Isolation and mutation rate of the spontaneous PUR box and *purR* mutants*

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Abstract In our previous research in purine metabolism in *Salmonella typhimurium*, it was observed that the mutation frequency of the PUR box was one order of magnitude higher than that of *purR* under mutagenesis. In order to investigate further into this phenomenon, large amounts of independent PUR box and *purR* spontaneous mutants were isolated on lactose minimal medium by using a super-repressing mutant of *purR* (*purR*^{s}). The mutational regions of 5 PUR box mutants and 4 *purR* mutants were cloned. Nucleotide sequence analysis showed that all the mutants had mutations at the expected sites. The comparison of the two types of mutations indicated that, although the *purR* gene was two orders of magnitude larger than the PUR box (100:1), the ratio of their mutation rate was only 7:3 (*purR*: PUR box). Thus, we concluded that high mutation frequency of the PUR box did not result from mutagenesis. Under spontaneous conditions, the mutation frequency of PUR box was also high. Some tentative explanations of this interesting phenomenon are given in this report.

Keywords: super-repressing mutant, PUR box, *purR*.

In *Escherichia coli* and *Salmonella typhimurium*, the *de novo* purine biosynthesis involves 11 enzymatic steps. The genes encoding these 11 enzymes occur mostly as unlinked single units and are controlled by the purine repressor, PurR[1,2]. PurR consists of 341 amino acids and is a homodimer. PurR binds to a 16 bp plaindromic seguence (PUR box) located within the operator region of pur regulon genes. The consensus binding sequence is 5′ ACGCAAACGTTTGCGT3′[3~6].

In our previous research in purine metabolism in *Salmonella typhimurium*, we used *purD*-MudJ and *purG*-MudJ fusion strains to isolate regulatory mutants by N-methyl-N′-nitro-N-nitrosoguanidine (NTG) mutagenesis. Among these mutants, a *purD* O^c mutant and a *purG* O^c mutant were found unexpectedly. The ratios of mutation rate of the *purD* operator or *purG* operator to that of *purR*$^-$ were 1:7 or 1:8, respectively[4]. It has been known that the *pur* operator (PUR box) is highly conserved at eight nucleotides (G_3, C_4, A_5, A_6, C_8, G_9, T_{12}, C_{14}), which are essential for recruiting PurR repressor[5~8]. Sequence analysis showed that the two O^c mutations occurred at C_{14} and G_3 in the PUR box[5,6]. As a mutation target, the 1.47 kb *purR* is about two orders of magnitude larger than the PUR box, but its mutation rate is only one order of magnitude higher than that of the PUR box. Why did the PUR box have such a high mutation rate? Did it happen because of mutagenesis?

One way to answer these questions is to study the spontaneous mutation of the PUR box and *purR*. Because the PUR box is very small, it would be very difficult to isolate a strain carrying O^c mutation theoretically. Reported here is a novel method to isolate large amounts of spontaneous PUR box and *purR* mutants by using a super-repressing mutant of *purR* (*purR*^{s}). First we estimated the spontaneous mutation rate of the PURbox and *purR*, and then analyzed the mutational sites of some mutants. Finally we present here some explanations for this interesting phenomenon.

1 Materials and methods

1.1 Materials

Strains and phages used in this study are listed in Table 1.

LB was used as the rich medium. NCE medium (no carbon or no citric acid E medium)[9] was supple-

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Table 1. Strains and phages

Strains and phages	Genotype	Source
Salmonella typhimurium		
LT2	Wild type	Ref. [9]
TT12306	*purD*::MudJ(*lacZ*, Kan^r)	Ref. [9]
TGM463	$purR^+$, zxx1891::Tn10d *tet* (*tet* 90% linked to $purR^+$) *purG*::MudJ(*lacZ*, Kan^r)	Ref. [9]
TGM464	$purR^-$, zxx1891::Tn10d *tet*, (*tet* 90% linked to $purR^-$) *purG*::MudJ(*lacZ*, Kan^r)	Ref. [9]
5-28	$purR^S$, *purD*::MudJ(*lacZ*, Kan^r)	This study
528-PB2	same as 5-28 but O^c *pur D*	This study
528-PB9	same as 5-28 but O^c *pur D*	This study
528-PB12	same as 5-28 but O^c *pur D*	This study
528-PB19	same as 5-28 but O^c *pur D*	This study
528-PB25	same as 5-28 but O^c *pur D*	This study
528-PR3	same as 5-28 but $purR^S purR^-$	This study
528-PR12	same as 5-28 but $purR^S purR^-$	This study
528-PR15	same as 5-28 but $purR^S purR^-$	This study
528-PR24	same as 5-28 but $purR^S purR^-$	This study
Phages		
P22	HT105/1 int-201	Ref. [9]
H5	P22c2	Ref. [9]

mented with 1% lactose, 10 μg/mL adenine and 0.06 mmol/L vitamin B1. EMB medium[9] was supplemented with 1% lactose, 0.06 mmol/L vitamin B1 and 100 μg/mL adenine. A solution of 100 μg/mL adenine was used to repress the purine nucleotide synthesis. In rich medium, final concentrations of kanamycin (kan), tetracycline and ampicillin were 50 μg/mL, 20 μg/mL and 50 μg/mL, respectively. In minimal medium, these concentrations were reduced to half of that in rich medium. *Taq* plus DNA polymerase used in PCR amplification and pUCm-T vector for T-A cloning were purchased from Sangon Company. Restriction enzymes were from MBI. Primers were synthesized by Sangon Company.

1.2 Methods

Isolation of the spontaneous PURbox and *purR* mutants was carried out as follows. A single colony of strain 5 ~ 28 was inoculated into 2 mL LB + Kan medium and grown to saturation at 30 ℃. Then cells were harvested, washed twice and diluted 10^4-fold in saline. Aliquots of 10 μL resuspension containing about 1000 cells were separately inoculated into 2 mL LB + Kan medium and grown to saturation at 30 ℃. Cells in 1 mL of these independent cultures were harvested, washed twice and resuspended in 1mL saline. Aliquots of 0.1 mL (about 10^8 cells) were plated on NCE medium with lactose as the sole carbon source, then incubated at 30 ℃. Colonies appeared in 48 h were the derepressed spontaneous mutants.

The preparation of P22 lysate and transduction cross were performed as described in Ref. [10]. The mutation rates were calculated using the method of median[11].

For PCR amplification, DNA cloning and sequencing refer to Ref. [12]. According to the nucleotide sequence of *purD*, a pair of primers was designed to amplify the DNA fragment containing the *purD* PUR box. The sequences of the primers are shown below (the shadowed are the first two bases of the PURbox):

Primer 1 (Forward) 5′-AGA AAGCTTCGCGAGCGTT GC-3′
*Hin*d III

Primer 2 (Reverse) 5′-GGC GAATTCGATGATACCGGC-3′
*Eco*R I

The chromosomal DNA of the spontaneous PUR box mutant was used as the template for PCR amplification. The 210 bp PCR product (Fig. 1) was digested by *Eco*R I and *Hin*d III and ligated with pUC19/*Eco*R I + *Hin*d III.

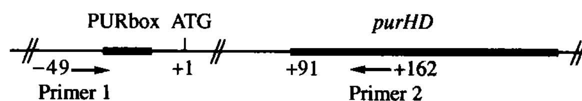

Fig. 1. Primers design for the PUR box amplification.

purR gene is 1467 bp in length. In order to reduce the errors in PCR reactions, we designed 3 pairs of primers according to the nucleotide sequence deposited in GenBank (Accession No. AF040636) and the *purR* gene was amplified in 3 fragments (Fig. 2). The primer sequences are shown below:

Primer 1 (C_{11} ~ A_{21}): 5′-CACAAAAAGTGATATAGCGCA-3′,

Primer 2 (complemented with A_{664} ~ C_{685}): 5′-GGTCTAGAATGGAAAGCAGAGGTT-3′;

Primer 3 (T_{537} ~ T_{556}): 5′-TTCCAGAAAGGCTATACGCT-3′,

Primer 4 (complemented with T_{1014} ~ C_{1037}): 5′-GCATCTAGATAATATCGCCGCCGCA-3′;

Primer 5 (G_{971} ~ C_{991}): 5′-GCAGCAAATTTTATCGCAGTC-3′,

Primer 6 (complemented with A_{1436} ~ T_{1455}): 5′-ACGTTAGAGATCGTTCCGAT-3′.

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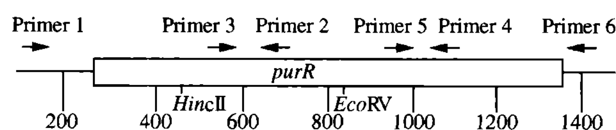

Fig. 2. Primers design for *purR* amplification. *purR* coding region is base pairs 282 ~ 1307; fragment 1 (675 bp) was amplified using Primers 1 and 2; fragment 2 (501 bp) was amplified using Primers 3 and 4; fragment 3 (485 bp) was amplified using Primers 5 and 6. *Hinc*II and *Eco*RV restriction sites are in fragment 1 and fragment 2, respectively.

The chromosomal DNA of the spontaneous *purR* mutant was used as the template for PCR amplification. The above three amplified fragments were cloned into pUCmT-vector respectively. The inserted DNA fragments were verified by enzyme digestion and their mutation sites were determined by DNA sequencing.

2 Results

2.1 Isolation of spontaneous mutants of the PUR box and *purR*

Strain 5 ~ 28 carries a super-repressing mutant of *purR* ($purR^s$). Because the expression of *lac* genes is strongly repressed by the $purR^s$ super-repressor, $purR^s$ mutant strain cannot grow on NCE medium with lactose as the sole carbon source[13], therefore once colonies appear on lactose minimal plates, they should be the derepressed mutants. Their growing is due to the $purR^s$ gene or the PUR box undergoing spontaneous mutations that relieve the repression by $PurR^s$. Based on the genetic instability of $purR^s$, we established a method to isolate spontaneous *purR* and PUR box mutants. According to the method described in 1.2, colonies appeared in 48 h were picked out and streaked on the repressed EMB + Kan plates. On this plate, the derepressed mutants are red. In order to keep the independency of each mutation, only one red colony was selected from each culture and ready for further research. By this procedure, we obtained 86 independent derepressed mutants. As the random mutants occurring during exponential growth need 48 h to form visible colonies on the minimal medium, these mutants are spontaneous mutants occurring randomly.

2.2 Identification of the spontaneous derepressed mutants

Strain TGM463 ($purR^+$) carries a copy of mini-Tn10 near *purR* (*tet* is 90% linked to $purR^+$). It forms white colonies on the repressed EMB plate. A P22 lysate grown on TGM463 was used as the donor to transduce the spontaneous derepressed mutants, and the Tet^r transductants were selected on repressed EMB tetracycline plates. If a mutation occurs in *purR*, about 90% Tet^r transductants will form white colonies on repressed EMB plates because *purR* is 90% linked to *tet*. If mutation occurs elsewhere, all the Tet^r transductants will emerge as red colonies, because *purR* and *purD* are respectively located at 31′ and 89′ of the genetic map of *S. typhimurium*. Our transduction analysis showed that among the 86 spontaneous mutants, 58 are *purR* mutants. In order to analyze the mutations that were not located in *purR* gene, the P22 lysate grown on each of the rest 28 mutants was used to transduce wild type strain LT2 separately. Kan^r transductants were selected on the repressed EMB kanamycin plates. Since the PUR box and *kan* are 98% linked, if mutations occur in the PURbox, 98% Kan^r transductants will form red colonies. As expected, all of the rest 28 mutants carry mutations in the PUR box. Thus the derepressed mutants can be divided into two sub-types, *purR* mutants and the PUR box mutants. Some of them are listed in Table 1.

2.3 Comparison of the mutation rates of *purR* and the PURbox

The spontaneous mutation rates of the derepressed mutants were calculated according to the method of median described by Von Borstel[11]. The results of two independent experiments are shown in Table 2.

Table 2. Comparison of the mutation rates of the PUR box and *purR*

Experiment	Mutation rate (cell/ generation)		Ratio of mutation rate
	purR	PUR box	(*purR* : PUR box)
1	6.88×10^{-9}	3.32×10^{-9}	6.8:3.2
2	4.69×10^{-9}	2.02×10^{-9}	7.0:3.0

These data shows that under spontaneous conditions, the PUR box also has a high mutation frequency. In contrast with the ratio 7/8:1 of mutation rate under mutagenesis, the frequency of the spontaneous PUR box mutations seems even higher. Thus, high mutation frequency of the PURbox does not result from mutagenesis. Its possible mechanism will be discussed below.

2.4 Nucleotide sequencing of the spontaneous PUR box and *purR* mutants

Five spontaneous mutants of the PUR box (528-

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PB1, 528-PB9, 528-PB12, 528-PB22 and 528-PB25) and four spontaneous mutants of *purR* (528-PR3, 528-PR12, 528-PR15 and 528-PR24) were randomly selected for cloning and DNA sequencing.

The sequence analysis showed that all the PUR box and *purR* mutants contained the corresponding mutation at the expected sites. The distribution of the mutation sites is shown in Fig. 3.

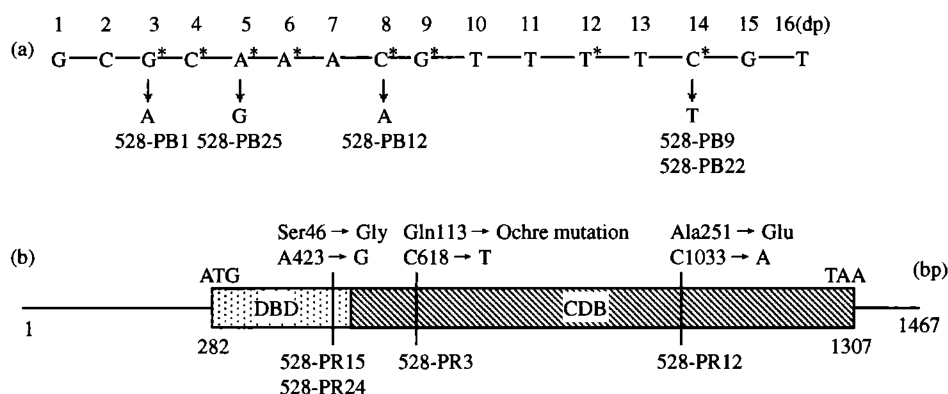

Fig. 3. Mutation sites in five spontaneous PUR box mutants (a) and in four spontaneous *purR* mutants (b). The conserved bases are marked by *. DBD denotes the DNA binding domain, amino acids 1~59 (base pairs 282~458); CDB denotes the co-repressor binding domain, amino acids 60~341 (base pairs 459~1307).

Fig. 3 shows that five spontaneous PUR box mutants have mutations at four sites: G3, A5, C8, and C14. All the mutations were base substitutions, including three transitions of G→A, A→G, C→T and a C→A transversion. The *pur* gene operator (PUR box) is a 16 bp consensus sequence and is highly conserved at eight nucleotides, which are essential for binding with PurR repressor. Our sequencing analysis of the PURbox showed the mutations at 4 out of 8 conserved bases. These mutations cause the loss of the binding function of the PUR box, which in turn rescind the repression of *lac* gene. These mutants can form normal colonies on lactose minimal medium and take on the phenotype of lac^+.

The mutations of four spontaneous *purR* mutants were also single base substitutions. Two of them were A_{423}→G, the other two were C_{618}→T and C_{1033}→A. These nucleotide mutations are corresponding to Ser46→Gly, Gln113→Ochre and Ala251→Glu mutations in PurR. Lu et al.[14] have demonstrated that S46G PurR mutants are defective in DNA binding *in vitro* and in repressor function *in vivo* as well. PurR is a typical allosteric homodimer, and each of its subunit is composed of an NH_2-terminal DNA binding domain connected by a hinge sequence to a larger corepressor binding domain[15]. There are two hydrogen bonds connecting the CO on the side-chain or main-chain of Ser46 to the NH1 or NH2 of Arg115′, respectively, and the third one connects the CO on the main-chain of Gln113 to the backbone NH of Ala49′. These 3 bonds connect the DNA binding domain (DBD) of one subunit with the CBD of the other. They are of great importance in the interdomain signaling[14]. Since 3 out of 4 mutations that we dectected are involved in this interdomain signaling, it can be deduced that there may be some "hotspots" of mutation in *purR*.

3 Discussion

As we know, it is very difficult to acquire O^c mutants because the bacterial operator is usually very small. In our early research of purine metabolism in *Salmonella typhimurium*, it was observed that the mutation frequency of the PURbox was one order of magnitude higher than that of *purR* under mutagenesis[5]. Did it happen because of mutagenesis? In order to answer this question, we studied the mutation rates of spontaneous *purR* and the PURbox mutants. Starting from a novel super-repressed mutant of *purR* ($purR^s$), we easily acquired large amounts of *purR* and PUR box spontaneous mutants. This experimental system not only solved the difficulty in isolating O^c mutants, but also provided important information for the study on the regulation in purine biosynthesis. Our result showed that the frequency of spontaneous mutations in the PUR box was also higher than expected. The ratio of mutation rate of the PUR box to that of *purR* was 3 : 7. This proved that the high mutation rate of the PUR box in our previous experiments did not result from NTG mutagenesis. It seems that a certain mechanism that produces high PUR box mutations under either spontaneous or mutagenic

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conditions exists. Based on our results, we try to explain this interesting phenomenon as follows.

First, the PUR box is a 16 bp palindrome and is highly conserved at 8 bases[7,8]. Our previous research demonstrated that any change of these consensus bases would lead to the loss of binding ability of PurR with the PURbox[5~8]. But as to *purR*, its coding region is 1307 bp in length, which encodes for a typical allosteric protein. The DNA binding affinity of PurR is modulated by guanine and hypoxanthine corepressors which bind with CDB. This process involves a series of allosteric conformational transitions[3,14]. It would be more effective to regulate DNA-protein binding by changing the 16 bp PUR box than the 1.4 kb *purR* gene. This is an economical choice for cells.

Second, PurR is a global regulatory protein[15]. It not only represses the expression of *purD* gene, but also controls the expression of all other genes in *de novo* purine biosynthesis. In addition, PurR regulates the transcription of *glyA*, *gcv*, and *prs*, which encode enzymes for the synthesis of glycine, one-carbon units, and 5-phosphoribosyl-1-pyrophosphate (PRPP), respectively[16]. If cells choose to escape the super-repressed condition by certain changes of $PurR^s$, it will certainly affect other metabolism pathways and the cell's living condition. In this aspect, to escape repression by changing of the 16 bp *purD* PUR box is of great advantages.

Finally, from another point of view, in the complex structure of PurR protein, key residues responsible for co-repressor binding, DNA binding and the interdomain signaling are only a very small part of it[3,14,15]. Mutations at other sites will have little influence on PurR's function. Our sequencing analysis also suggests that there may be some "hotspots" of mutations in *purR* gene. The real target sequence for mutations in *purR* may be much smaller than expected, which leads to the observed high mutation rate of the PUR box.

Besides, in our research in spontaneous mutation, we noticed that in contrast to its spontaneous mutation rate, *purR* has a higher mutation rate in mutagenesis. This may be due to the effect of NTG. NTG is an alkylating agent, and it can add an alkyl to G and T bases. Mutagenesis is a kind of chemical process. The larger the DNA target is, which contains more G and T, the higher the mutation rate would be. Since the mutation target of *purR* is larger than the PURbox, it may have more chances to be mutated in mutagenesis. Thus its mutation frequency is higher under mutagenesis than in spontaneous condition.

Above are only some tentative explanations. It needs further study to clarify the mechanism of this interesting phenomenon and its significance in evolution.

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