ 3′---AAATTTA---5′	→	No changes in tRNA encoding genes

Table 2. Potential mutagenic targets for UV and MMS modification and mechanisms of mutation creation in *glnU*, *glnV* amber, *lys*-tRNA and *argE* genes, leading to Arg$^+$ phenotype in *E. coli* K-12 AB1157. Nucleotide triplets corresponding to tRNA anticodon in *glnU*, *glnV* amber and *lys*-tRNA genes are in italics. Underlined sequences and shadowed bases show potential sites of photoproducts (6-4 photoproducts and thymine dimers) formation and targets for methylation, respectively.

2.3. The *trpE65* → Trp$^+$ and *tyrA14* → Tyr$^+$ reversion systems in *E. coli* B/r derivatives

It is thought that *E. coli* B is the clonal descendant of a *Bacillus coli* strain used by Felix d'Herelle from the Pasteur Institute in Paris, in his studies performed on bacteriophages almost 100 years ago. *B. coli* was isolated from human feces as a normal commensal of the human gut. *B. coli* was renamed to *E.coli* strain B and published by Delbrück and Luria in 1942 (Delbrück & Luria in 1942). The history of *E.coli* B was excellently presented by Daegelen and co-workers (Daegelen et al., 2009). *E. coli* B/r (B resistant to radiation) is one of the mutants obtained from *E. coli* B after irradiation with UV light by Evelyn Witkin in 1942 (Witkin, 1946). *E. coli* B was found to be very sensitive to UV irradiation due to La protease (Lon protein, product of the *lon* gene) deficiency. *E. coli* B/r strain owns its UV-resistance to *sulA* mutation (Studier et al., 2009). SulA protein is synthesized in bacterial cell during the SOS response induction and is a substrate for the Lon protease (Goldberg et al., 1994).

Reversions of *trpE65* to Trp$^+$ phenotype and *tyrA14* to Tyr$^+$ in *E. coli* B/r WP2 (Ohta *et al*., 2002) and WU3610 derivatives (Bockrath *et al*., 1987), respectively, are analogous to *E.coli* K12 AB1157 mutation detection systems. Both *trpE65* and *tyrA14* are ochre mutations in genes coding for enzymes involved in tryptophane and tyrosine biosynthesis, respectively. The Trp$^+$ or Tyr$^+$ phenotype may be recovered by (i) any point mutation at *trpE65* or *tyrA14* leading to the formation of any sense nucleotide triplet, and (ii) ochre suppressor mutations. In the WP2 (*trpE65*) system the examined suppressors are *supB*, *supC*, *supG* and *supM*, formed in the genes coding for tRNA: *glnU*, *tyrT*, *lysT* and *tyrU*, respectively (Ohta *et al*., 2002). In the WU3610 (*tyrA14*) strain *de novo* ochre suppressor mutations in glutamine tRNA are studied. The WU3610-11 derivative bears an amber suppressor created from another glutamine tRNA gene that can be converted to an ochre suppressor (Bockrath and Palmer, 1977; Bockrath *et al*., 1987). Both systems have been used in MFD studies (Bridges *et al*., 1967; George & Witkin, 1974; Bockrath and Palmer, 1977; Bockrath *et al*., 1987).

Besides *E. coli* WP2 strain, its derivatives are widely used: WP2 carrying pKM101 plasmid from *S.typhimurium*, WP2 *uvrA* mutant and WP2 *uvrA* bearing pKM101. *E. coli* WP2 and its derivatives are recommended to be used in conjunction with Ames *S.typhimurium* tester strains to screen various compounds for mutagenic activity (Mortelmans & Riccio, 2000), (see chapter 2.8).

2.4. Lac$^+$ reversion system for determination of base substitutions and frameshift mutations

A commonly used and convenient *E. coli* K-12 *lacZ*→Lac$^+$ reversion system allows rapid detection of specificity of mutation. The β-galactosidase encoding *lacZ* gene is a part of the lactose operon. Mutants in *lacZ* gene are unable to grow on a medium containing lactose as the sole carbon source. A set of 11 mutants (*E.coli* K12 CC101-111 strains) with a *lacZ* deletion in the chromosome and F′ episome with cloned *lacZ* gene bearing defined mutations (six base substitutions in CC101-106 strains, and five frame shift mutations in CC107-111 strains) have been constructed (Coupples and Miller, 1989; Coupples et al., 1990)

Glutamine at 461 position is essential for β-galactosidase activity. In CC101-CC106 strains coding position 461 was changed. Reversion to the Lac$^+$ phenotype is due to a specific base substitution at 461 position restoring the glutamic acid codon. CC107-CC111 carry mutations in the *lacZ* gene that revert to Lac$^+$ *via* specific frameshifts. The altered sequences contain monotonous runs of each of the four bases or a run of –G-C- sequences on one strand. Addition or loss of a single base pair or loss of –G-C- sequence lead to reversion of the *lacZ* mutation. In the case of CC101-CC111 strains the marker is episomal, in contrast to e.g. the AB1157 strain where the marker is situated on the chromosome. Figure 3 presents the idea of the CC101-CC106 and CC107-CC111 strains construction. The *lacZ*→Lac$^+$ reversion system in *E.coli* K12 CC101-111 strains is very handy and used all over the world for studying specificity of mutations in genes under investigation.

A.

Glu-461

TGG TCG CTG GGG AAT GAA TCA GGC CAC GCC GCT AAT CAC GAC GCG CTG TAT CGC TGG ATC AAA TCT CTC GAT CCT TCC CGC CCG GTG CAG TAT GAA GGC GGC GGA GCC GAC ACC ACG GCC ACC GAT ATT ATT TGC CCG ATG TAC GCG CGC GTG GAT GAA GAC CAG CCC TTC CCG GCT GTG CCG AAA TGG TCC ATC AAA AAA TGG CTT

B.

	Codon at position 461 in *lacZ* gene	Mutation leading to Lac⁺ phenotype
wild type Lac⁺	-GAG-	-
CC101	-TAG-	AT → CG
CC102	-GGG-	GC → AT
CC103	-CAG-	GC → CG
CC104	-GCG-	GC → TA
CC105	-GTG-	AT → TA
CC106	-AAG-	AT → GC

C.

	The wild type and altered sequence	Mutation leading to Lac⁺ phenotype
wild type Lac⁺	GGC GGC GGA GCC	
CC107	GGC GGG GGG CC	+ 1 G
wild type Lac⁺	GGC GGC GGA GCC	
CC108	GGG GGG CGG AGC C	- 1 G
wild type Lac⁺	TAC GCG CGC GTG	
CC109	TAC GCG CGC GCG TG	- 2 CG
wild type Lac⁺	ATC AAA AAA TGG	
CC110	ATA AAA AAT GG	+ 1 A
wild type Lac⁺	ATC AAA AAA TGG	
CC111	ATC AAA AAA ATG G	- 1 A

Figure 3. The idea of the *E. coli* CC101-CC106 and CC107-CC111 strains construction. **A.** A fragment of the *lacZ* sequence - the underlined sequences have been altered to create the tester strains: Glu-461 to yield strains CC101-CC106, the remaining sequences rich in –G-, -C-G- and A, respectively, to yield strains CC107-CC111. **B**. Altered codon at position 461 in *lacZ* gene in six different strains (CC101-CC106) and base substitutions recovering the Lac⁺ phenotype. **C.** Altered sequences in *lacZ* gene in five strains (CC107-CC111) and frameshifts recovering the Lac⁺ phenotype.

Fijałkowska and Shaaper with colleagues constructed a series of *lacZ* strains allowing studies on replication fidelity based on analysis of frequency of Lac⁺ revertans. The entire *lacIZYA* operon from F′*pro lac* plasmid of Coupples and Miller's strains containing specific *lacZ* mutation has been inserted into the chromosome of the *E. coli* MC4100 (*lac*⁻) in two possible orientations with regard to the chromosomal replication origin *oriC*. This system

enables investigation of frequencies of base pair substitutions and frame-shift mutations. It has been used to show that during chromosomal DNA replication in *E. coli* two DNA strands, the leading and the lagging, are replicated with different accuracy in *w.t.* as well as in various mutants in genes involved in replication or DNA repair (Fijałkowska et al., 1998; Maliszewska-Tkaczyk et al., 2000; Gaweł et al., 2002).

2.5. Forward mutation system with the use of *E. coli lacI* strain

A forward mutational system, in contrary to reversion systems, monitors the mutation of a wild type gene. The *E.coli lacI* nonsense system described by Miller and Coulondre (Coulondre & Miller, 1977; Miller, 1983) is a forward system playing an important role in the examination of specificity of numerous mutagens. The *lacI* gene encodes the repressor of *lac* operon required to metabolize lactose. The base of *lacI* system is the analysis of nonsense mutations in the *lacI* gene (present on an F′ episome). The system also involves several techniques to identify each nonsense mutation. There are over 80 sites within *lacI* gene where a nonsense mutation can arise by a single base change. Nonsense mutations constitute 20-30% of all mutations induced by many mutagens. Since the *lacI* gene encodes the repressor of the *lac* operon, *E.coli lacI*$^-$ cells express the operon constitutively. In this way *lacI* mutants can be selected on the plates containing phenyl-β-galactosidase (a lactose analog) as the only source of carbon. These mutants can metabolize the analog but cannot induce the operon. Further mutant analysis involves ability to be suppressed by various tRNA suppressors and subsequent genetic analyses.

The distribution of *lacI* mutations can be arranged according to base substitution generated by each nonsense mutation, creating a map of mutational hot and cold spots, for places where the number of mutations exceeds or is smaller, respectively, in comparison to other sites. Created map of mutational spectra is characteristic for different mutagens. The *lacI* nonsense system shows limitations of detecting only base substitution mutations and not detecting AT→GC transitions. Nevertheless, it became possible to determine the nature of the *lacI* mutations directly by DNA sequencing.

2.6. The *trpA* → Trp$^+$ reversion system in *E.coli* K12

An important approach to determine mutagen specificity based on reversion of an auxotrophic *trpA* mutation to the Trp$^+$ phenotype in *E. coli* K12 was developed by Yanofsky and co-workers (Berger et al., 1968). The *trpA* gene is a part of the *trp* operon and codes for the tryptophan synthetase α chain in *E.coli*. There is a set of the *trpA* alleles that enable to monitor all possible base substitutions (*trp88, trp46, trp23, trp3, trp223, trp58, trp78, trp11, trp446*) and frame shifts (*trpE9777, trpA21, trpA540, trpA9813* alleles) and allow studying mutagen specificity. The Trp$^+$ revertants are divided into classes based upon colony size and two physiological tests: 5-methyl tryptophan (5-MT) sensitivity and indole glycerol phosphate (IGP) accumulation. Moreover, full Trp$^+$ revertants (FR) and partial Trp$^+$ revertants (PR) are distinguished. The PR group is divided into 3 more classes: PRI, PRII, PRIII. To enhance the frequiences of spontaneous and induced Trp$^+$ revertants pKM101

plasmid from *S. typhimurium* (described in chapter 2.8) was introduced to *E. coli trp*⁻ strains by conjugation (Fowler et al., 1979; McGinty & Fowler, 1982; Persing et al., 1981; Fowler & McGinty, 1981). Table 3 shows characterization of UV-induced Trp⁺ revertants.

***trpA* allele**	**Revertant class**	**5-MT sensitivity**	**IGP accumulation**	**Inferred base-pair substitutions**
3	I FR PR	R S	- +	AT → TA -
11	I FR PR	R S	- +	GC → CG - AT → TA AT→ GC
23	I FR II PR III PR IVPR	R S S S	- + + +	AT → CG GC → CG CG → AT -
46	I FR II PR III PR	R S S	- + +	AT → GC AT → CG AT → TA -
58	I FR II PR III PR	R S S	- + +	AT → GC AT → CG GC → AT
78	I FR PR	R S	- +	AT → CG -
88	I FR PR	R S	- +	AT → CG -
223	I FR PR	R S	- +	AT → GC AT → CG -

Table 3. The characterization of UV-induced *trpA* base pair substitutions based on Fowler et al., 1981

2.7. Adaptive mutations

Adaptive mutations (also called "directed", "stationary phase" or "starvation associated") are a special kind of spontaneous mutations that occur in non-dividing or slowly-growing stationary-phase cells. Mutations of this type are detectable after exposure to a non-lethal selection and allow growth under these conditions.

As a tool for studying stationary phase mutations, *lacI33* →Lac$^+$ reversion has been used. The *lacI33* marker (+1 G frame shift) is carried on the F'sex plasmid in the FC40 *E.coli* K12 strain and its derivatives. The reversion to Lac$^+$ phenotype is due to a -1G frameshift mutation (Foster and Trimarchi, 1995; Rosenberg et al., 1994). The *lacI33* →Lac$^+$ reversion, if plasmid born, depends on *recABC* encoded proteins (Foster and Trimarchi, 1994). When *lacI33* mutation is localized on the chromosome it reverts adaptively at a much lower rate and the event is *recA* independent.

The systems searching for stationary phase mutations operating on bacterial chromosomal loci use reversion to prototrophy of auxotrophic *E.coli* strains. Prototrophic revertants are able to grow on minimal plates lacking required compounds. These systems often use reversions in genes such as *trpE* (Bridges, 1993) or *tyrA* (Bridges, 1996) in *E.coli* B/r (see chapter 2.3) and *trp* operon in *E.coli* K12 (Hall, 1990).

The *argE3*→Arg$^+$ reversion system in *E. coli* K12 AB1157 strain can be also used for selection bacteria mutated adaptively on minimal plates lacking arginine. Colonies of stationary phase mutations appear on these plates after four and more days of incubation at 37^{o}C. Further phenotypic analysis and susceptibility to a set of amber and ochre T4 phages allowed the identification of stationary phase mutations in AB1157 *mutY*$^-$ strain defective in the ability to remove adenine from A-8-oxo-G and A-G mispairing (Nowosielska and Grzesiuk, 2000), and in AB1157*dnaQ*$^-$ mutated in proofreading subunit of *E.coli*, main replicative DNA polymerase PolIII (Nowosielska et al., 2004a; 2004b). It has been shown that in the *dnaQ*$^-$ strain two repair polymerases, PolIV and PolV, influence the frequency and specificity of starvation-associated mutations.

In bacteria there is no single mechanism for the generation of stationary-phase mutations. Under starvation conditions mutations can arise as a result of oxidative and other DNA damage, errors occurring during DNA replication, defects or inefficiency of DNA repair systems but also DNA repair synthesis by itself may be a source of mutagenesis under conditions restricted for growth. *Pseudomonas* possesses a different set of specialized DNA polymerases compared with enetrobacteria, also its DNA repair systems involved in stationary-phase mutagenesis differ. To study stationary-phase mutagenesis in *Pseudomonas* the *pheA*→Phe$^+$ reversion system described in chapter 2.9. is used the most.

2.8. The Ames test with the use of *Salmonella* strains

The *Salmonella* mutagenicity assay was introduced by Bruce Ames and co-workers in the early 1970s, later modified (Ames *et al.*, 1975) and constantly improved. The test involves reversion of histidine auxotrophs of *Salmonella enterica* serovar Typhimurium mutation to

prototrophy by base substitutions in the *hisG46* allele or by frame-shift in the *hisD3052* allele. In addition, the tester strains carry: (i) additional mutations, such as *rfa*, increasing the permeability of the bacterial cell wall and enabling better penetration of mutagenic agents to the cell, or *uvrB*, disturbing DNA repair; (ii) plasmid pKM101 – a mutagenesis-enhancing plasmid bearing *mucA* and *mucB* genes that code for proteins corresponding to *E.coli* UmuC and UmuD encoding SOS-induced, repair polymerase V (PolV), responsible for translesion synthesis (TLS) (Mortelmans, 2006).

Ames *Salmonella* test has been developed to screen chemicals for their potential mutagenicity and genotoxicity. It is used routinely as a screening assay to predict animal carcinogens. Since many compounds show genotoxicity only after enzymatic conversion to active form, a method was discovered to imitate mammalian metabolism in a bacterial system by adding an extract of rat liver. Rats are first injected with a polychlorinated biphenyl (PCB) mixture, Aroclor 1254, inducing expression of enzymes involved in activation of chemicals. The livers of these rats are homogenized, centrifuged, and supernatans (microsomal fraction), termed S9 mix, are collected. Test method involves histidine auxotroph *S. typhimurium* TA98 for testing frameshift mutations and TA100 for testing base-pair substitutions (several other strains have also been constructed). Cultures of these strains are mixed with a chemical tested, incubated in the presence (or absence) of S9 mix, and plated on solid minimal medium lacking histidine. A two-day incubation allows His^+ revertants to form colonies on minimal plates. The frequency of His^+ revertants indicates mutagenic potency of tested chemical (Figure 4).

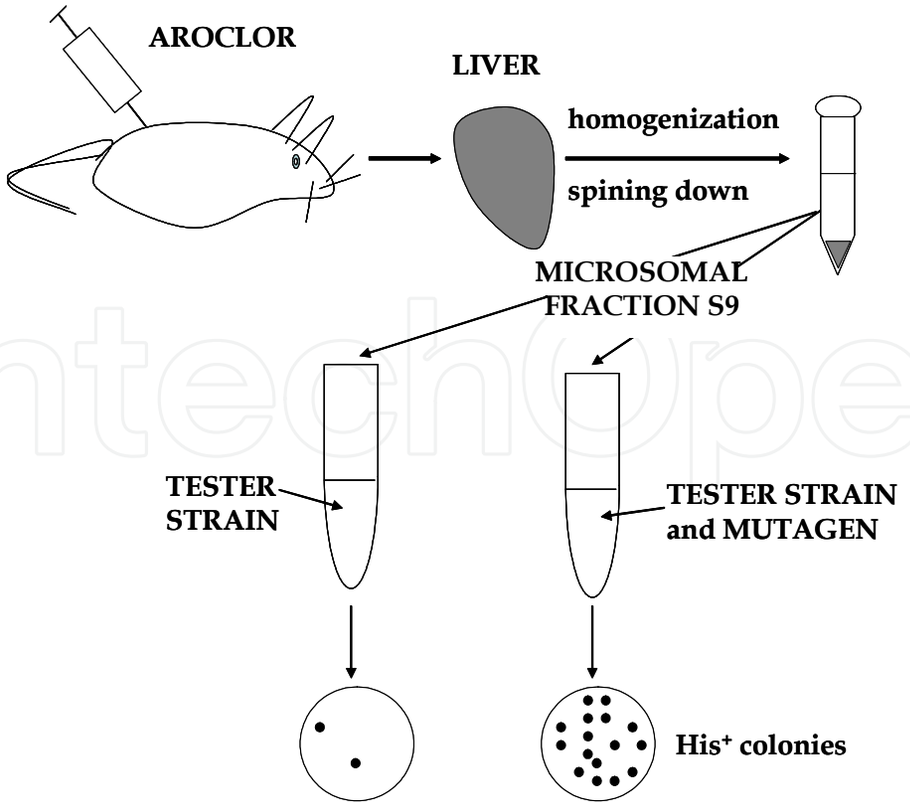


Figure 4. An idea of Ames test – description in the text.

The following *S. typhimurium* strains are recommended for general mutagenesis: TA97 (*hisD6610*, *rfa*, Δ*uvrB*, +R), TA98 (*hisD3052*, *rfa*, Δ*uvrB*, +R), TA100 (*hisG46*, *rfa*, del*uvrB*, +R), and TA102. The last strain, except the plasmid pKM101bearing R-factor, contains multicopy plasmid pAQ1 carrying the *hisG428* mutation and a tetracycline resistance gene. Strains containing R-factor are much better tester strains for a number of mutagenes that weakly or not at all revert strains devoid of pKM101plasmid. In *E.coli* and *S. typhimurium* the presence of pKM101 increases mutagenesis by inducing SOS response leading to error-prone DNA repair.

The *hisG* gene encodes for the first enzyme of histidine biosynthesis. Mutation in this gene, *hisG46*, present in TA1535 strain and its R-factor$^+$ derivative, TA100, substitutes $\frac{-GGG-}{-CCC-}$ (proline) for $\frac{-GAG-}{-CTC-}$ (leucine). Both strains serve to detect mutagens that cause base-pair substitutions. Mutation in *hisD* gene, *hisD3052*, present in TA1538 strain and its R-factor$^+$ derivative, TA98 detect various frameshift mutagens. Frameshift mutagens can stabilize the shifted pairing occurring in repetitive sequences or hot spots resulting in frameshift mutation which restores the correct for histidine synthesis reading frame. The *hisD3052* mutation has 8 repetitive –GC- residues next to -1 frameshift in the *hisD* gene. This mutation is reverted by such mutagens as 2-nitrosofluorene and daunomycin.

The Ames II assay is a liquid microtiter modification of the Ames test. It involves new set of *S. typhimurium* strains TA7001-6 (Gee *et al*., 1994) and also a mixture of these strains called TAMix (Fluckiger-Isler *et al*., 2004). Table 4 contains genotypes of mentioned above strains, and mutation specificity of indicated mutagens.

Strain	Genotypes	Mutagen	Mutation detected
TA98	*hisD3052* Δ*ara9* Δ*chl008* (*bio chl uvrb gal*) *rfa1004*/pKM101		frameshifts
TA7001	*hisG1775* Δ*ara9* Δ*chl004* (*bio chlD uvrb chlA*) *galE503 rfa1041*/pKM101	STN, N4AC	A:T → G:C
TA7002	*hisG9138* Δ*ara9* Δ*chl004* (*bio chlD uvrb chlA*) *galE503 rfa1041*/pKM101	STN, MMS	T:A → A:T
TA7003	*hisG9074* Δ*ara9* Δ*chl004* (*bio chlD uvrb chlA*) *galE503 rfa1041*/pKM101	STN, ANG/UVA	T:A → G:C
TA7004	*hisG9133* Δ*ara9* Δ*chl004* (*bio chlD uvrb chlA*) *galE503 rfa1041*/pKM101	NQNO, MNNG	G:C → A:T
TA7005	*hisG9130* Δ*ara9* Δ*chl004* (*bio chlD uvrb chlA*) *galE503 rfa1041*/pKM101	NQNO, MMS	C:G → A:T
TA7006	*hisG9070* Δ*ara9* Δ*chl004* (*bio chlD uvrb chlA*) *galE503 rfa1041*/pKM101	NQNO, 5azaC	C:G → G:C

Table 4. Detection of mutation specificity of selected mutagen with the use of indicated *S. typhimurium* strains. STN, streptonigrin; N4AC, N^4-aminocytidine; MMS, methyl methanesulfonate; ANG, angelicin; NQNO, 4-nitroquinoline-1-oxide; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; 5azaC, 5-azacytidine.

As mentioned in chapter 2.3, *E. coli* B/r WP2 system complements the *Salmonella* assay and both strains should be used in combination when potential mutagens are examined. It has appeared that some chemicals such as nitrofurans are nonmutagenic in the *Salmonella* strains, whereas caused mutation in *E. coli* WP2 and in human cell cultures. The procedures described for the Ames *Salmonella* assay and *E. coli* WP2 tryptophan reverse mutation test are similar (Mortelmans & Riccio, 2000).

In *S. typhimurium* an another test for mutation detection was developed. It includes examination of forward mutations leading to resistance to the purine analog 8-azaguanine. The mutants are unable to convert enzymatically 8-azaguanine to the toxic metabolite. Base-pair mutations, frameshift mutations and deletions in different genes are expected to render 8-azaguanine resistance (Skopek et al., 1978).

2.9. Test system to study mutations in *Pseudomonas putida*

The genus *Pseudomonas* constitutes a large and diverse group of mostly saprophytic bacteria occurring in soil, water, plants and animals. They are known for ability to metabolize toxins, antibiotics, organic solvents and heavy metals present in environment, thus playing an important role in the development of soil microorganism community. The metabolic versatility of these organisms has been used to degrade waste products (bioremediation) and to synthesize added-value chemicals (biocatalysis) (Pieper and Reineke, 2000; Wackett *et al.*, 2002).

There is only a limited number of test systems that allow studying mutagenic processes in *Pseudomonas*. Rifampicine (Rif) resistance is used as one out of two such systems. The antibacterial action of Rif bases on binding to β subunit of RNA polymerase (RNA pol) and blocking the RNA elongation. Rifampicine resistance (Rif ^{r}) mutants harbor substitutions in β subunit of RNA pol that either make direct contacts with Rif or are located near the binding pocket (Campbell *et al.*, 2001). Mutations decrease the binding of Rif to RNA pol, making the enzyme, to different degree, not sensitive to the antibiotic (Jin and Gross, 1988). Except for few mutations located at the 5' end, other Rif ^{r} mutations in *E.coli* are found in three clusters in a central region of *rpoB* gene. Sequence analysis in the *rpoB* region harboring Rif ^{r} mutations indicate a high level of conservation among prokaryotes (Campbell *et al.*, 2001). For that reason *rpoB*/Rif ^{r} system can be used as a mutation testing system in distinct bacterial species including *Pseudomonas*.

Using *rpoB*/Rif ^{r} system it has been found that isolated mutants of *P. putida* and *P. aeruginosa* express different levels of resistance to Rif, depending on the localization of mutations in the *rpoB* sequence (Jatsenko *et al.*, 2009). The spectrum of mutations strongly depends on temperature of growth. Thus, the usage of the same growth temperature is very important in mutation research in *Pseudomonas* while employing the *rpoB*/Rif ^{r} system.

Except *rpoB*/Rif ^{r} system, phenol utilization as growth substrate has been used to measure different types of point mutations in *P. putida*. The presence of *pheA* gene encoding phenol monooxygenase enables bacteria to utilize phenol as growth substrate and form colonies on

selective plates. The reporter gene *pheA* was modified in RSF1010-derived plasmids by +1 frameshift mutation (Saumaa *et al.*, 2007). Assay system that allows base substitution detection uses two steps PCR technique. Mutant oligonucleotides contained specific base substitutions replacing the CTG for Leu-22 in the *pheA* gene with a TGA, TAA, or TAG stop codons. In the first step, the PCR with oligonucleotides pheAup and pheA22TGA, pheA22TAA or pheA22TAG, complementary to the positions 42 and 71 relative to the coding sequence of the *pheA* gene was performed. After treatment with restriction enzyme ExoI, PCR products were purified and used in a second PCR with the oligonucleotide pheAts complementary to *pheA* nucleotides 295 to 313. The amplified DNA fragments were cloned into the EcoRV site of pBluscript KS (+) (the mutations were verified by DNA sequencing). As a result, plasmids: pKTpheA22TGA, pKTpheA22TAA, and pKTpheA22TAG were obtained (Tegowa *et al.*, 2004).

2.10. *Bacillus subtilis* as a model for mutation detection

Bacillus subtilis is a Gram-positive bacterium commonly occuring in soil. It shows the ability to form endospores allowing the organism to tolerate extreme environmental conditions. The sporulation of *B. subtilis* provides a system for the detection of forward mutations in many genes whose products are responsible for spore formation. Mutants form non-sporulating or oligosporogenous colonies lacking brown pigment presented in normally sporulating cells. Using this mutation detection system mutagenic activity of many compounds, e.g. acridine orange, acriflavin, nitrous acid, 2-nitrosofluorene, nitrogen mustard, aflatoxin B1, 4-nitroquinoline-*N*-oxide, ethidium bromide, have been shown (Macgregor & Sacks, 1976).

B. subtilis HA101 strain and its derivatives (e.g. excision-repair deficient derivative, TKJ5211 strain) are other mutagen-tester bacteria. They carry suppressible nonsense mutations in *his* and *met* genes. The presence of suppresors is detected by examination of sensitivity of His^+ and Met^+ revertants to tester SPO1 phages (sus-5 and sus-11). This system has been used to examine mutagenic properties of various compounds, e.g. nitrofurazone, 4-nitroquinoline 1-oxide (4NQO), 4-aminoquinoline 1-oxide (4AQO), α- and β- naphthylamine (Tanooka, 1977) or triethanolamine (Hoshino & Tanooka, 1978).

2.11. Resistance of bacteria to antibiotics

Antibiotic resistance to streptomycin, rifampicin or nalidixic acid is often used for determination of spontaneous and induced mutagenesis in bacteria. It is a universal, rapid and simple test used in many species. The frequency of an antibiotic resistant bacteria usually is determined on plates supplemented with respective antibiotic. Rifampicin-, streptomycin- and nalidixic acid-resistant mutants arise due to spontaneous or induced mutations in chromosomal DNA. The mechanisms leading to rifampicin resistance was described in chapter 2.9. Resistance to streptomycin involves mutational changes in the 30S subunit of the ribosome, whereas resistance to nalidixic acid results from point mutations in structural genes encoding gyrase (topoisomerase II) subunit A (*gyrA E. coli*) or topoisomerase IV (*parC E. coli*). Further analysis of antibiotic resistant mutants is also possible, e.g., by sequencing of *gyrA*

gene from nalidixic acid-resistant mutants of *B. subtilis* spores (Munakata et al., 1997). Genes responsible for resistance to ampiciline, tetracycline, carbenicyline, chloramphenicol, spectinomycine are usually harbored by plasmids or transposons and used as markers of new features/mutations introduced to the bacterial strains.

Resistance to other agents such as mentioned 8-azaguanine (see chapter 2.8) or phages, e.g. T1 phages, has been also used in mutagenesis tests, particularly in the 20th century studies. Resistance to T1 is due to *tonA* or *tonB* mutation (Miller, 1972).

3. Conclusions

Living organisms are continuously exposed to damaging agents both from the environment and from endogenous metabolic processes, whose action results in modification of proteins, lipids, carbohydrates and nucleic acids. The knowledge on DNA modifications leading to mutations is critical to our understanding of how and why the genome is affected during the lifespan of the organism, and how the DNA repair systems efficiently work *via* several different pathways. Bacterial systems for testing mutations are informative, cheap, and quick methods to study these processes. Connected with modern molecular biology methods, such as sequencing, RT PCR, side directed mutagenesis etc., bacterial systems constitute extremely valuable tools for studying metabolic processes, DNA repair systems, mutagenic and anticancer properties of chemicals etc. Two of the systems described here are of special value: *lacZ* $^{-}\rightarrow$Lac^{+} reversion system for determination the specificity of mutations and Ames test for studying mutagenic properties of chemicals. The latter, improved constantly e.g. by construction of new indicator strains and modern techniques, is extremely important in searching for new anticancer drugs.

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Acknowledgement

We thank to the Editorial Board of *Acta Biochimica Polonica* for permission to published parts of the article entilted Reversion of *argE3* → Arg^{+} in *Escherichia coli* AB1157 – a simple and informative bacterial system for mutation detection by Anna Sikora and Elzbieta Grzesiuk (*Acta Biochimica Polonica,* 2010, 57: 479-485).

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