行政院國家科學委員會專題研究計畫成果報告
突變種大腸桿 Thioredoxin 支持噬菌體生長之研究

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一、中文摘要

大腸桿菌的 thioredoxin 在細胞中催化重要的氧化還原反應，例如核甘酸的還原。除此之外，此蛋白質對噬菌體 f1, M13, T3/7 的生長亦是必要的。對 T3/7 而言，thioredoxin 是去氧核醣核酸聚合酵素的一個次單元，負責噬菌體去氧核醣核酸的複製。在噬菌體 f1, M13 的組裝過程中，thioredoxin 為必要的蛋白質。本研究將 thioredoxin 的氨基酸 33 與 34 做定點突變，以探討活性區序列對這些噬菌體生長的影響。我們發現 P34G 、P34Y 、及 G33V/P34Y 三種突變株皆降低 f1 和 M13 於 37^o C 生長的平盤效應(E.O.P.)，但下降程度最大只到.3.6 倍。其中 P34Y 突變對 M13 的影響比對 f1 嚴重 3 倍，而其他兩突變對此兩種噬菌體的影響較類似。三種突變對 T3/7 則有較大的影響: P34G 或 G33V/P34Y 突變下降 E.O.P. 10^2 倍，而 P34Y 降低 E.O.P.達 10^9 倍。因此 33 與 34 位置氨基酸的改變對於噬菌體生長有特殊的影響。我們將更進一步研究這些現象。

關鍵詞：Thioredoxin、噬菌體、突變

Abstract

E. coli thioredoxin is important for oxidoreduction reactions in the cells, such as the reduction of ribonucleotides. In addition, the protein is essential for the growth of bacteriophage T3/7, f1and M13. It is a subunit of T7 DNA polymerase that is responsible for the replicatin of phage DNA. The protein is also required for the assembly of phage f1 and M13. In this study, we mutate the residues 33 and 34 in order to investigate the effects of amino acid sequence in the active site of thioredoxin on the growth of these phages. We found that P34G, P34Y, and G33V/P34Y mutants all decrease the plating efficiency (E.O.P.) of f1 and M13 at 37^o C. However, the largest effect is a 3.6-fold decrease in E.O.P.. M13 infects P34Y mutant with three times less efficiency than when f1 does, while both these phages infect another two mutants with quite similar efficiency. On the other hand, all three mutations lower E.O.P.s to a greater extent for T3/7: P34G and G33V/P34Y decrease E.O.P. by 10^2-fold, while P34Y reduces it by 10^9-fold. Therefore, amino acid change at residues 33 and 34 imposes special effect on the growth of bacteriophages. We plan to make further investigation on such phenomena.

Keywords: Thioredoxin, Bacteriophage, Mutation

二、緣由與目的

E. coli thioredoxin is an essential

protein for the growth of certain bacteriophages (1-4). This function of thioredoxin does not rely on the redox property of the protein. The T7 DNA polymerase holoenzyme carries out the DNA synthesis by employing the reduced thioredoxin and T7 encoded gene 5 protein in a 1 to 1 complex (5). Reduced thioredoxin is also required for the assembly of filamentous phages (4). It has been demonstrated that mutant thioredoxin lacking one or both active site cysteines can still support f1 growth (6, 7). For the other two active site residues, residues 33 and 34, Minarik et al. (8) showed that replacement of the conserved Gly33 with Trp resulted in a decrease of E.O.P. to a value of $2*10^{-10}$ for T7, and 10^{-12} for f1 at 37^{o} C. A double mutant Leu33/Gln34 yielded an E.O.P. of 10^{-5} for T7, and 10^{-8} for f1. Russel and Model (4) isolated an *E. coli* thioredoxin P34S mutant strain that had a normal efficiency of plating at 37^{o} C. However, the E. O. P. decreases to 10^{-7} at 42^{o} C (6). In view of the drastic effect of Trp at position 33, and no effect of Ser at position 34 at 37^{o} C, we generated P34/Y, P34/G, and G33V/P34Y three mutants to study the effects of the amino acid change on the growth of bacteriophages.

三、結果

Cloning of trxA

An approximately 400 bp fragment of *trx*A with a 5' *Eco*RI site and a 3' *Bam*HI site was obtained by PCR from pBHK8 plasmid, which has *trx*A and its promoter cloned. The 5' primer (newtrx5') is AGAATTCCCAACACGCCAGGTT (*Eco*RI site underlined) and the 3' primer (trx3') is ACGGATCCGCGACGGGGCACCC (*Bam*HI site underlined). The fragment was cut by *Eco*RI and *Bam*HI, and then ligated to pKS#5(-) vector. The plasmid, named pKS#5(-)trxA, was used to transformation *E. coli* strain XL1-blue. White colonies were selected on X-gal plates with IPTG induction. Plasmids were isolated and sequenced for confirmation.

Site-directed mutagenesis for P34Y mutant

PKS#5(-)trxA was used as template to generate all the mutants by the mutagenesis method of Deng and Nickoloff (9) in this study. For generation of P34Y mutant, the mutagenesis primer consists of sequence 5'GCGATCATTTTGCA*GTA*ACCGCA. The selection primer (called K-B) has a sequence of 5'CGACCTCGAGGGGGGGCCCAGATCTCAGCTTTTGTTCCC3' (*Bgl*II site underlined) that will convert the *Kpn*I site of the pKS#5(-)trxA to *Bgl*II site. A third primer (Trxnde) of sequence 5'GCTCATATGTAACTCCACAGG3' was also added for generating *Nde*I site at the beginning of the coding region. These three primers were phosphorylated by T4 polynucleotide kinase, and annealed to heat-denatured pKS#5(-)trxA. The new mutant DNA strand was synthesized by T4 DNA polymerase and T4 DNA ligase. The primary selection was done by *Kpn*I restriction digestion and transformation of *E. coli* strain BMH71-18. Ampicillin resistant transformants were subjected to the second

selection by digestion of the isolated plasmid with *Kpn*I first and then transformation of DH5α strain. The plasmid, called pKS#5P34Y was isolated from amp-resistant colonies and sequenced to verify the existence of *Nde*I site and the P34Y mutation. This mutagenesis method outlined here also applies to the following mutagenesis work.

To place the mutant *trx*A on pET vector, pKS#5P34Y was cut with NdeI and BamHI restriction enzymes. A 354 bp fragment was recovered and ligated between the *Nde*I and *Bam*HI sites of pET32c(-)-stop to obtain pET32P34Y.

Site directed mutagenesis for P34G mutant

For generation of P34G mutant, the mutagenesis primer consists of sequence 5'TTTGCAC*CC*ACCGCACCACTC. Trxnde and K-B were also used in the mutagenesis reaction as described in the above paragraph. The plasmid that contains the correct mutation as verified by dideoxy DNA sequencing was named pKS#5P34G. The p34G mutant gene was obtained from pKS#5P34G by *Nde*I and *Bam*HI digestion, and then ligated to pET32(-)-stop that has been digested with the same enzymes to generate pET32P34G.

Site directed mutagenesis for G33V/P34Y

The mutant was generated by using a mutagenesis primer of sequence 5'TTTGCA*GTA*A*AA*GCACCACTCTGC as well as primers Trxnde and K-B. The plasmid that contains the correct mutation was confirmed by the DNA sequence, and called pKS#5G33V/P34Y. pKS#5G33V/P34Y was cut with *Nde*I and *Bam*HI to yield the mutant *trx*A fragment. Ligation of this fragment to pET32c(-)-stop generated pET32G33V/P34Y.

Determination of E.O.P.

PET32c(-)-stop carrying wild-type trxA as well as each of the mutants was used to transform *E. coli* strain A179(p1-3) and SK3967 that lack the chromosomal trxA. The resulting transformants A179(p1-3, pET32trxA), A179(p1-3, pET32P34Y), A179(p1-3, pET32P34G), and A179(p1-3, pET32G33V/P34Y) were infected by phage f1 and M13 to determine the change in E.O.P.. The results (Table 1) show that the mutations decrease E.O.P. for both phages. Similarly, SK3967(pET32trxA), SK3967(pET32P34Y), SK3967(pET32P34G), and SK3967(pET32G33V/P34Y) were infected by phage T3/7 to determine the E.O.P.. P34G and G33V/P34Y mutations decrease E.O.P. by 10^2-fold, whereas P34Y reduces E.O.P. by 10^9-fold (Table 2).

四、討論

All three thioredoxin mutants, P34Y, P34G, and G33VP34Y reduce the efficiency

of phage growth. Their effects are larger when infected by T3/7 than by f1 and M13. Plating of f1 on the three mutants gives E.O.P.s 2-fold or less than 2-fold deviation from that on the wild type. Comparison of f1 and M13 shows that P34Y mutation has a larger effect on the infection by the latter; their E.O.P.s differ by approximately three times. However, all these mutational effects are relatively small on f1 and M13 as compared to those on T3/7, which demonstrated a 10^{-9}-fold decrease of E.O.P. for the P34Y mutation. For the other two mutations, E.O.P.s are also at least 10 folds lower for T3/7 than for f1 or M13.

Expression of thioredoxin in A179(p1-3) carrying pET32trxA, pET32P34Y, pET32P34G, or pET32G33V/P34Y has been investigated. When induced by 0.4 mM IPTG and heat shock, their amounts of expression are about the same for the four strains. In determination of E.O.P., the bacteria (SK3967 and A179(p1-3)) carrying the wild-type or the mutant pET plasmid, were induced by 0.4 mM IPTG, albeit no heat treatment, before the infection. We have examined the expression of wild-type thioredoxin under this condition and found that the amount of protein was about the same with or without induction in SK3967. This amount was also similar to the level of thioredoxin expressed by *E. coli* strain BL21. Induction of wild-type *trx*A on pET by 0.4 mM IPTG increased the expression by 2.8-fold in strain A179(p1-3) compared to the same strain without induction or *E. coli* strain K91. Therefore, the over 10-fold difference between E.O.P. of T3/7 and f1 (or M13) is attributed more to the effects of the properties of the mutation than to the amount of the protein expression. It is particularly clear that P34Y mutation is devastating to T3/7. More studies will be carried out in the near future to investigate effects of these mutations.

五、參考文獻

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Table 1. Effects of *trx*A mutations on the E.O.P. of phage f1 and M13.
Wild type and mutant strains were grown to OD_{600} of 0.2-0.3, and then IPTG was added to a concentration of 0.4 mM. The culture was grown to OD_{600} of 0.5, infected by phage, and the plates were incubated at 37° C. The initial titer of the phages were 10^{12} PFU/ml. Results were the average observation from three independent experiments.

	E.O.P.	
	Phage f1	Phage M13
A179(p1-3, pET32trxA)	1	1
A179(p1-3, pET32P34Y)	0.82±0.03	0.28±0.04
A179(p1-3, pET32P34G)	0.61±0.03	0.67±0.03
A179(p1-3, pET32G33V/P34Y)	0.44±0.03	0.33±0.03

Table 2. Effects of *trx*A mutations on the E.O.P. of phage T3/7.
Wild type and mutant strains were grown to OD_{600} of 0.3-0.4, and then IPTG was added to a concentration of 0.4 mM. The culture was grown to OD_{600} of 0.5-0.6, infected by phage, and the plates were incubated at 37° C. The initial titer of the phages were 10^{10} PFU/ml. Results were the average observation from three independent experiments.

	E.O.P.
	Phage T3/7
SK3967(pET32trxA)	1
SK3967(pET32P34Y)	$6x10^{-9}±4x10^{-10}$
SK3967(pET32P34G)	$2.3x10^{-2}±4x10^{-2}$
SK3967(pET32G33V/P34Y)	$1.3x10^{-2}±9x10^{-3}$