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Growth-dependent and adaptive mutation rates to *ebgR* and IS30 transposition in the bacteria *Escherichia coli* K-12 at different extracellular Mg^{2+} concentrations

Stopnja rastno odvisnih in adaptivnih mutacij v *ebgR* in transpozicija IS30 v bakteriji *Escherichia coli* K-12 pri različnih zunajceličnih koncentracijah Mg^{2+}

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Abstract: During starvation on carbon and energy *Escherichia coli* K-12 cells, modified to possess EbgA51 as the only β-galactosidase enzyme, experience adaptive mutations in the *ebgR* repressor gene. In this way, cells acquire the capacity to utilize the lactulose as the only source of carbon and energy and begin to grow. Adaptive mutations at *ebgR* are mediated largely by insertion sequences, 40% of adaptive mutants contain IS30 insertions. Also, besides sensing extracellular Mg^{2+}, a PhoP-PhoQ system decreases the adaptive mutation rate to *ebgR* in a to-date unknown way. By performing fluctuation tests and genetic analyses, we tested the hypothesis that Mg^{2+} plays an important role in the adaptive mutation at *ebgR*. Results gathered with *phoP* and *phoQ* mutant strains demonstrated that the adaptive, but not the growth-dependent, mutation rate is increased by a high extracellular Mg^{2+} concentration. In an Mg^{2+}-rich environment, the *phoQ* cells experience a nearly identical adaptive mutation rate as the wild-type strain. Results with the wild-type strain show that the relation between the levels of PhoP-PhoQ expression and the adaptive mutation rate is not as straightforward as expected and that different Mg^{2+} concentrations do not affect IS30 transposition. We discuss the possible role of magnesium in the adaptive mutation process.

Keywords: Adaptive mutation, magnesium, *Escherichia coli*, PhoP-PhoQ, *ebg* operon, IS30, gene regulatory network

Ključne besede: Adaptivna mutacija, magnezij, *Escherichia coli*, PhoP-PhoQ, *ebg* operon, IS30, genska regulatorna mreža

Izvleček: Med stradanjem ogljika in energije pride v celicah *Escherichia coli* K-12, modificiranih tako, da kodirajo EbgA51 kot edino beta-galaktozidazo, do adaptivnih mutacij v represorskem genu *ebgR*. Na ta način celice pridobijo sposobnost uporabe laktuloze kot edinega vira ogljika in energije ter začnejo z rastjo. Adaptivne mutacije v *ebgR* so uravnane v veliki meri z insercijskimi sekvencami, 40% adaptivnih mutant vsebuje IS30. Prav tako dvokomponentni regulatorni sistem PhoP-PhoQ – poleg zaznavanja zunajceličnega Mg^{2+} – znižuje stopnjo adaptivnih mutacij *ebgR* na še vedno neznan način. S pomočjo fluktuacijskih testov in genetskimi analizami smo preverili hipotezo, da ima Mg^{2+} pomembno vlogo v procesu adaptivne mutacije. Dobljeni rezultati s *phoP* in *phoQ* mutantami kažejo, da se ob povečani zunajcelični koncentraciji Mg^{2+} zviša stopnja adaptivnih mutacij, stopnja rastno odvisnih mutacij pa ostane nespremenjena. V okolju z veliko Mg^{2+} imajo *phoQ* mutante skoraj enako stopnjo adaptivnih mutacij kot celice divjega tipa. Rezultati poskusov z divjim sevom pa kažejo, da odnos med izražanjem PhoP-PhoQ in adaptivno mutacijo ni preprost, različni koncentraciji Mg^{2+} pa ne vplivata na transpozicijo IS30. V članku razpravljamo o možni vlogi magnezija v procesu adaptivne mutacije.

Introduction

The monumental study on phage resistance in *Escherichia coli* (Luria and Delbruck 1943) demonstrated that mutations can result from rare spontaneous events prior to the application of selective pressure by a phage. More than 40 years later, Cairns and co-workers (1988) added an equally important finding in the quest for the origin of mutations. They proved that during non-lethal and non-mutagenic stress, spontaneous adaptive mutations emerge which relieves the selective pressure in non-dividing or slowly dividing cells (Foster 1999). It was found that, in contrast to mutations during growth (or growth-dependent mutations), adaptive mutations take place only in the presence of a specific selective pressure and not in other non-specific forms of stress. It was established that the adaptive mutation process can lead to frameshift mutations (Layton and Foster 2003), small deletions (Saumaa et al. 2002) or to various genome reorganizations (Lamrani et al. 1999, Saumaa et al. 2002).

We studied an adaptive mutation process in *E. coli* K-12 strains which lack the LacZ β-galactosidase enzyme. For this reason, the silent *ebg* operon (Fig. 1) codes the only β-galactosidase in the cell (Hartl and Hall 1974). The expression of Ebg β-galactosidase is under the control of the repressor EbgR. Cells with active EbgR do not produce a sufficient amount of enzyme for growth on minimal medium with lactulose as the only source of carbon and energy (Hall 1995). EbgR is not induced by the sugar lactulose or the synthetic inducer IPTG. Non-dividing cells enter a stationary phase and produce a colony only if an *adaptive mutation* lowers the synthesis, activity or efficiency of EbgR. This allows RNA polymerase to transcribe the *ebg* operon and in this way, a sufficient amount of Ebg β-galactosidase is produced for growth. Adaptive mutations are known to occur weeks after the $EbgR^+$ cells are plated on the selective medium (Hall 1995).

When Hall (1998) disrupted *phoP* and *phoQ* genes, the adaptive mutation rate to *ebgR* was reduced by more than one order of magnitude. The PhoP-PhoQ system demonstrates an influence on the mutation process only in the stationary phase and not during growth. The PhoP-PhoQ two-component regulatory system is comprised of the regulatory protein PhoP and the periplasmic sensor PhoQ (Groisman 2001). PhoQ has been demonstrated to specifically bind a divalent Mg^{2+} ion (Garcia-Vescovi et al. 1996), and besides detecting extracellular changes in Mg^{2+} concentration, it determines the phosphorylation state of PhoP (Castelli et al. 2000). Active phosphorylated PhoP mediates the activity of at least 50 genes in *E. coli* (Groisman 2001). It was established (Soncini et al. 1996) that extracellular Mg^{2+} in *millimolar* concentrations reduces the transcription rate of PhoP target genes. When the environmental Mg^{2+} concentration is lowered to *micromolar* levels, expression of the PhoP-PhoQ regulon is induced. In particular, PhoP-PhoQ allows *E. coli* to grow in a low-Mg^{2+} environment, which results from PhoP-activated expression of the *mgtA* and *mgtB* genes encoding Mg^{2+} transporters (Soncini et al. 1996).

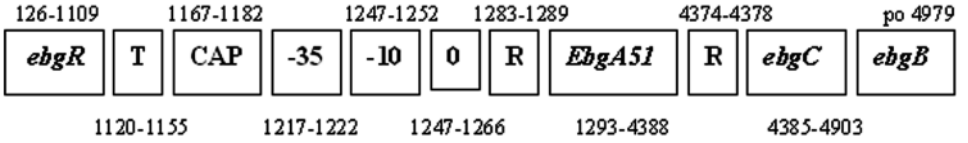

Fig. 1: Structure of *ebg* operon. It has a typical hierarchical structure comprised of sequences that code the repressor EbgR, two β-galactosidase subunits (EbgA51 and EbgC) and protein EbgB as well as a mosaic of binding sites for ribosomes (R), repressor (O), cAMP receptor protein (CAP) and RNA polymerase sigma factor (-35 and -10) (Hall et al. 1989). T site is a transcripton terminator for the repressor. Numbers above/below elements represent the position within the *ebg* operon expressed in base pairs.

Slika 1: Struktura *ebg* operona. Ima tipično hierarhično strukturo, saj poleg zaporedij, ki kodirajo represor EbgR, obe podenoti β-galaktozidaze (EbgA51 in EbgC) in protein EbgB, vsebuje tudi mozaik vezalnih mest za ribosome (R), represor (O), cAMP receptorski protein (CAP) in sigma faktor RNA polimeraze (–35 in –10) (Hall in sod. 1989). Mesto T je transkripcijski terminator represorja, številke pod/nad elementi pa predstavljajo pozicijo znotraj *ebg* operona izraženo v baznih parih.

It is particularly important to point out that the PhoP-PhoQ system does not operate in a totally independent manner. In fact, PhoP-PhoQ is integrated into a complex *gene regulatory network* that enables bacterial cells to rapidly adapt to various stressful conditions and to survive in them (Utsumi et al. 2008). PhoP-PhoQ is activated by a small protein B1500 whose gene expression is regulated by another two-component regulatory system EvgA-EvgS (Eguchi et al. 2007). The signal for the EvgS sensor is unknown. There is also a cascade between PhoP-PhoQ and two other systems: BasS-BasR and RcsC-YojN-RcsB (Hagiwara et al. 2004). BasS senses a high Fe^{3+}concentration whilst RcsC senses low temperature, glucose and osmotic shock. In addition, activated PhoP directly promotes expression of the RstB-RstA system (Minagawa et al. 2003). The signal that activates RstB in *E. coli* is still unidentified.

Hall (1999) found out that 80% of adaptive mutations at *ebgR* are mediated by insertion sequences (IS). This is a significantly higher IS contribution than 61% detected in growth-dependent mutations at *ebgR*. Spectra of other non-IS mediated mutations do not differentiate between growth-dependent and adaptive mutants. Hall (1999) also realized that relative contributions of different IS varied between adaptive and growth-dependent mutations. It is particularly important that 40% of adaptive mutants were IS30-mediated, whilst IS30 inserted in *ebgR* only in 6% of cases during growth. During starvation, more than 50% of IS30 insertions in *ebgR* were positioned at a single site, at bp 472, that corresponds to the IS30 target sequence or hot-spot (Olasz et al. 1998). It is intriguing that the 24 bp region centered at bp 472 is such a hot spot for IS30 transposition during starvation, but it is not a hot spot in growing cells. The inference is that IS30 transposition is regulated by some factors specifically induced by starvation (Hall 1999a, Foster 1999). This conclusion is also supported by the fact that *E. coli* K-12 cells placed under nutritional deprivation experienced a burst of IS30 activity (Naas et al. 1994, Naas et al. 1995). Authors stated that stationary phase cells have a highly plastic genome and they suggest that such plasticity might play an adaptive role.

Our main aim is to identify factors that influence IS30 transposition during starvation and show that IS30 is not just a "selfish" element, for it can be controlled by the integrated gene regulatory network of the cell. In this way, the adaptive mutation process will be finally seen as regulated stress response comparable to the heat-shock response or the stringent response – the notion strongly argued by Foster (2005). We would like to stress that the organization of the pathway that starts with specific stress signal sensing and ends up with an adaptive mutation has not yet been identified in any experimental system studied so far (McKenzie et al. 2000). Yet, it is definitely known that adaptive mutations need a sigma factor RpoS or some other element that is under its control (Lombardo et al. 2004, Saumaa et al. 2002, Gomez-Gomez et al. 1997, Bjedov et al. 2003). RpoS enables bacterial cells to differentiate in the stationary phase and it regulates up to 10% of *E. coli* genes (Weber et al. 2005). Taking into account the immense complexity of the gene regulatory network, it seems most likely that adaptive mutations at *ebgR* are in some way regulated by some part of the gene regulatory network that operates in accordance with Mg^{2+} homeostasis. The recognition that the adaptive mutation process might be mediated by the cells' gene regulatory network will definitely leave a footprint in the sculpturing of evolutionary theory where the central tenet teach us that "mutations are random insofar as organisms cannot direct the production of particular mutations in response to their particular needs" (Lenski 2008). Also we will get fresh insights into the mechanisms of antibiotic resistance knowing for example that adaptive mutations can produce resistance to antibiotic ciprofloxacin (Riesenfeld et al. 1997). We started our quest with the hypothesis that magnesium might be one of the factors playing an important role in the adaptive mutation at *ebgR*. Here we present the results obtained so far.

Materials and Methods

Bacterial strains

***Escherichia coli* K-12 strains**. The wild-type strain SJ134 is F^- *ΔlacZ4680 lacY*$^+$ *ebgR*$^+$ *ebgA51 rpsL*. Strain MH242 is F^- Δ*lacZ4680 lacY*$^+$ *ebgR*$^+$ *ebgA51 rpsL phoQ*. Strain MH076 is F^- Δ*lacZ4680 lacY*$^+$ *ebgR*$^+$ *ebgA51 rpsL phoP* (Hall 1998).

Media

The mineral salts medium (MM), a limiting glycerol medium (GM) and a lactulose- selection medium (LM), were prepared according to Hall (1999). The source of magnesium or other supplementary compounds was added to a final concentration in LM cooled at 55 ˚C. Rich medium (RM) consisted of the Luria-Bertani mixture. All solid media were solidified with 15 g of agar (Difco) per liter. Initially, we performed tests with all three strains in LM supplemented with various concentrations of Mg^{2+} ($MgSO_4$).

Fluctuation test

To monitor the accumulation of *ebgR*$^-$ colonies, we performed a fluctuation test as described by Hall (1998). First we prepared 80, 86 and 58 independent cultures of the strains SJ134, MH242 and MH076, respectively. They were grown from the inoculum of 2–2.5×10^3 cells for exactly 24 hours at 30 ˚C (250 rpm^{-1}) in GM to ensure the same density. We randomly selected two independent cultures of each strain and determined the optical density at 600 nm; the expected OD_{600} value was 0,460. We spread an average of 1.9×10^7, 2.2×10^7 and 1.6×10^7 SJ134, MH242 and MH076 cells onto separate LM plates. The plates were then incubated at 30 ˚C for at least six days.

Viability test

Viability tests were performed as described by Hall (1999). On days 1, 2 and 3, cells were washed from five plates, suitably diluted, and plated onto RM to determine the number of viable cells.

Reconstruction test

Reconstruction tests were performed as described by Hall (1998). They showed that under these conditions, more than 98% of pre-existing *ebgR*$^-$ mutants formed detectable colonies in three days. Consequently, colonies that appeared on day 3 were the result of mutations that had been present in the initial population and were treated as growth-dependent mutants. The colonies that appeared on days 4, 5 and 6 were treated as adaptive mutants; adaptive mutations occurred after days 1, 2 and 3 of incubation, respectively.

Genetic analysis

The relative frequency whereby IS30 causes adaptive mutations in SJ134 was calculated as follows: first we performed colony PCR on adaptive mutants from three separate LM plates containing 400 µM or 5 mM Mg^{2+} that acquire a mutation at *ebgR* within the first 24 hours of incubation. Adaptive mutants were initially amplified using a three-primer cocktail consisting of forward primer 1 corresponding to bp 73 to 94 (5'-AAGCACTTCCTCTACAATGGGG-3') of *ebgR* (GenBank accession no. M64441) and primers 2 and 3 corresponding to bp 970 to 989 (5'-CGGGGAACAAATGAGAACAC-3') and the complement of bp 130 to 149 (5'-GGTTT-TATGCCGCCAGTATC-3') of IS30 (GenBank accession no. X00792). The 25-µl reaction also contained 2.5 mM $MgCl_2$, 200 µM (each) deoxynucleoside triphosphates (dNTP), 0.1 U of Taq polymerase (Applied Biosystems) and 2.5 µl of PCR buffer (Applied Biosystems). Eight adaptive mutants that on the initial amplification generated PCR product were sequenced using forward primer 1 and the complement of bp 1144 to 1168 (5'-AAGCACTTCCTCTACAATGGGG-3') of *ebgR* (GenBank accession no. M64441). They were all shown to contain the IS30 insertions.

Statistical analysis

The frequency of adaptive mutants of each independent trial was calculated from five up to nine replicates from the same environment after the 1st, 2nd and the 3rd day of incubation. We put together frequencies of adaptive mutants per day for every environment and calculated the average frequency. Statistical calculations were performed by SPSS for Windows version 14.0. The Kolmogorov-Smirnov test was used to test for normality of data. A comparison of two groups of data was performed using an unpaired Student's t-test (two-sided) and Mann-Whitney U test, depending on whether the data showed a normal distribution or not.

Calculation of mutation rates

To estimate the average rate of mutations that occurred prior to plating and during the growth of each culture (growth-dependent mutations), we

used the computer program implemented by Frank Stewart that uses the Ma-Sandri-Sarkar algorithm (Ma et al. 1992) with a small modification simplifying and speeding up the calculation.

By dividing the average frequency of new *ebgR*$^-$ colonies from the environment with the average number of cells at the time the mutation occurred, we obtained data points presenting cumulative *ebgR* mutations per 10^8 viable cells per day. The average adaptive mutation rate was the slope of the best-fit line calculated as the least squares fit to the data points (Hall 1998).

Results

Adaptive mutations at ebgR in the wild-type (WT) strain

To see the effect of different levels of PhoP-PhoQ expression on the adaptive mutation process, we first spread a total of 45 independent cultures of WT cells onto LM containing a reference Mg^{2+} concentration (400 μM $MgSO_4$ and medium level of PhoP-PhoQ expression) and 46 cultures onto LM plates containing a high Mg^{2+} concentration (5 mM $MgSO_4$ and a low level of PhoP-PhoQ expression). After the first day, we observed a small (29%) but statistically significant ($p<0.05$) decline with 5 mM Mg^{2+} in the number of new *ebgR*$^-$ colonies in comparison to 400 μM Mg^{2+} (Jerman et al. 2005). After the 2nd day, the frequency drop was 14% but on the 3rd day the frequency moved up by 3% in 5mM Mg^{2+}. Both results are not statistically significant.

Since a significant decline in the number of adaptive mutants appeared only after the first 24 hours and since the difference was not quite satisfying, we performed fluctuation tests at an even lower Mg^{2+} concentration where PhoP-PhoQ is highly expressed (Soncini et al. 1996). We plated 28, 11 and 12 cultures on LM supplemented with 50 μM $MgSO_4$, 4 μM $MgSO_4$ and 0.4 μM $MgSO_4$, respectively. After three days in 50 μM Mg^2, the WT strain produced the average cumulative number of adaptive mutants that was exactly the same as in 400 μM Mg^{2+}. On the other hand, after two days (experiments lasted only two days) in 4 μM Mg^{2+} and 0.4 μM Mg^{2+}, the cumulative number of *ebgR*$^-$ mutants decreased by 19% and 31%, respectively.

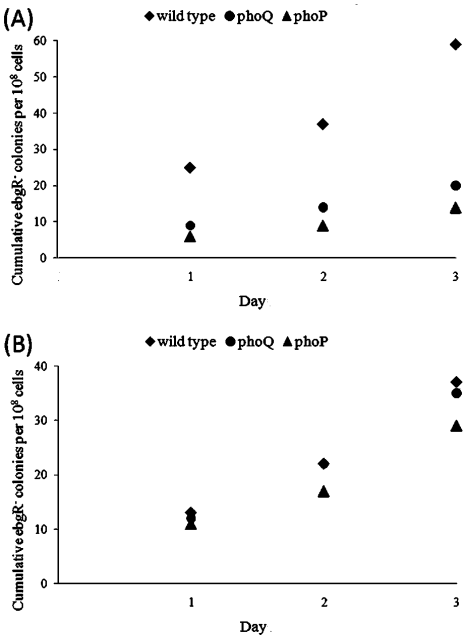

Fig. 2: Appearance of *ebgR* mutant colonies on lactulose-selective medium during incubation at 30˚C. (**A**) – Fluctuation tests were performed in the lactulose-selection medium containing 400 μM Mg^{2+}. The colony counts in the wild-type and the *phoQ* and *phoP* mutant strains, signify the average for 13 (Σ61 plates), 5 (Σ49 plates) and 3 (Σ29 plates) independent trials, respectively (**B**) – Fluctuation tests were performed in the lactulose-selection medium containing 5 mM Mg^{2+}. The colony counts in wild-type, *phoQ* and *phoP* mutant strains signify the average for 5 (Σ19 plates), 4 (Σ37 plates) and 3 (Σ29 plates) independent trials, respectively.

Slika 2: Pojavljanje *ebgR* mutant na laktuloznem selekcijskem gojišču pri inkubaciji na 30˚C. (**A**) – Fluktuacijski testi so opravljeni v selektivnem gojišču z laktulozo, ki vsebuje 400 μM Mg^{2+}. Število kolonij v divjem sevu, *phoQ* in *phoP* mutantah je povprečje izračunano iz 13 (Σ61 plošč), 5 (Σ49 plošč) in 3 (Σ29 plošč) neodvisnih poskusov. (**B**) – Fluktuacijski testi so opravljeni v selektivnem gojišču z laktulozo, ki vsebuje 5 mM Mg^{2+}. Število kolonij v divjem sevu, *phoQ* in *phoP* mutantah je povprečje izračunano iz 5 (Σ19 plošč), 4 (Σ37 plošč) in 3 (Σ29 plošč) neodvisnih poskusov.

Both falls are significant ($p<0.05$). Clearly, the correlation between the PhoP-PhoQ expression levels and the number of adaptive mutants, if it exists at all, is not straightforward.

Adaptive mutations at ebgR in phoQ and phoP mutant strains

On the contrary, when cells are completely devoid of functional PhoP or PhoQ, a highly significant decline in the adaptive mutation frequency is observed after three days ($p<0.00001$) (Fig. 2A). The average adaptive mutation rates in 400 μM Mg^{2+} calculated for strains SJ134, MH242 and MH076 were 1.7×10^{-7}, 5.6×10^{-8} and 3.9×10^{-8} per viable cell per day, respectively. It turns out that after the first two days but not after day 3, *phoP* cells experienced a significantly stronger reduction in the number of adaptive mutants compared to *phoQ* cells ($p<0.001$). When we spread *phoP* and *phoQ* cells on 5mM Mg^{2+}, we clearly observed that the number of *ebgR*$^-$ cells increased after three days (Fig. 2B) ($p<0.001$). In 5mM Mg^{2+}, the average adaptive mutation rate of *phoP* and *phoQ* cells was very similar to the WT strain: 1.2×10^{-7}, 1.1×10^{-7} and 9.1×10^{-8} per viable cell per day for SJ134, MH242 and MH076, respectively. Again, *phoP* mutants in 5mM Mg^{2+} showed a lower frequency of adaptive mutations compared to *phoQ* mutants ($p<0.05$). When compared to the WT strain, the *phoP* cells showed a 22% lower cumulative number of adaptive mutants after three days ($p=0.052$). On the other hand, in 5mM Mg^2, the *phoQ* cells have a nearly identical (non-significant 2% reduction) cumulative number compared to the WT strain.

Viability tests performed on all three strains in LM supplemented with 400 μM Mg^{2+} and 5mM Mg^{2+} demonstrated that the population densities and the death rates of both mutant strains were comparable to those of the WT cells (Fig. 3). We also observed that in 5mM Mg^{2+}, both mutant strains required the same amount of time to form a detectable colony as did the WT cells, namely three days. However, this is not the case for 400 μM Mg^{2+} – both mutant strains required 4 days to form detectable colonies.

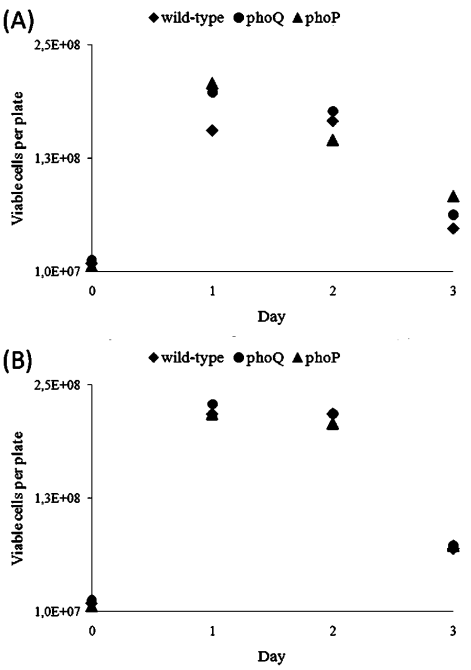

Fig. 3: The number of viable cells on lactulose-selective medium during incubation at 30˚C. (A) lactulose-selection medium containing 400 μM Mg^{2+} (B) lactulose-selection medium containing 5 mM Mg^{2+}. The viable counts are the averages for 5 plates.

Slika 3: Število viabilnih celic na laktuloznem selekcijskem gojišču pri inkubaciji na 30˚C. (**A**) selektivno gojišče z laktulozo, ki vsebuje 400 μM Mg^{2+} (**B**) – selektivno gojišče z laktulozo, ki vsebuje 5 mM Mg^{2+}. Števila viabilnih celic so povprečja petih plošč.

Growth-dependent mutation rate to ebgR

To ascertain if extracellular Mg^{2+} also plays a role in the *dividing phoQ* mutants we tested its effect on the growth-dependent mutation rate. For the *phoQ* strain, 10 cultures (400 μM Mg^{2+}) and 29 cultures (5 mM Mg^{2+}) at 2.1×10^7 cells per culture produced an average of 1.45 mutations and 1.05 mutations per culture prior to plating, respectively. The average growth-dependent mutation rate was 6.9×10^{-8} and 5.0×10^{-8} per cell division in 400 μM Mg^{2+} and 5mM Mg^{2+}, respectively. Clearly, millimolar concentrations of Mg^{2+}in *phoQ* mutants affect only adaptive, but not growth-dependent, mutation at *ebgR*.

IS30 transposition at two different Mg^{2+} concentrations

Next we tried to find out if different Mg^{2+} concentrations effect the IS30 transposition. Accordingly, we tested 78 adaptive mutants isolated from the 400 μM Mg^{2+} cultures and 95 adaptive mutants from the 5mM Mg^{2+} cultures in order to define the relative contribution of IS30 at two different Mg^{2+} concentrations. In 400 μM Mg^{2+}, the IS30 element was inserted in 27 out of 78 adaptive mutants (35%); the result is comparable to the 40% IS30 share measured by Hall (1999). In 5mM Mg^{2+}, the number was 42 out of 95 mutants (44%). The difference was not significant (p=0.2), which indicates that the relative contribution of IS30 in the WT strain is probably independent from the level of PhoP-PhoQ expression.

Discussion

Hall (1998) argued that the adaptive mutation rate to *ebgR* is shaped by some proteins that are under the control of the PhoP-PhoQ regulon. If this is the case, then the high levels of PhoP-PhoQ expression in the WT strain should affect the adaptive mutation rate differently from the one at low levels. However, our results with the WT strain illustrate that the high expression of PhoP-regulated proteins evidently does not produce more adaptive mutation events. In other words, the correlation between levels of PhoP-PhoQ expression and the adaptive mutation process is not so straightforward, if the connection exists at all. We are aware that it is definitely premature to rule out the possibility that a protein, directly or indirectly linked with PhoP, regulates elements responsible for the adaptive mutation at *ebgR*.

If we concentrate only on the IS30 element which has always been a favourite candidate for the role of the regulated entity during starvation (Hall 1999a, Foster 1999), we can seek out very interesting clues. For example, when the IS30 transposase (an enzyme coded by the element itself) was fused with a bacterial or a eukaryotic protein, the IS30 element broadened the arsenal of target sequences and increased the integration frequency at a specific site in *E. coli* cells (Szabo et al. 2003). It has also been established that the IS30 element codes a decanucleotide that is a potential binding site for the cell's regulatory protein (Szabo et al. 2008). If this protein is expressed only in the stationary phase, then we could elegantly explain why the hotspot is active only during starvation and not during growth. As Hall (1999b) put it "*E. coli* has managed to take advantage of IS elements for its own benefit".

Although our preliminary results did not find evidence that IS30 is directly linked to different levels of PhoP-PhoQ expression, it can be due to the fact that our research has basically just started. The determination of the adaptive mutations spectra in *phoP-phoQ* mutants in 400 μM Mg^{2+} and 5 mM Mg^{2+} is underway. It could be that Mg^{2+} influences other adaptive mutations at *ebgR*, such as IS1, IS2 or IS4 transposition or any other non-IS mediated adaptive mutation known to occur (Hall 1999a).

However, probably the strongest result we collected was the finding that in the 5mM Mg^{2+} culture, the *phoQ* cells experienced a nearly identical adaptive mutation rate as the WT strain. In other words, enough extracellular Mg^{2+} is a guarantee that the adaptive mutation process will run almost unimpeded, at least as regards the number of adaptive mutation events. As we have already mentioned, the spectra of adaptive mutations in 5mM Mg^{2+} remained undetermined in any strain so we really cannot say which type of adaptive mutations at *ebgR* were present in the *phoQ* cells. We should also keep in mind that in a high Mg^{2+} environment, the *phoP* mutants noticeably did not recover as well as the *phoQ* ones; both strains were otherwise isogenic. It is known that at a high concentration, PhoP is able to activate its target genes independently of its phosphorylation status, in a concentration-dependent manner (Lejona et al. 2004). The same authors also emphasise that, under physiological conditions, PhoQ is required for PhoP activation which makes this explanation less likely. However, other authors emphasize that extracellular Mg^{2+} suppresses the cell's activity by some unknown elements that are independent of the PhoQ periplasmic domain (Regelmann et al. 2002). Discovering the elements which are responsible for the different responses of otherwise genetically identical cells is one of the future research tasks.

Nonetheless, our results strongly indicate that if *phoP* and *phoQ* cells have a sufficient extracellular Mg^{2+} concentration, then the adaptive mutation rate is more or less close to the rate in the WT strain; here the MgtA-MgtB makes sure that cells always have a sufficient quantity of *intracellular* Mg^{2+}. However, in the *phoP* or *phoQ* strain, the *mgtA* and *mgtB* genes are insufficiently expressed which means that intracellular Mg^{2+} could be a critical factor for the adaptive mutation process at *ebgR*.

There are at least two possible scenarios in which this could work. Scenario A is that a high Mg^{2+} concentration only enables *phoQ* and *phoP* cells to grow and make a colony and has otherwise no effect on adaptive mutations *per se*. In other words, adaptive mutations are ostensibly linked with Mg^{2+} and the highly significant decline in number in the 400 μM Mg^{2+} by the *phoP* and *phoQ* cells is then just a consequence of poor growth. It is a fact that $Mg^{2+}$is essential for bacterial growth; in a low Mg^{2+} (10 μM Mg^{2+}) environment, *phoP* or *phoQ* mutant strains of *Salmonella typhimurium* lose the ability to form colonies (Soncini et al. 1996). Also, *E. coli* cells need 20-50 μM of intracelullar Mg^{2+} for active growth (Smith and Maguire 1998). At the same time, the finding that the *phoP* and *phoQ* mutants in 5mM Mg^{2+} need as much time to make a colony as the WT strain, partially supports this scenario. But by performing reconstruction tests we observed that mutant strains form a colony with the same efficiency as the WT cells. In addition, the viability tests (Fig. 3) showed that the growth curve of mutant strains was practically the same as that of WT cells. Therefore, according to our estimation, which is in accordance with Hall's conclusions (1998), this scenario is less likely to suit the factual processes.

The second scenario is that intracellular Mg^{2+}, directly or indirectly, influences the number of adaptive mutations by affecting the dynamics or kinetics of gene regulatory elements or insertion sequences that participate in the adaptive mutation process. It is generally accepted that Mg^{2+} plays an active role in catalysis as a co-factor or directly by binding to an enzyme (Cowan 2002, Sreedhara and Cowan 2002). Moreover, under normal conditions, 88–90% of ATP molecules form bonds with Mg^{2+} (Grubbs 2002); 75% of all intracellular Mg^{2+} is bound to ATP. Therefore, when the ATP concentration decreases, the quantity of free Mg^{2+} automatically increases; the concentration of free intracellular Mg^{2+} in *E. coli* amounts to an average between 0.3 and 0.5 mM which is around 1% of all the intracellular Mg^{2+}. Recent data showed that as cells differentiate from the logarithmic to the stationary phase, there is a solid drop in ATP concentration. After the cells' entrance into the stationary phase, a quick increase of ATP follows after which a progressive decrease of ATP is once again observed (Buckstein et al. 2008). This means that just by entering a stationary phase, the cells experience a dynamic fluctuation of free intracellular Mg^{2+}. If, at the same time, the Mg^{2+} transporters are less efficient, as is the case in the *phoP* and *phoQ* mutants, then an intricate concentration pattern of free intracellular Mg^{2+} arises. In our opinion, this is a factor that needs thorough study in order to comprehend the role of Mg^{2+} in shaping the adaptive mutation rate to *ebgR*.

Conclusions

The results presented in this paper demonstrate the following: (i) in the WT strain, the link between levels of PhoP-PhoQ expression and the adaptive mutation rate to *ebgR* is not straightforward, if it exists at all; (ii) in the *phoP* and *phoQ* mutants, the adaptive but not the growth-dependent mutation rate to *ebgR* is increased by 5mM extracellular Mg^{2+}; (iii) in the 5mM Mg^{2+} environment, the *phoQ* cells experience a nearly identical adaptive mutation rate as the WT strain; (iv) *phoP* mutants in 5mM Mg^{2+} show a significantly smaller increase of the adaptive mutation rate as the *phoQ* mutants; (v) in the WT strain, high levels of PhoP-PhoQ expression do not affect the relative frequency whereby IS30 causes adaptive mutations.

Finally, adaptive mutation is a strategy used by non-dividing cells. This means that knowing which factors determine the adaptive mutation rate to *ebgR* does not necessarily give us an understanding of the Cairns system where only 4% of adaptive mutations are IS-mediated. We need novel findings and insights to truly understand the causal background of the adaptive mutation phenomenon. Only then can we recognise the wider

relevance of adaptive mutations, for example, in evolutionary theory or in antibiotic resistance.

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Povzetek

Študija odpornosti *Escherichia coli* na fag T1 je pokazala, da so mutacije lahko rezultat redkih spontanih dogodkov v času rasti, ki se zgodijo pred izpostavitvijo fagom. V 90. letih so temu klasičnemu spoznanju dodali enakovreden uvid, da v času neletalnega in nemutagenega selekcijskega pritiska v bakterijskih celicah nastajajo t.i. adaptivne mutacije, ki sprostijo selekcijski pritisk in celici omogočijo rast. Adaptivne mutacije se za razliko od rastno odvisnih mutacij pojavljajo samo v navzočnosti selekcije za te mutacije in ne v drugih ne-specifičnih oblikah stresa.

Poskuse smo izvajali na treh sevih *Escherichia coli* K-12, ki imajo nefunkcionalen gen za LacZ β-galaktozidazo, zato tih ali kriptičen *ebg* operon kodira edino β-galaktozidazo v celici. Celice zaradi aktivnega represorja EbgR ne sintetizirajo zadostne količine Ebg β-galaktozidaze, zato se na minimalnem gojišču z laktulozo, kot edinim virom ogljika in energije ne delijo oziroma se delijo zelo počasi. Celice tako vstopijo v stacionarno fazo ter tvorijo kolonijo le v primeru, če so sinteza, aktivnost ali učinkovitost represorja EbgR znižane. Fluktuacijski testi so nedvoumno pokazali, da se na selekcijskem gojišču z laktulozo več tednov pojavljajo adaptivne mutante, ki imajo prekinjen gen *ebgR*. To omogoči RNA-polimerazi prepisovanje *ebg* operona in tako nastane zadostna količina Ebg β-galaktozidaze za rast in delitev.

Adaptivna mutacija v *ebgR* je v 80 % uravnana z insercijskimi sekvencami. Vse kaže, da je insercija IS30 elementa v času stradanja vsaj delno usmerjena s še neznanimi dejavniki okolja. IS30 ima namreč naravno vročo točko, konsenzusno zaporedje dolgo 24 baznih parov, znotraj *ebgR* gena, ki pa je aktivna zgolj v času stradanja in ne v času rasti. Na adaptivno mutacijo vpliva tudi PhoP-PhoQ sistem, ki je sestavni del integrirane gensko regulatorne mreže. Ali drugače, PhoP-PhoQ sistem ne deluje povsem avtonomno, ampak interagira z drugimi dvokomponentnimi regulatornimi sistemi, kot so EvgA-EvgS in BasS-BasR idr. Primarni signal za senzorni protein PhoQ je določena koncentracija Mg^{2+} v periplazmi. V milimolarni koncentraciji magnezija se *phoQ* in *phoP* izražata konstitutivno, v mikromolarni koncentraciji pa jima izražanje močno naraste. PhoP-PhoQ celicam *E. coli* predvsem omogoča rast v nizki koncentraciji Mg^{2+}, saj PhoP med drugim aktivira proteina MgtA in MgtB, ki magnezij učinkovito transportirata v celico.

V raziskavi je bil naš glavni namen identifikacija faktorjev, ki vplivajo na proces adaptivne mutacije. Še posebej smo želeli ugotoviti, ali je IS30 element podvržen regulaciji s strani elementov gensko regulatorne mreže. Na ta način bi ugotovili, ali je adaptivna mutacija reguliran stresni odgovor povsem ekvivalenten na primer odzivu na težavne razmere idr. Hkrati bi lahko ponovno potrdili, da insercijske sekvence oz. na splošno mobilni genetski elementi kljub navidezni samostojnosti niso povsem avtonomni in »sebični« genetski elementi. S pomočjo fluktuacijskih testov in genetskimi analizami smo preverjali hipotezo, da ima Mg^{2+} pomembno vlogo v procesu adaptivne mutacije *ebg* operona.

Primerjali smo stopnjo adaptivnih mutacij na selektivnem gojišču z laktulozo v okolju z različnimi koncentracijami Mg^{2+}. Kot referenčno vrednost smo uporabili 400μM $MgSO_4$, okolje z visoko koncentracijo Mg^{2+} je vsebovalo 5mM $MgSO_4$, okolja z nizko koncentracijo Mg^{2+} pa 50 μM, 4 μM in 0.4 μM $MgSO_4$. Pri celicah divjega tipa nismo odkrili jasne povezave med izražanjem PhoP-PhoQ in stopnjo adaptivnih mutacij v *ebgR* genu. Pri *phoP* in *phoQ* mutantah pa stopnja adaptivnih mutacij v referenčnem okolju značilno

pade, v okolju s 5 mM magnezijem pa se število adaptivnih mutant poveča. Pri *phoQ* mutantah stopnja adaptivnih mutacij doseže skoraj enako stopnjo kot pri celicah divjega tipa, drugače je pri *phoP* mutantah, kjer je število adaptivnih mutacij značilno nižje od *phoQ* mutant. Magnezij ne vpliva na stopnjo rastno odvisnih mutant v celicah z okvarjenim PhoQ proteinom. Ugotavljamo tudi, da magnezij v divjem sevu ne vpliva na frekvenco transpozicije IS30.

Vse skupaj kaže, da bi lahko bil znotrajcelični Mg^{2+} ključni faktor za proces adaptivne mutacije v *ebgR* genu. Pred nami sta dve hipotezi. Prva pravi, da magnezij zgolj omogoči adaptivni mutanti, da naredi kolonijo, kar bi pomenilo, da je adaptivna mutacija *per se* proces neodvisen od magnezija. Druga pa predpostavlja, da magnezij preko vpliva na gensko regulatorne elemente ali insercijska zaporedja, ki sodelujejo v procesu adaptivne mutacije, posredno spreminja število mutacijskih dogodkov. Zato trenutno med drugim poteka analiza spektra adaptivnih mutacij v različni koncentraciji okoljskega magnezija v vseh treh sevih. To bi lahko prineslo dodatna spoznanja o adaptivni mutaciji, katere izvor je še vedno skrajno slabo razumljen.

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