Antisense 기법을 이용한 대장균에서의 단백질 생산수율 향상

김 영환, 차 형준
포항공과대학교 화학공학과

Development of Improved *Escherichia coli* system for high-yield protein production using antisense technique

Jaoon Y.H. Kim and Hyung Joon Cha
Department of Chemical Engineering, POSTECH

Introduction

Here we report that an *E. coli* vector system, capable of improving noticeably the yield of protein expression, was newly devised. It has been assumed that the accumulation of acetate has been troublesome on protein production and cell growth in the high density culture of E.coli due to its lipophilic and acidic properties. Various trials such as mutation of genes coding enzymes of acetate pathway or redirection of carbon flux from pyruvate to another metabolite have been done up to now (1, 2, 4). In this study, we used antisense gene coding an enzyme of major acetate pathway to regulate acetate formation. This strategy has been targeted on the construction of microbial system that is able to use efficiently its energy and material resources for protein expression by antisense down-regulation of overproducing acetate at the specific time. Three kinds of plasmid with gene each coding antisense *ack* (acetate kinase), *pta* (phosphotransacetylase), and both were constructed. Green fluorescent protein (GFP) was used as a foreign protein for monitoring protein expression level under *trc* promoter. These plasmid systems were tested for several host strains. The protein expression level increased 2.5times more than the control system with only GFP under *trc* promoter. The cell growth level also showed better tendency in the case of plasmid with antisense. Acetate decreased to below 50% of control system that does not have antisense gene at the stationary phase. Different with the previous assumption (2), it can be deduced that acetate itself could not be the major inhibition on protein expression. It could be concluded that the energy for overproduction of acetate is redistributed to higher expression of foreign protein. These results have also demonstrated that the antisense regulation can be a better alternative strategy as a metabolic engineering tool than mutation or flux-redirection method.

Materials and Methods

E. coli Top10 was used for cloning *GFPuv* gene and antisense strand for *ackA* and *pta* into plasmid. BL21 and W3110 harboring constructed plasmids were used as host strain for investigating of protein expression level. Each antisense strand of ackA and pta, promoter of ackA-pta operon and terminator gene were obtained from K-12 chromosome by PCR. *GFPuv* gene was taken from the plasmid pGFPuv (Clontech) also by PCR. All of these genes cloned into the multi-cloning site of the plasmid pTrcHisC (Invitrogen).

Host strains were grown at 37℃ in a defined medium M9(6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl per liter) with 50㎍/㎖ ampicillin, and 10g/L glucose. Overnight cultures(18hrs) at 37℃ in LB medium were used to inoculate into 500 ㎖ erlenmeyer flask containing 150 ㎖ of M9 media above. strains were cultivated at 37℃, 250rpm. At OD ≅ 0.6, *E.coli* strains were induced by the addition of 1 mM IPTG for the protein expression.

Cell growth was monitored by optical density (at 600 ㎚, OD 600) using a UV/VIS spectrophotometer(UV-1601PC, shimadzu). The GFPuv assay was performed by measuring fluorescent intensity using a fluorospectrophotometer(RF-5301, shimadzu) at an excitation wavelength of 395 ㎚ and emission of 509 ㎚. The samples used for measurement were obtained directly from M9 culture broth. Acetate concentration was measured on a gas chromatograph equipped with a flame ionization detector and INNOWAX(Hewlett-Packard) column. The samples used for this measure were prepared by filtering through 0.45㎛ filter paper. The column temperature was 170℃, injector temperature was 245℃, detector temperature was 245℃. The carrier gas was N_2 (40 ml/min).

Results and Discussion

Figure 1 shows the photo image comparing fluorescent intensity of each plasmid-harboring *E.coli* W3110 strain. The image was photographed using UVP image capture system under the UV illuminator. As showed in this image, the host with plasmid containing antisense gene emitted more intense fluorescent light than the wild type with just *GFPuv* gene. Especially the case of anti-*ackA* gene expressed the most

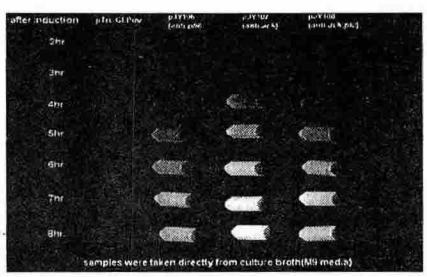

Fig. 1. compared intensity of green fluorescence illuminated by UV

intense light.

We investigated quantitative intensities using fluorescence spectrophotometer. The expression level in the cases of antisense *ackA*, *pta* and both contained plasmid was 1.7(~2.5) times higher than wild type with just *GFPuv* expressed (Fig.2.). Concentration of Acetate was checked in the case of W3110 strain. Antisense host regulated with antisense *ackA*, *pta* and both showed less excreted acetate level. The concentration is below the 50% of wild type W3110 (Fig.3.).

We observed that cell growth was not influenced negatively even in the case of 1.7 times more expression than wild type (Fig.4.).

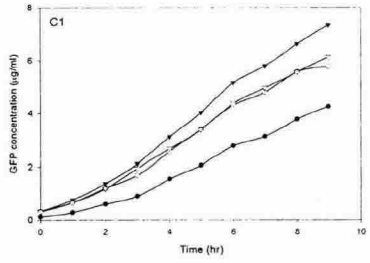

Fig. 2. GFP expression level of 4 kinds of strain

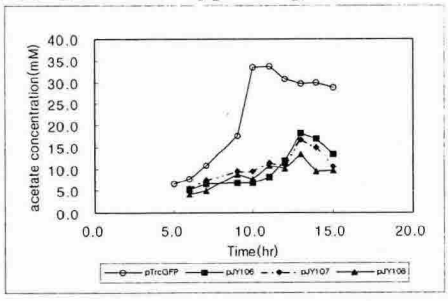

Fig.3. concentration profile of acetate for W3110

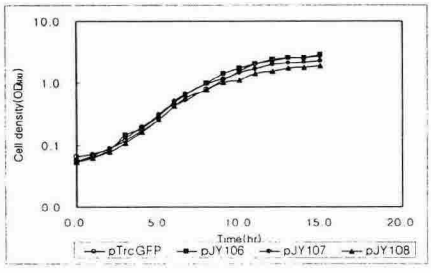

Fig. 4. growth profile of *E.coli* W3110

These experiments were done for another strain, *E.coli* BL21 known as suitable for the expression of recombinant protein. We also found that our vector system is worked for this strain. BL21 showed similar tendencies with W3110 in the profile of growth and protein expression although it was reported these strains have different characters (W3110 is derivative of K-12 and BL21 is derivative of B strain.) (5). The improvement is less than the case of W3110. In the case of BL21 the fluorescent intensity was improved up to 1.7 times

more than wild type (Fig. 5. & 6.).

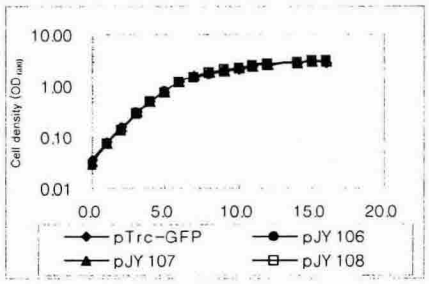

Fig. 5. growth profile of BL21

Fluorescent intensity in 9hrs after induction

pTrc/GFPuv	167.05
pTrc/GFPuv:AsPta	258.82
pTrc/GFPuv:AsAck	271.85
pTrc/GFPuv:AsAck:AsPta	259.44

Fig. 5. GFP expression level in BL21 harboring each plasmid

It has been reported previously that culture of *E. coli* to the high cell density is prohibited by the acetate accumulation and assumed that it is due to the properties of acetate itself. Thus various mutant strains for acetate pathway and other pathway for the purpose of reduction of acetate itself or redirection from acetate path to another were investigated for improving production of recombinant protein (1, 2, 4). However the effects of these trials were less than expected. It has been reported that 10~30% of carbon flux is excreted to acetate at the late growth or stationary phase (1). It is certain that the acetate is not good for cell, but the path plays an important role in the cell physiology as considering the high portion which it holds. From the results of our experiment, the inhibibitory effect of acetate on protein production is caused by overconsuming of cellular energy due to overproduction of specific metabolite. It was also reported that Acetyl phosphate, the intermediate material of acetate pathway, important factor in the cellular process (3). Thus in this case, the knock-out mutation may not be a proper tool for solving the problem. The results showed that antisense regulation can be a better method as a metabolic engineering tool. It is considered that this method is suitable for elucidate the detailed metabolism.

References

1. Farmer, W.R. and Liao, J., *Appl. Environ. Microbiol.*, **63**, 3205-3210 (1997).
2. Bauer, K. A., Ben-bassat, A., Dawson, M., Neway, J. O., *Appl. Environ. Microbiol.*, **56**, 1296-1302 (1990).
3. Prűß, B. M., *Arch. Microbiol.* **170**, 141-146 (1998).
4. Aristidou, A. A., San, K. Y., Bennett, G. N., *Biotechnol. Prog.*, **11**, 475-478 (1995).
5. Shiloach, J., Kaufman, J., Guillard, A. S., Fass, R., *Biotechnol. Bioeng.*, **49**, 421-428 (1996).