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QUARTERLY

Reversion of *argE3* ochre strain *Escherichia coli* AB1157 as a tool for studying the stationary-phase (adaptive) mutations

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Adaptive (starvation-associated) mutations occur in non-dividing cells and allow growth under the selective conditions imposed. We developed a new method for the determination of adaptive mutations in *Escherichia coli*. The system involves reversion to prototrophy of the $argE3_{OC}$ mutation and was tested on AB1157 strains mutated in the *mutT* and/or *mutY* genes. The bacteria that mutated adaptively grow into colonies on minimal medium plates devoid of arginine (starvation conditions) when incubated longer than 4 days. Using the replica plating method we solved the problem of discrimination between growth-dependent and adaptive *argE3*→Arg^+ revertants. Phenotype analysis and susceptibility of the Arg^+ revertants to a set of T4 phage mutants create an additional possibility to draw a distinction between these two types of Arg^+ revertants.

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 have been found only in genes whose functions were selected for (e.g. [1–5]). The last part of this definition is rather controversial especially as one of the most extensively used experimental systems for the investigation of adaptive mutations has recently been shown not to be strictly adaptive because, by using appropriate methods, mutations in non selected loci could also be found [6]. This system uses the reversion of the *lacI33*-1 frameshift mutation carried on the F' sex plasmid in the FC40 strain of *Escherichia coli* [7, 8]. The *lacI33*→Lac^+ reversion, if plasmid borne, depends on *recABC* genes [9]. When *lacI33* mutation is localized on the chromosome it reverts adaptively at a much lower rate and the event is *recA* independent. This means that the system is a special case which

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is related to plasmid metabolism rather than to chromosomal DNA.

The systems operating on bacterial chromosomal loci use reversion to prototrophy of auxotrophic *E. coli* strains. Prototrophic revertants are able to grow on minimal medium plates lacking the previously required components (e.g. [10]). Adaptive mutations have been reported, i.e., for *trpA, trpB* [4], *trpE* [10] and *tyrA* [11, 12].

In this report we present a new system for adaptive mutation investigation in *E. coli,* the $argE3_{OC} \rightarrow Arg^+$ reversion to prototrophy, supplemented with the replica plating. This replica plating method described by Miller [13] was applied for a new purpose, namely, the discrimination between colonies which grew on selective plates as a result of creation of growth-dependent mutations, and those formed after prolonged starvation in the absence of regular DNA replication. An additional advantage of the system, verified on two *E. coli* mutants: *mutT* and *mutY*, is the simple way of estimation of the specificity of mutations.

MATERIALS AND METHODS

Bacterial strains. All bacteria were derivatives of *E. coli* K12 strain AB1157 (genotype: F^- *thr-1 leuB6 proA2 his4 thi1 argE3 lacY1 galK2 rpsL supE44 ara-14 xyl-15 mtl-1, txs-33* [14]). AB1157*mutT* was obtained by P1 transduction of *leu*::Tn*10 mutT* from JM105*mutT;* AB1157*mutY* by transduction of *mutY68::*miniTn*10* from BH980. Bacterial donors of *mutT* and *mutY* were from Drs. S. Boiteux (Dept. de Radiobiologie et Radiopathologie, France) and J. Laval (Institut Gustave Roussy, France), respectively. The double mutant AB1157*mutTmutY* was constructed by A. Wójcik [15].

Mutation experiments. Bacteria were grown overnight at 37°C with shaking in E medium (E medium consisted of C-salts [16] supplemented with glucose (0.5%), casamino acids (0.2%), Thi (10 μg/ml) and arginine, histidine, threonine, proline and leucine at 25 μg/ml each), centrifuged and resuspended in C-salts. Aliquots, at $3–4 \times 10^8$ and $3–4 \times 10^7$ cells/plate (the number of viable bacteria was estimated by dilution and plating on LB medium solidified with 1.5% Difco Bacto agar) were plated onto E–Arg (E minus arginine) solid medium. Plates were sealed with Parafilm and incubated at 37°C. Arg^+ colonies were counted first after 48 h and then their number was checked each day up to 10 days. The number of viable cells on starvation plates was determined as follows: the colonies which appeared on E–Arg plates were removed with a cork borer and the bacteria were washed off with 5 ml of E medium, diluted and plated on LB-agar plates. Arg^+ revertants were analyzed as described before [17, 18]. Briefly, 100 colonies were tested for arginine, histidine and threonine requirements, and 50 colonies from class I, Arg^+ His^- $Thr^{-/+}$ and class II, Arg^+ His^+ $Thr^{-/+}$ were tested for their susceptibility to a set of amber and ochre T4 phages (Table 1) to identify tRNA suppressors and deduce the specificity of mutations

Replica plating was a modification of the method described by Miller [13]. Overnight cultures of AB1157 strains were plated on E–Arg plates devoid of arginine (master plates) and incubated at 37°C for 2 days, the time necessary for growth-dependent Arg^+ revertants to grow into colonies on minimal medium plates. After 2 days three or four E^-Arg replica plates were prepared from each master plate (for replica plates a replicating block covered with velvet was used). These plates were incubated for 3 days. The distribution of the Arg^+ growth-dependent colonies was identical on the master and replica plates because a large number of non-starved, vigorously replicating cells were transferred from the colonies already existing on a master plate to replica plates. In addition to these colonies, single cells were transferred through the vel-

vet to replica plates but each of the cells has mutated at different time under starvation conditions and, as a result, the bacteria which had mutated adaptively grew on each plate in different places.

RESULTS AND DISCUSSION

A method for the discrimination between growth-dependent and adaptive Arg$^+$ revertants

We used the replica plate test to distinguish between growth-dependent and adaptive colonies. Figure 1 presents a picture of a master plate (after 2 and 5 days of incubation at 37°C) and three replica plates. The master plate contained 10^7 cells of *E. coli* strain AB1157*mutT*. On the plate photographed after two days of incubation only growth-dependent Arg$^+$ colonies can be seen, but after the next 3 days of incubation additional, small colonies appeared both on the master plate and on the replica plates. Large, growth-dependent colonies formed exactly the same pattern on all plates (the circles in Fig. 1 surround three growth-dependent colonies on every plate). Small colonies which appeared after the 5^{th} day of plate incubation were distributed in a different way on the master and replica plates (the circle in Fig. 1C shows three colonies which did not appear on the remaining plates). This can be explained as follows: during the slow process of DNA turnover individual cells randomly distributed on each plate mutate in the *argE* gene which allows them to form Arg$^+$ colonies. Since the adaptive *argE3*→Arg$^+$ reversions arise independently on each replica plate the "adaptive" colonies are located on each plate in different places. This test seems to be especially useful in the case of slow growing growth-dependent colonies, which, unlike the adaptive ones, were pre-formed on the master plate and consequently create an identical pattern on all replica plates.

Adaptive *argE3*$_{OC}$→Arg$^+$ reversion in *mutT* and *mutY* strains

In this study we used *argE3*→Arg$^+$ reversion to prototrophy as an indicator of the spontaneous mutagenesis rate under starvation conditions and two other reversions: *hisG4*→His$^+$ and *thr*$^-$→Thr$^+$ to estimate the adaptive mutation specificity.

To test the system on a higher number of revertants, additional mutations increasing the level of spontaneous reversions had to be introduced into the bacterial chromosome, because the level of spontaneous Arg$^+$ reversions in the AB1157 strain is very low (3–5 colonies/10^8 cells). In the system of reversion to prototrophy of *tyrA14* [11], *trpA23* [12] or *trpE65* [19], mutations in *mutT* or *mutY* genes lead to an elevated mutation rate in starved bacteria. The product of the *mutT* gene, the MutT protein, hydrolyses 8-oxo-dGTP, generated by active oxygen species, to the nucleoside diphosphate, thus cleansing the triphosphate pool of this contaminant [20]. The lack of the MutT protein leads to a high rate of spontaneous mutations. The MutY protein is an adenine DNA-glycosylase that removes adenine from an A:8-oxoG pair. 8-OxoG is a DNA lesion resulting from attack by various oxidizing species and its presence leads to GC→TA transversions by directing the incorporation of adenine [21].

For the investigation of adaptive mutations, AB1157 bacteria from an overnight culture were plated on minimal medium plates lacking arginine (E–Arg). The Arg$^+$ colonies started to appear after 2 days of incubation at 37°C. There were not more than 2–3 colonies at the beginning of the experiment and 14–20 at the end of the 10-day period (Fig. 2). The presence of *mutT* or *mutY* mutations increased the number of growth-dependent as well as adaptive Arg$^+$ revertants.

Figure 3 shows the effect of the *mutT* mutation on the appearance of the *argE3*$_{OC}$→Arg$^+$ revertants during 10 days of incubation under starvation conditions. AB1157*mutT* bacteria

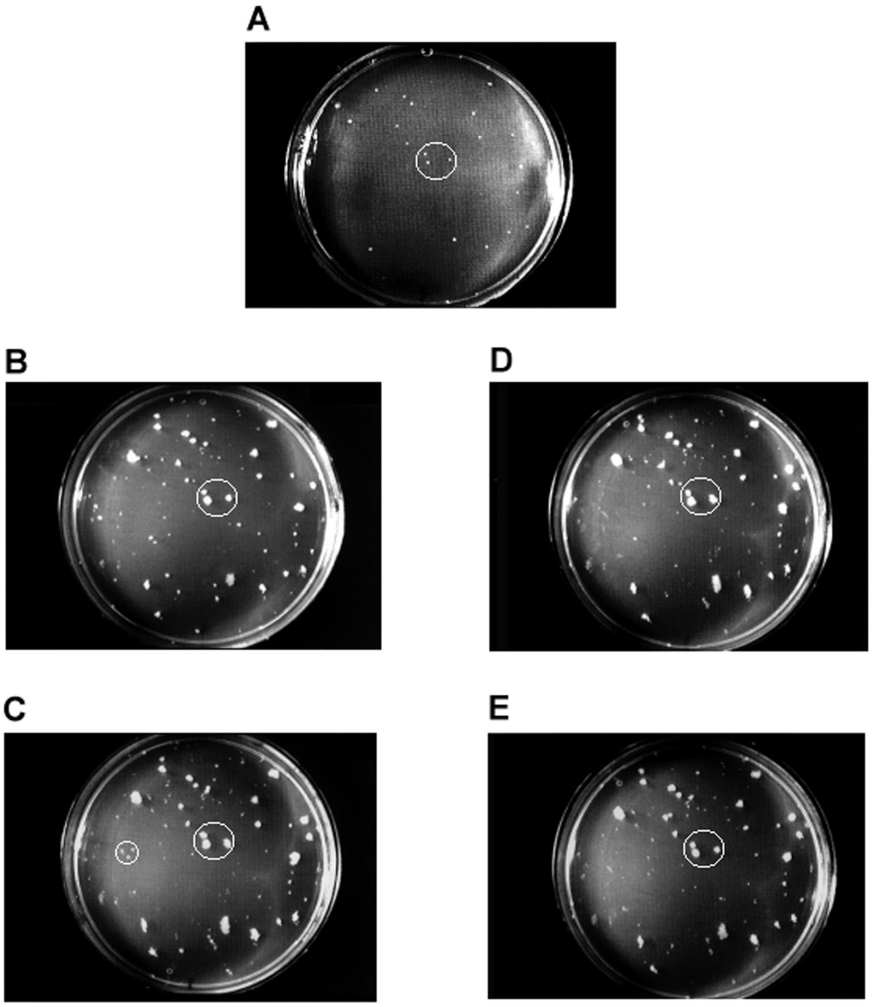

Figure 1. Replica plate test on AB1157*mutT* strain.

A, master plate after 2 days of incubation at 37°C. B, master plate after 5 days of incubation at 37°C. C, D, E, replica plates after 3 days of incubation at 37°C. The 3 colonies surrounded by a circle on each plate are an example of Arg^+ growth-dependent revertants. The 3 colonies in a smaller circle on plate C are an example of Arg^+ adaptive revertants.

were plated at two densities: 10^8 and 10^7 cells/plate. In resting cells under selection conditions Arg^+ revertants appeared at a rate between 100 and 190 colonies per day (average 153 ± 35) when 10^8 bacteria were plated, and between 46 and 200 (average 94 ± 17) when the density of 10^7 was used. The overall increase in the mutation rate was 3- and 14-fold for 10^8 and 10^7 cells/plate, respectively.

The kinetics of adaptive mutation growth rate in the AB1157*mutY* strain was different (Fig. 3). The density of 10^7 bacteria/plate gave not more than 10 colonies at the end of the incubation period, but 10^8 cells produced over 150 colonies/plate. The large number of starvation-associated mutations in the absence of MutY protein could originate from the presence of unrepaired bases such as 8-oxoG which occur frequently in starved cells. The results obtained here, in the *argE3*→Arg^+ reversion to prototrophy system are similar to those reported for *mutT* in *tyrA14* and *trpA23* [12] and for *mutY* in *tyrA14* [11] systems.

Surprisingly, in the AB1157*mutTmutY* double mutant the level of adaptive mutations was low and comparable to that observed in

wild type AB1157 (Fig. 2). As expected, the level of growth-dependent Arg^+ revertants in the *mutTmutY* strain was very high (about 770 ± 15 mutants per 10^8 cells).

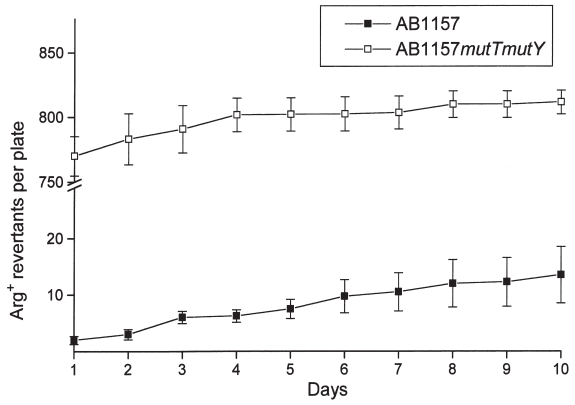

Figure 2. Arg^+ revertants of AB1157 and AB1157 *mutTmutY* strains in 10^8 cells as a function of the duration of incubation at 37°C on E–Arg plates.

Points represent means, and bars – standard errors of three or four experiments.

In all the described experiments it was important to check whether the cells on starvation plates were alive. After removal of all visible colonies, the plates were washed and the number of viable cells was estimated by diluting the obtained suspensions and plating onto LB-agar plates. Figure 4 shows the viability of AB1157 wild type strain and its *mutY* and *mutT* mutants on E–Arg plates (10^8 cells were placed on each plate). The number of viable cells was estimated at the beginning of the experiment, on the 1st day and after 5 and 10 days of plate incubation. Viable counts of *mutY* and *mutT* strains were not significantly changed during the first 5 days of incubation. However, after 10 days the number of living *mutY* cells decreased to reach a 10-fold lower number in comparison to the initial counts, while the number of viable *mutT* cells stayed more or less on the same level. Since the drop in the number of living cells in *mutY* mutant is very similar to that in wild type strain it can be concluded that the mutation in *mutY* gene has no influence on cell viability and that the mutation in *mutT* gene helps bacterial cells to survive under prolonged starvation conditions.

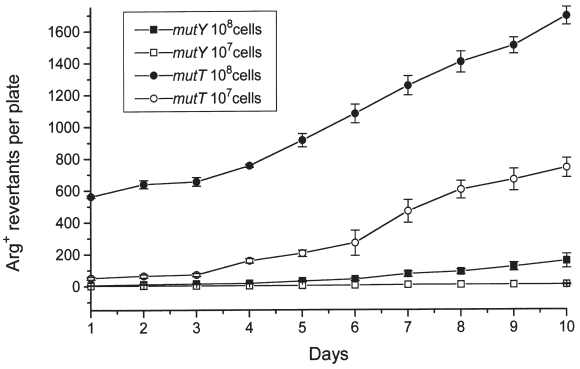

Figure 3. Accumulation of Arg^+ adaptive mutations over time (incubation at 37°C) on E–Arg plates in AB1157 *mutT* and *mutY* strains at 10^7 and 10^8 cells/plate.

Points represent means, and bars – standard errors of three or four experiments.

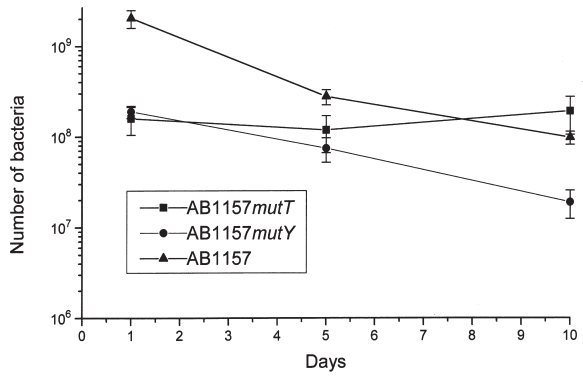

Figure 4. Viable counts of AB1157 wild type, *mutT* and *mutY* strains.

Bacteria were washed off from of E–Arg plates after 1, 5 and 10 days of incubation at 37°C.

Specificity of adaptive mutations

Previously, we have described a method for estimating mutagenic specificity in *E. coli* AB1157 strain carrying three mutations: *argE3* (ochre), *hisG4* (ochre) and *thr-1* (amber) [18]. The method is based upon analysis of the Arg^+ revertants which may arise by back mu-

tations at the mutated site (UAA), or by ochre suppressor formation. One of the advantages of the method is that it allows investigation of the mutagenic processes on the intact chromosomes.

In the present experiments two sets of Arg^+ colonies were tested for their specificity: the colonies which appeared after 2 days of incubation at 37°C (growth-dependent revertants), and those which appeared between the 4^{th} and 10^{th} day of incubation (stationary-phase mutations). First, the Arg^+ revertants selected on E–Arg plates were examined for their requirements for histidine and threonine by plating them on E–His and E–Thr plates. All the Arg^+ revertants can be divided into two phenotypic classes: class I: Arg^+His^- $Thr^{+/-}$; class II, Arg^+His^+ $Thr^{+/-}$. The suppressor activity of the Arg^+ revertants can be examined by testing their sensitivity to a set of amber (B17) and ochre (oc427, ps292, ps205) mutants of the T4 bacteriophage. T4 mutants are able to grow only on the Arg^+ revertants creating a suppressor (the results of phage typing precisely point to the suppressor present, see Table 1). The Arg^+ revertants which are not sensitive to the set of T4 ochre mutants arose by back mutation in *argE3* gene.

Table 2 summarizes the results of specificity tests for growth-dependent and starvation-associated Arg^+ revertants of the AB1157 wild type, *mutY* and *mutT* strains. In the AB1157 strain Arg^+ growth-dependent revertants were almost evenly distributed between phenotypic class I and II. Class I revertants were a result of *supB* suppressor formation by GC→AT transitions while class II were due to *supL* suppressor formation by AT→TA transversions. Arg^+ adaptive revertants of the same strain belonged mainly to class I and were a result of the formation of back mutations. Back mutations may be caused by AT→TA transversion at the 3′ATT5′ sequence in the TAA ochre codon but also by AT→GC transition at the last thymine of the same sequence. In the latter case, mRNA formed on this sequence contains the nonsense UAG amber codon recognized by $supE44_{am}$ suppressor, normally present in AB1157 strain.

Phenotypically, growth-dependent Arg^+ revertants of the AB1157*mutT* strain belonged mainly to class II but both classes, I and II, consisted of back mutations at the *argE3* locus. Adaptive Arg^+ revertants of this strain were back mutations too but, in opposition to the growth-dependent revertants, they belonged mainly to class I and within this class a small fraction (around 12%) of GC→AT transitions was also observed.

In the AB1157*mutY* strain the level of growth-dependent Arg^+ revertants was low and phenotypic distribution of these two classes was similar to that characteristic for

Table 1. Nucleotide sequence change associated with the Arg^+ phenotype of revertants of AB1157*argE3* strain

Site of mutation (suppressor)	Lysis of Arg^+ revertants by T4 phages *					Mutation
	WT	B17	oc427	ps292	ps205	
supB	+	+	+	+	+	GC→AT
$supE_{(oc)}$	+	–	+	+	+	GC→AT
argE3	+	+	–	–	–	**
supL	+	+	+	+	–	AT→TA

*Lysis (+) or nonlysis (–) was determined by placing 1–1.5 × 10^4 PFU of phage suspension on bacterial stripe on LB plate and incubating overnight. The T4 phages were: wild type (WT), amber (B17) or ochre (oc427, ps292, ps205).
**Mutations in *argE3* locus (back mutations) could be caused by transversions or transitions at the AT base pairs in the TAA *ochre* codon.

Table 2. Specificity of growth-dependent and adaptive Arg^+ revertants of the AB1157 strain

Strain	Phenotypic class	Type of mutations							
		Arg^+ growth-dependent revertants per 10^8 cells				Arg^+ starvation-associated revertants per 10^8 cells			
		Distribution among phenotypic classes (%)	back	*supB* by GC→AT or $supE_{oc}$	*supL* by AT→TA	Distribution among phenotypic classes (%)	back	*supB* by GC→AT or *supEoc*	*supL* by AT→TA
AB1157	I	44	–	1.0	0.26	84	11	1.3	–
	II	56	–	0.17	1.5	16	2.2	-	0.24
AB1157 *mutT*	I	28	157	–	–	64	870	218	–
	II	72	403	–-	–	36	612	–	–
AB1157 *mutY*	I	27	1.5	0.38	–	99	79.2	63.4	15.8
	II	73	2.6	1.0	1.5	1	1.6	–	–

the *mutT* strain (class I, 27%; class II, 73%). Class I of the revertants of AB1157*mutY* strain consisted mainly (80%) of back mutations and a much lower level (20%) of GC→AT transitions. Class II consisted of back mutations (50%) and equal amounts of GC→AT transitions and AT→TA transversions (25% each). However, almost all (99%) of the starvation- associated Arg^+ revertants of the *mutY* strain belonged to class I and half of them were back mutations. The other half consisted of GC→AT transitions (80%) and AT→TA transversions (20%).

The results presented here are in agreement with the observation that adaptive mutations occur in the gene whose function was selected: phenotypically, colonies growing on E–Arg plates were Arg^+ but still His^- and Thr^-.

The adaptive class I Arg^+ reversions of the *mutT* strain consisted of back mutations (80%) and mutations which arose as a result of *supB* suppressor formation by GC→AT transitions (20%). All of the adaptive Arg^+ revertants of the *mutY* strain belonged to class I; half of them consisted of back mutations and the other half resulted from *supB* (40%) and *supL* (10%) suppressors formation by GC→AT transitions and AT→TA transversions, respectively. This means that the adaptive and growth-dependent reversions of arginine auxotrophy in the *mutY* strain arose by the formation of different suppressors.

CONCLUSIONS

The presented here *argE3*→Arg^+ reversion to prototrophy is a valuable system for investigation of adaptive mutations. An additional advantage of the system is that, using a set of T4 phage mutants and two additional markers: *hisG4* and *thr-1,* it is possible to estimate the specificity of the mutations (type of suppressor leading to Arg^+ mutation) and distinguish between Arg^+ growth-dependent and adaptive revertants. This was demonstrated for the AB1157 *mutY* strain. The AB1157 *mutT* we used here as a tester strain, it did not show the advantage of the system because almost all of its Arg^+ revertants resulted from back mutations in the *argE3* gene. Nevertheless, there are strains (for instance AB1157 mutated in *dnaQ* gene encoding the ε subunit of DNA polymerase III, Grzesiuk & Nowosielska, in preparation), which revert to prototrophy by forming suppressors and in those strains the specificity of mutations can be precisely estimated.

The application of replica plating for adaptive mutation investigation is a new and very useful idea. It shows directly the difference between growth-dependent (even small, slow growing) colonies and those which arose due to an adaptive mutation.

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