Chloromycetin resistance of clinically isolated *E. coli* is reversed by using EGS technique to repress the chloromycetin acetyl transferase

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AIM: To explore the possibility of repression of chloromycetin (*Cm*) acetyl transferase by using external guided sequence (EGS) in order to reverse the clinical *E. coli* isolates from *Cm*-resistant to *Cm*-sensitive.

METHODS: EGS directed against chloromycetin acetyl transferase gene (*cat*) was cloned to vector pEGFP-C1 which contains the kanamycin (*Km*) resistance gene. The recombinant plasmid pEGFP-C1+EGScat1+cat2 was constructed and the blank vector without EGS fragment was used as control plasmids. By using the CaCl₂ transformation method, the recombinant plasmids were introduced into the clinically isolated *Cm* resistant but *Km* sensitive *E. coli* strains. Transformants were screened on LB agar plates containing Km. Extraction of plasmids and PCR amplification showed the existence of EGS plasmids in these four transformed strains. These results indicated that the Cat of the four clinical isolates had been suppressed and the four strains were converted to *Cm* sensitive ones.

CONCLUSION: The EGS directed against Cat is able to inhibit the expression of Cat, and hence convert *Cm*-resistant bacteria to *Cm*-sensitive ones. Thus, the EGS has the capability of converting the phenotype of clinical drug-resistant isolates strains to drug-sensitive ones.

Key words: External guide sequence; Drug-resistant bacteria; Conversion of drug resistance

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INTRODUCTION

Drug resistance in pathogenic bacteria is a problem of major clinical importance, which has not been effectively solved yet. The traditional approach to this problem is searching for novel antibiotics. However, this is expensive and time consuming, and the bacteria can quickly develop drug resistance to these novel antibiotics. With advances in gene therapy, ribozyme technology[7-10] appeared following antisense RNA technique[11-13], which though is abstract of the activity of catalysis and cleavage. Then, external guide sequence (EGS) technique[7] was found, with the advantages of both ribozyme and antisense RNA. EGS, a synthetic gene coding for small oligoribonucleotides, is able to form complexes with the mRNA encoded by target gene. The complexes are recognized as substrates of RNase P. Then, EGS directs RNase P to cleave and inactivate the target mRNA. Therefore, the EGS technique is a method to block a particular gene by inhibiting the translation of its mRNA[12-14]. In 1997, by designing EGS directed against chloromycetin (*Cm*) acetyl transferase (*cat*), Sidney Altman successfully applied EGS technique in engineered bacteria to convert the *Cm*-resistant bacteria into *Cm*-sensitive.
The competence cells of testing bacteria were mock-transformed (without plasmid) and served as strain controls. Competent cells were inoculated on LB plates containing 50 μg/mL of Km after transformation, followed by incubation at 37 °C for 12-16 h; (2) 

**Extraction of plasmids and total DNA**

Plasmid was extracted by using alkaline cleavage method[19] and total DNA was extracted by using the boiling method[20]. Positive clones were selected and transferred into Eppendorf tubes containing 20 μL sterilized water and boiled for 10 min. The supernatant was collected after centrifugation; (3) 

**PCR amplification**

The primers were designed specifically for the inserted EGS on the vector. The amplified fragment containing EGScat1 and cat2 was 391 bp. The primer pairs used for PCR amplification were 5'-AGCCTGACCGATGATGTTG-3' and 5'-TCCTCAGGGACTCTC AGAC-3'. The PCR reactions were performed at 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1.5 min, total 30 cycles. The PCR products were electrophoresed on a 1.5% agarose gel; (4) 

**Cm sensitivity test of the transformants**

After transformation, the colonies grown on plates and the original colonies were re-inoculated on the LB plates containing 200 μg/mL of Cm and plates containing 50 μg/mL of Km, and incubated at 37 °C for 8-12 h. Then, their growth conditions on these two plates were checked and recorded; (5) 

**Growth determination of transformants in liquid broth**

The colonies (K3, K plasmid transformants and original bacteria) were separately inoculated into 200 mL of broth culture containing Cm (200 μg/mL) with the inoculating concentration A0 = 0.05, and incubated in an orbital shaker at 37 °C and 200 r/min. A 0.5 mL bacterial suspension was sampled hourly and its A value was determined. Growth curve was plotted according to the constantly collected A values. The transformants were also incubated at the same concentrations into PA bottles at 37 °C and the bacterial growth was observed hourly as well.

## RESULTS

### Study of the partial phenotypes of the clinically isolated E coli strains' rates of spontaneous mutation

Sixty-one Cm-resistant but Km-sensitive E. coli strains were clinically isolated. Of them, 16 strains were selected as test strains for their comparatively low simultaneous mutation rate. However, the test strains sensitive to Km were still suitable to present the spontaneous mutation and hence obtain Km resistance again, and then Gram stained for morphology check. These steps were repeated until pure bacterium strains were obtained[16]; (2) 

**Extraction of plasmids of the test bacteria and determination of plasmid incompatibility**

The methods for plasmid extraction and incompatibility determination were described by Huang[17]; (3) 

**Determination of spontaneous mutation rate of the test bacteria**

The spontaneous mutation rate of testing bacteria to Km was determined for colonies grown on LB and LB+Km plates by using dilution plate counting method[18]; (4) 

**Determination of logarithmic growth stage of test bacteria**

Growth curve was plotted based on bacterial optical density (A00) [10].

### Transformation of test bacteria by pEGFP-C1-EGScat1+cat2 recombinant plasmids and identification:

(1) 

**Transforming test**

The competent cells of testing bacteria were prepared by using CaCl2 method, and were transformed respectively with plasmids K3 and K[20]. The transformants that received K3 plasmid were designated as testing bacteria, and those transformed with K plasmid without EGS were used as plasmid controls. The competent cells from original testing bacteria were mock-transformed (without plasmid) and served as strain controls. Competent cells were inoculated on LB plates containing 50 μg/mL of Km after transformation, followed by incubation at 37 °C for 12-16 h; (2) 

**Extraction of plasmids and total DNA**

Plasmid was extracted by using alkaline cleavage method[19] and to...
plasmid into the cells. Furthermore, the DH5α derivatives transformed with the plasmids extracted from test bacteria were hard to be transformed again with K3 and K. This suggested the plasmid incompatibility between original plasmids and K or K3 plasmids.

**Determination of logarithmic phase** The bacterial growth curve revealed that the logarithmic growth stage was from 4 to 8 h after being inoculated in the broth. In this study, the fresh culture of test strains was harvested in this period to prepare competitive cells.

**Growth rate of the transformed bacteria in liquid broth culture** Four strains, 16, 20, 3,900, and 6,470 were transformed with K3 successfully. Kinetic growth study demonstrated that these four strains transformed with K3 plasmid exhibited growth inhibition in the broth containing 200 μg/mL of Cm (Figure 1). The growth inhibition became apparent at 2 h and peaked at 3 and 4 h, but active growth resumed at 5 h. In contrast, neither bacteria transformed with K plasmid nor original bacteria with mock-transformation showed any growth inhibition in the incubation condition described above. Their concentrations increased steadily as demonstrated by As600 values. Although the number of bacteria transformed with EGS began to elevate at 5 h, the degree of growth was much lower than that of the original bacteria. In PA bottles, the K3 plasmid transformed bacterial culture was still clear at 6 h to the naked eye and started to be turbid at 8 h, while the culture for K plasmid transformed bacteria or mock-transformed original bacteria started to be turbid at 3 h and was highly turbid at 6 h. The above findings demonstrated that EGS gene was able to convert bacteria from Cm-resistant to Cm-sensitive phenotype. All of the four strains that obtained their phenotypic conversion of drug resistance showed a lower spontaneous mutation rate, which may contribute to their easy transformation because of their stability. They exhibited very few variant strains when cultured on Km plates, and thus the transformants could be easily identified and collected.

**Drug sensitivity test of transformed bacteria** Drug sensitivity test showed that the four strains transformed with K3 plasmid not only obtained sensitivity to Cm in liquid broth culture but also became sensitive to Cm in LB+Cm (200 μg/mL) solid culture (as indicated in Figure 2). The bacteria transformed with K plasmid and the original isolates grew well in LB+Cm (200 μg/mL) solid culture.

**Determination of plasmid extraction from testing bacteria transformed by pEGFP-C1-EGS cat1+cat2 and identification of clones by PCR**

**Determination of the plasmids in transformed bacteria** Four strains of transformed bacteria were subjected to the determination of the plasmid extraction. Figure 3 is the map of rapid extraction of plasmid and Figure 4 is the map of plasmid extraction after the transformants were cultured for 2 d. Figure 3 shows that the K and K3 can be detected in the strains transformed with K and K3, respectively. This indicated that K3 and K plasmids had been introduced into the cells successfully. In Figure 4, TK had the same bands as K, while TK3 had no similar bands as K3, indicating that the K3 plasmid was lost or had been degraded after 2 d of culturing. This may explain why the transformant retained its growing capacity gradually after several hours of culturing in the broth medium.

**Identification of EGS gene by PCR** The colonies transformed with K3, which did not grow on LB+Cm (200 μg/mL) plate (restoration of Cm sensitivity) but grew well in the corresponding LB+Km (50 μg/mL) plate, were
Mutation is a common phenomenon present in microorganisms. The clinically isolated bacterial strains are characterized with even higher spontaneous mutation rate than lab strains. In the early period of our experiments, it was found that the competent cells of the control bacteria sensitive to Km not transformed with plasmids and originally sensitive to Km still grew on the Km plates. At first, it was mistakenly rendered as the result of impurity of test strain. After further purification and spontaneous mutation study, we understood that it has resulted from high mutation rate of the clinically isolated bacteria. Mutated test strains obtained Km resistance, lost Km-sensitiveness, and therefore made selecting transformants difficult. To solve this problem, our efforts focused on selecting stable strains with low spontaneous mutation rate. At the end of the procedure, 16 comparatively stable strains were screened from the clinically isolated bacterial strains.

In this study, only 4 of 16 test strains were converted from drug-resistant to drug-sensitive phenotype. The low efficiency of conversion may be ascribed to the low transformation frequency. Firstly, as *Enterobacteriaceae*, test strains were hard to be transformed for their native low transformation frequency. Secondly, the low transformation rate can be contributed to the existence of host plasmids in test strain which may result in plasmid incompatibility. Thirdly, although the criteria for screening positive transformants in the culture medium containing Km were set up, not all of the transformed bacteria were desired transformants due to the spontaneous mutation of the testing bacteria. We believe that even if we select other sensitive antibiotics to testing bacterial strains, spontaneous mutation would also happen. So suitable methods to screen positive transformants were abstract. Km resistance in this study did not function as an effective selection marker due to the spontaneous mutation of the test strains. To increase the efficiency of conversion, we tried to apply one-step method and electroporation transformation method, but the results were also not satisfactory. Although we can construct recombinant plasmids and insert a fluorescence enzyme's gene into the vector (EGFP site at pEGFP-C1 vector) to aid in screening, it may increase the transforming difficulty or render the transformed bacteria. In order to reduce the false positive clone in transformation assay, the original bacteria with high spontaneous mutation rate were excluded. At the same time, we found that the efficiency of transformation could be enhanced by using SOC medium and by collecting cell culture at early logarithmic phase.
PCR amplification was used to identify positive clones. In contrast to the spontaneously mutated bacterial strains, the transformants obtained through transformation grew slowly on the Km plates. On the other hand, long time culturing resulted in the loss of plasmids. Thus, it was difficult to identify the EGS transformants by plasmid extraction. In this study, PCR was applied to identify the presence of EGS in the EGS transformed bacteria and it worked effectively. Certainly, further study is to perform Northern blot to know the attenuation of mRNA transcribed from cat gene, which could further definitely verify the roles of EGS. As our conditions are limited, the experiment was not done.

EGS as a sort of synthesized oligonucleotides can combine the target RNA to form the substrate similar to pre-tRNA, which can be cleaved by RNase P and lose the ability of further gene expression. The targeted mRNA is cleaved but EGS itself is not cleaved[1]. EGS can function continuously. In this study, however, we found that the positive clones regained the Cm resistance after being cultured in broth containing Cm for 8 h and then on solid culture for 2 d. No EGS was detected and no plasmid could be extracted in clones that regained Cm resistance. This demonstrated that EGS plasmid had been lost in the host because of continuous culture. The reason might be due to an incompatibility between indigenous and exogenous plasmids.

Our experiments demonstrated that culturing on Km plates facilitated EGS plasmid inheritance, while culturing on the Cm plates was prone to result in the loss of EGS plasmid. This study also suggested the need for more effective methods to introduce EGS into the bacterial cells and to maintain its stability. Though a lot of challenges were involved in this study, transformants were still obtained. The results reflected the effectiveness of the EGS transforming drug-resistant bacteria, and provided a foundation for the clinical application of EGS.

In recent years, although RNA interference technique has been developed, no RNA interference phenomenon was observed in prokaryotes[27-30]. Thus, the EGS technique is still the most prospective way to solve bacterial drug resistance. As to the development of EGS technique, Sidney Altman put forward that enhancing the promoter, increasing the copy of EGS, augmenting the binding sites of EGS and stabilizing the EGS-mRNA complex are the potential methods. Furthermore, combined with nanotechnology, the EGS technology could be more promising. Conclusively, to improve this study, more measures should be explored for further investigation.

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