# High Glucose Inhibits the Expression of EtcABC Which Resulted in Negative Regulation of Type III Fimbriae in *Klebsiella pneumoniae* Isolates

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**Abstract.** *Klebsiella pneumoniae* is one of abundant normal flora in human, but also well-studied as an opportunistic pathogen due to its ability to form biofilm. Biofilm could be formed by biofilm-forming bacteria as its survival method. Valid example for biofilm-associated medical issue is catheter-related diseases, such as urinary tract infection (UTI). In order to form the biofilm, bacteria utilize the fimbriae in order to form the attachment of bacterial cell and surface of medical equipments. *Klebsiella pneumoniae* has two types of fimbriae, type I and type III, which play big role in regulating its biofilm formation. We previously found that the expression of type III fimbriae and biofilm formation in *K. pneumoniae* strain STU1 were tightly regulated by cAMP-CRP pathway, and significantly increased by the overexpression of EtcABC, the component of bacterial phosphoenolpyruvate (PEP):carbohydrate phosphotransferase systems (PTS). Interestingly, by RT-qPCR and Western Blotting, we found that the expression of EtcA in STU1 (the homologue of KPN00353 in *K. pneumoniae* strain MGH78578) was reduced significantly by high glucose. The reduction of EtcA which was predicted to have role as an EIIA in PTS, significantly reduced the expression of MrkA, the main protein for type III fimbriae assembly and formation. We therefore took *K. pneumoniae* isolates isolated from Tzu Chi hospital in Hualien, Taiwan to follow the observation we found in STU1 and found the same pattern that the higher the concentration of glucose added in the bacterial culture medium, the less the expression of EtcA and MrkA. In diabetic mellitus patients, *K. pneumoniae* was often to be the causing agents of pyogenic liver abscess (PLA). From this point of view, we found interesting scenario that probably could be the mechanism of *K. pneumoniae* infection in diabetic mellitus patients. Our *in vitro* data strongly suggested that high glucose levels negatively affected the type III fimbriae expression and biofilm formation in *K. pneumoniae* STU1 and clinical isolates. Further study is indeed worth to be done for unraveling the unknown molecular mechanisms of *Klebsiella* biofilm formation and infections in certain type of patient conditions.

**Keywords:** biofilm formation · bacterial type III fimbriae · MrkA protein · virulence factor

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# 1 Introduction

Biofilm formation plays an important role in regulating the virulence of *K. pneumoniae* [1]. *Klebsiella pneumoniae* is a Gram-negative, non-motile, encapsulated, rod-shaped bacterium, and one of the lactose-fermenting bacteria. *K. pneumoniae* is one of the most frequent resistant bacteria worldwide. The ability of some *Klebsiella* organisms to produce extended-spectrum beta-lactamases (ESBL) will cause the resistance to beta lactam antibiotics [2]. The infections caused by *K. pneumoniae* occur in humans of all ages. However, the highest risk groups appear to be infants, the elderly and the immunocompromised. There are several infection diseases caused by *K. pneumoniae*, such as pneumonia, bronchitis, upper respiratory tract infection, urinary tract infections (UTIs), diarrhea etc. [3]. There are several factors in bacteria and its growth environment which promote the formation of bacterial biofilm [4–6]. The expression of bacterial fimbriae is one of the most important factors which support the surface-bacterial adhesion or attachment in the very early stage of biofilm formation [7]. *K. pneumoniae* possesses two types of fimbriae, type I (encoded by *fimAICDFGH*) and type III fimbriae (encoded by *mrkABCDF*) [8]. According to previous reports, type III fimbriae enhanced the biofilm formation of *K. pneumoniae* [1, 9]. In our previous study, we have demonstrated that the overexpression of *etcABC* enhanced the formation of biofilm in *K. pneumoniae* STU1 and two clinical isolates [10, 11]. Our previous data strongly proved that the enhanced biofilm formation was due to the increased expression of type III fimbriae in *K. pneumoniae* overexpressing *etcABC* [11]. Strongly proven, the overexpression of EtcABC increased both mRNA and protein expression of MrkA, the major unit of type III fimbriae in *K. pneumoniae* strain STU1. EtcA, EtcB, and EtcC in *K. pneumoniae* STU1 were found as the homologues of KPN00353, KPN00352, KPN00351 respectively in *K. pneumoniae* MGH 78578 [12]. Via utilizing the genomic information of randomly selected ten *K. pneumoniae* isolates in NCBI database, we have previously demonstrated that the locus of *etcABC* genes is common in *K. pneumoniae* isolates. However, we found a difference in the upstream area of open reading frame *etcA*. EtcA, the homologue of KPN00353 in *K. pneumoniae* MGH 78578, was predicted as the homologue of EIIA of PTS components in *K. pneumoniae* [12]. Crr, the common glucose specific EIIA ($EIIA^{Glc}$) in bacteria, was reported to have interaction to and activate adenylyl cyclase (AC, encoded by *cyaA*) [13]. The activation of AC will cause the increase in cyclic-AMP (cAMP) level which synthesized from ATP [13, 14]. In *K. pneumoniae* STU1, we demonstrated that the overexpression of EtcABC increased intracellular cAMP levels [11]. The effect of EtcABC overexpression showed that EtcABC could compensate the function of Crr in *K. pneumoniae*. The higher cAMP levels also affected cAMP receptor protein (CRP) which was reported to regulate type III fimbriae of *K. pneumoniae*. Shortly, we have proven that the overexpression of EtcABC in *K. pneumoniae* could promote and strengthen the positive roles of cAMP-CRP in regulating type III fimbriae [11]. The relation between cAMP and glucose concentration is well studied, that the higher glucose present in the medium and uptaken to the bacterial cells, the lower intracellular cAMP levels [13, 14]. Due to this previous finding of ours, we finally decided to observe any specific changes on *Klebsiella* biofilm formation caused by low and high concentration of glucose purposely provided in the culture. We also observed the changes caused by different levels of glucose concentration on MrkA protein expression

in *K. pneumoniae* isolates, and showed few of the results in this proceeding article. We found that different type of *K. pneumoniae* isolates reacted differently on their MrkA protein expression and biofilm formation due to glucose concentration added to the culture medium. We hypothesized that the differences on *etcA* upstream sequences (the sequences of *etcA* predicted promoter area) may cause this effect differently on *Klebsiella* biofilm. Probably, this area is the binding site of transcription factor that affect the transcription of *etcA*, *etcB*, and *etcC* which might be glucose concentration dependent.

## 2 Material and Methods

### 2.1 Bacterial Strains, Plasmids, Primers and Growth Conditions

The *Klebsiella pneumoniae* strain and isolates used in this study are summarized in Table 1. Unless otherwise indicated, bacteria were routinely in Luria Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) with or without the addition of 0.1% glucose in the medium for low concentration of glucose and 1% glucose for high glucose concentration.

### 2.2 Quantification of Biofilm Formation

In brief, 3 mL of a bacteria suspension was inoculated into a Falcon polystyrene tube (Medclub Scientific Co LTD, Taiwan) after dilution of the bacteria from an overnight culture with fresh LB to optical density at 600 nm (OD600) of 0.1. After incubation at 37 °C for 24 h, the value of OD600 of the bacterial culture in the tube was measured. Thereafter, the tube was rinsed twice with water. We added 5 mL of 0.1% crystal violet and incubated it at room temperature for 20 min, then rinsed twice with water followed by air-drying. The biofilm formation was quantified by addition of 95% ethanol. The absorbance of the ethanol solution was measured and determined at 595 nm.

### 2.3 Western Blotting

For subtraction of antibodies cross-reacting with *E. coli* proteins, cell lysates of *E. coli* was used to subtract antibodies as described previously [15]. Prior to bacterial harvest, the absorbance of OD600 of each sample was detected and the volume of bacterial culture by centrifugation at 17,000 × g for 3–5 min. Each pellet was solubilized in distilled water with protein sample buffer, followed by incubation for 10–15 min at 100 °C. Samples were separated on a 12% SDS polyacrylamide 63 gel and transferred to a nitrocellulose membrane, Hybond-C Extra (Amersham, U.S.A). Electroblotting was performed using a Mini Trans-Blot semiphor transphor unit (Amersham, U.S.A) run at a constant 0.45 mA/mm2 for 1 h at room temperature using a PowerPac 500/200 power supply (Bio-Rad, U.S.A). The membrane was incubated in blocking reagent (5% skim milk with 1 mL of Tween 20 in 1000 mL of 1 × PBS) at 4 °C for an overnight. The membrane was incubated in blocking reagent added with 5 μL of rabbit anti MrkA as the first antibody with 50 rpm agitation for an hour at room temperature. Then, the membrane

was washed with PBST buffer (1 mL of Tween 20 in 1000 mL of 1 × PBS) under 50 rpm agitation for 15 min at room temperature for three times. The membrane was incubated with PBST buffer added with 0.5 μL of anti-rabbit IgG (whole molecule)-Peroxidase antibody produced in goat (Sigma-ALDRICH®, United States). Then, the membrane was washed with PBST buffer (1 mL of Tween 20 in 1000 mL of 1 × PBS) under 50 rpm agitation for 15 min at room temperature for three times. The protein of interest was detected by the ECL chemiluminescence Western Blotting kit (Amersham, U.S.A). The intensities of the bands will be detected using the gel catcher 2850 chemiluminescence camera system (CLUBIO, Taipei, Taiwan).

### 2.4 Quantitative Reverse Transcription PCR (RT-qPCR)

The purified RNA was treated with 50 U/mL RNase-free DNase I (New England Biolabs, USA) for 30 min at 37 °C to remove residual genomic DNA. The mRNA was reverse transcribed using a QuantiNova reverse transcription kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). To quantify the cDNAs from the transcripts of *16S rRNA*, *recA* or *etcA*, *etcB*, and *etcC*, probe based qPCR was performed in triplicate using fluorescein-labeled and dual-quenched probes (Integrated DNA Technologies, Coralville, IA, USA) and TaKaRa Taq DNA polymerase (TaKaRa Bio, Shiga, Japan). Real-time PCR was performed by a Rotor-Gene real-time genetic analyzer (Qiagen, Hilden, Germany). The gene expression levels were normalized to those of 16S rRNA following the $2^{-\Delta\Delta CT}$ method. The housekeeping gene *recA*, encoding recombinase A, was used as a reference.

## 3 Results

### 3.1 Glucose Concentration in the Culture Medium Affected mRNA Levels of etcA, etcB, etcC and Biofilm Formation of *K. pneumoniae* STU1

Since we previously had found and proven that the biofilm formation in *K. pneumoniae* STU1 required cyclic AMP receptor protein (CRP) and was increased by the overexpression of EtcABC, we thought to try the effect given by glucose in the culture medium on biofilm formation of *K. pneumoniae* STU1 [11]. The higher glucose up taken by the bacterial cells, the lower intracellular cAMP levels will be. Based on our previous finding and the well-known bacterial phenomenon of glucose and cAMP levels, we found our biofilm measurement from *K. pneumoniae* STU1 interesting and promising. The biofilm formation of STU1 in culture medium containing low glucose concentration (0.1% v/v) was significantly higher than that formed in culture medium containing high glucose concentration (1% v/v). Because of this data, we then thought to measure the mRNA levels of *etcA*, *etcB*, and *etcC* of STU1 in different glucose concentration. We found that the mRNA levels of *etcA*, *etcB*, and *etcC* of STU1 were significantly decreased in high glucose concentration condition than those measured from STU1 cultured with culture medium containing low glucose concentration (Fig. 1A). The detection and quantification of mRNA levels of *etcA*, *etcB*, and *etcC* of STU1 in low and high glucose concentration were not supporting the concept of EtcABC overexpression increased bacterial biofilm formation (Fig. 1B).

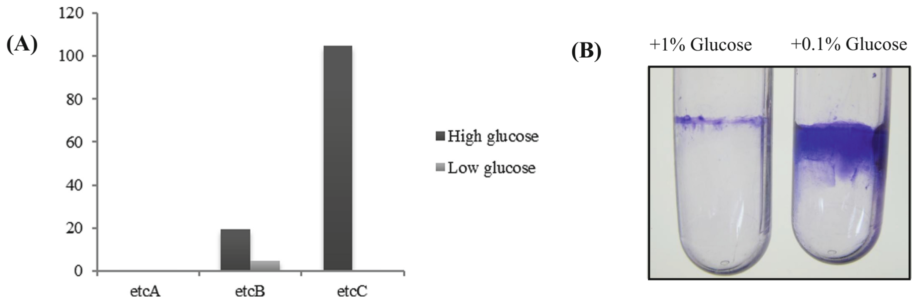


**Fig. 1.** The high glucose concentration (1% v/v) added to the culture medium repressed the mRNA expression levels of *etcA*, *etcB*, *etcC* (**A**) and bofilm formation (**B**) in *K. pneumoniae* STU1. High concentration of glucose referred to LB medium containing 1% glucose. Low concentration of glucose referred to LB medium containing 0.1% glucose. The mRNA expression of *recA* is used as loading control.

### 3.2 High Glucose Concentration Added into the Bacterial Culture Medium Repressed or Negatively Affected the Expression of MrkA, The Major Protein of Klebsiella Type III Fimbriae

We found that the expression of MrkA protein expressed by *K. pneumoniae* STU1 was significantly lower in high glucose concentration than that in low glucose concentration detected by specific MrkA antibody for Western Blotting, while the total protein loaded was similar confirmed by SDS-PAGE (Fig. 2). Therefore high glucose concentration inhibits the expression of MrkA. This phenotype is explainable due to the inhibition of CRP activation in high glucose concentration which reduces the level of cAMP [11]. Thinking about the role of EtcABC overexpression in increasing the cAMP level, we therefore compared the expression of MrkA protein of *K. pneumoniae* STU1 overexpressing EtcABC cultured in medium containing high (1%) and low (0.1%) concentration of glucose. By western blotting, we found that the level of MrkA expressed by *K. pneumoniae* overexpressing EtcABC grown in high glucose concentration is higher than that expressed by the vector control (data not shown). From this current data, we predicted that the overexpression of EtcABC in *K. pneumoniae* stabilized the expression of MrkA protein in LB medium containing high concentration of glucose.

### 3.3 Evaluation of the Importance of the Polymorphisms Found on etcA Promoter Area and Its Relation to etcABC Expression in *K. pneumoniae* Isolates

To strengthen our analysis, we amplified and sequenced the whole upstream region of *etcA* homologues from twenty chosen *K. pneumoniae* isolates in Soo's laboratorium in Tzu Chi University, Taiwan. According to the sequencing analysis that we got, we found that one third of our sequenced *K. pneumoniae* isolates could be grouped in the first group which own AATATATA at the sigma factor RpoD binding site in the promoter area of *etcA* homologues. The other two third isolates own AACATATA or AACTTATA sequences at the promoter area of *etcA* homologues in their chromosomes. Recently, by observing the expression of MrkA protein from different *K. pneumoniae* isolates, we

**Table 1.** The *Klebsiella pneumoniae* strain and isolates used in this study.

| **No.** | Strains of *Klebsiella pneumoniae* | Source of bacterium |
|---|---|---|
| **1** | STU1 | Soo's lab (TCU, Taiwan) |
| **2** | 78012 | Sputum |
| **3** | 78431 | Sputum |
| **4** | 83687 | Blood |
| **5** | 83535 | Blood pyrexia |
| **6** | 105 M 88613 | Blood open w'd |
| **7** | 105 M 90624 | Blood Acidosis |
| **8** | 105 M 89753 | Blood UTI |
| **9** | 105 M 90208 | Sputum pneumoniae |
| **10** | 105 M 97062 | Sputum |
| **11** | 105 M 90249 | Blood APN |
| **12** | 105 M 93687 | Liver abscess |
| **13** | 105 M 92176 | Blood Liver failure |
| **14** | 105 M 92183 | Blood UTI |
| **15** | 105 M 97496 | Sputum |
| **16** | 105 M 91622 | Blood abdominal pain |
| **17** | 105 M 97070 | Sputum |
| **18** | 105 M 16096 | Blood UTI |
| **19** | 105 M 105148 | Blood AMI |
| **20** | 105 M104024 | Blood DM |
| **21** | 105 M105525 | Blood Fever |
| **22** | 105 M 91908 | Blood abdominal pain |
| **23** | 105 M 103759 | Blood UTI |

found the pattern of this particular nucleotide polymorphism according to their grouping (Fig. 3). The first group consisted of *K. pneumoniae* isolates whose promoter area of the *etcA* homologue consists of AATATATA sequences, as observed in *K. pneumoniae* STU1, could not express MrkA protein in high glucose concentration as that expressed in low glucose concentration. *K. pneumoniae* STU1 owns the same sequence and shows the same phenotype. Meanwhile, *K. pneumoniae* isolates whose promoter area of the *etcA* homologue consists of AACATATA or AACTTATA, could express similar expression of MrkA protein in both high and low concentration of glucose. We are currently

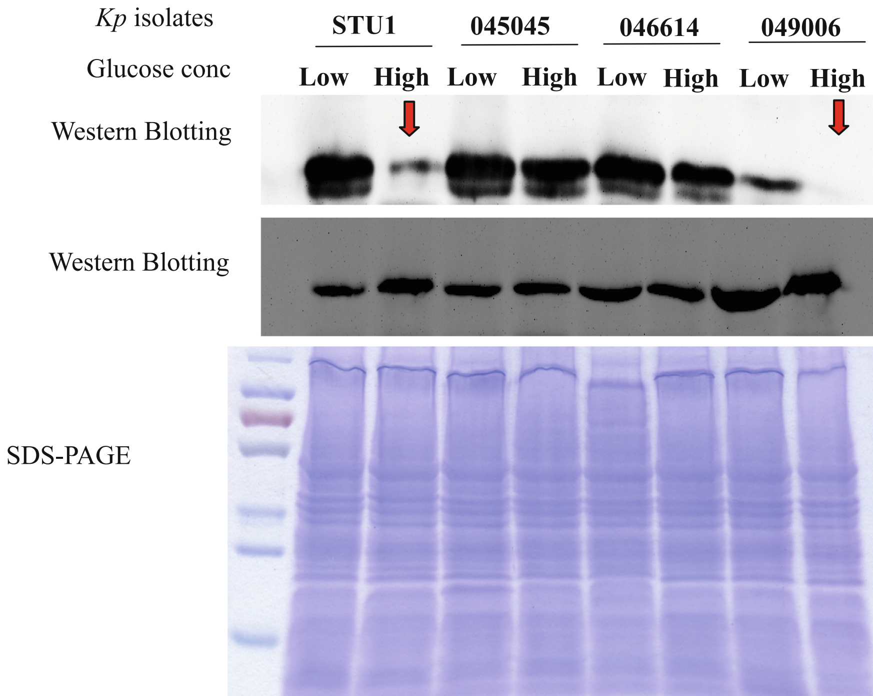


**Fig. 2.** The expression of MrkA from *K. pneumoniae* isolates in medium containing either high or low concentration of glucose. High concentration of glucose referred to LB medium containing 1% glucose. Low concentration of glucose referred to LB medium containing 0.1% glucose. *K. pneumoniae* STU1, 045045, 046614, and 049006 were chosen as the presentative of *K. pneumoniae* isolates. ManA protein expression is used as loading control.

questioning any association between the nucleotide polymorphisms found in the promoter area of *etcA* and the different expression of MrkA among *K. pneumoniae* isolates. We hypothesized that this promoter area of etcA may play some role to affect the mRNA expression of *etcA*, *etcB*, and *etcC*, which also was affected by the presence of glucose in the culture medium.

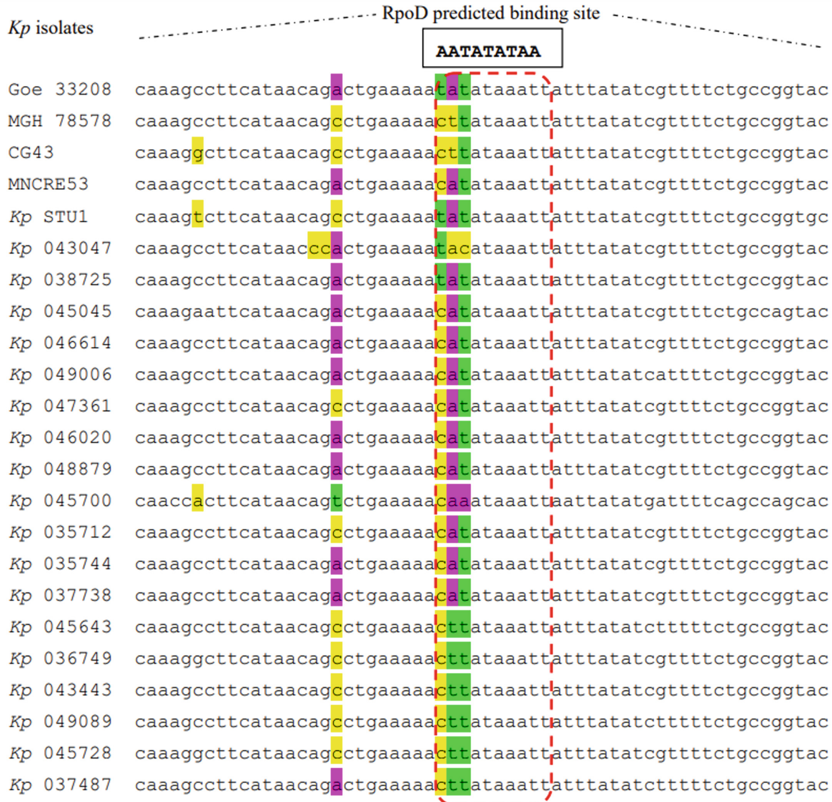


**Fig. 3.** The sequences of the promoter area of *etcA* homologues among *K. pneumoniae* isolates. The sequences of the predicted sigma factor RpoD binding site on the promoter area of *etcA* homologues were found vary (boxed sequences) in *K. pneumoniae* isolates. The data of etcA promoter from *K. pneumoniae* strain Goe 33208, strain MGH 78578, strain CG43, strain MNCRE53 were found from NCBI database. Other than those strains, the data of *etcA* promoter from *K. pneumoniae* STU1 and all the isolates were gotten from the sequencing results.

## 4 Discussion

The expression of PTS related proteins is dependent on their own cognate sugar existence in the growth medium [16]. Our group in Soo's lab in TCU, Taiwan previously had predicted and found that EtcA is the homologous of Crr, the EIIA for glucose uptake based on Phosphoenolpyruvate Phosphotransferase System (PTS) in *Klebsiella pneumoniae* STU1. Therefore, we predicted that the expression of EtcABC could be induced by its cognate sugar also. However, until now, we haven't had enough data to support the cognate sugar for EtcABC system in *Klebsiella pneumoniae.* In our preliminary data, the *etcABC* deletion mutant growed much slower than the wild-type *K. pneumoniae* STU1 in M9 medium with glucose as the sole carbon source (data not shown). However, the growth of *etcABC* mutant was similar with the growth of wild-type in M9 with other

sugar types, such as lactose, mannose, or fructose. Our preliminary data showed that glucose might be the cognate sugar of EtcABC.

The effect of EtcABC overexpression on MrkA expression is dependent on the activated form of CRP, cAMP-CRP (11). We had found CRP binding site on the upstream area of *mrkA* gene. However, in *K. pneumoniae* STU1, we did not figure out whether the analyzed upstream area of *mrkA* was in *mrkA* promoter area or not. However, as the response to the environmental condition, the enhanced EtcABC could affect CRP activation which then regulates type III fimbriae via regulating the expression of *mrkH*. In 2017, Luo et al. has proven that CRP inhibits the expression of *KP1_4563* gene (Luo et al., 2017). Therefore, Luo et al. also predicted that CRP positively regulates the expression type III fimbriae. But, they did not do any work directly to address this matter. In this proceeding article, we showed that the addition of high glucose concentration (to 1% v/v) into the bacterial culture medium decreased significantly the mRNA levels of *etcA*, *etcB*, and *etcC*. Since the overexpression of EtcABC was found to increase MrkA expression and biofilm formation of STU1, we thought to detect this phenotype again in different glucose condition. However, after testing few of *K. pneumoniae* isolates isolated from different patients from Tzu Chi hospital, Hualien-Taiwan, we found that the addition of high glucose concentration did not negatively affect the expression of MrkA of every K. pneumoniae isolates. Therefore, we thought to analyze the promoter area of *etcA t*o understand more the molecular level based on the bioinformatic analysis result. Our analysis showed that in *etcA* promoter area, RpoD binding site was found. However, in several sequenced *K. pneumoniae* isolates, we could notice some pattern of the differences. We are still trying to understand more and unravel the importance of this RpoD binding site in regulating *etcA* expression which probably could answer the difference effect on MrkA protein expression levels cause by high glucose.

## 5 Conclusion

Based on our current data, we found that high glucose concentration in the bacterial culture medium negatively affected the *etcA*, *etcB*, and *etcC* mRNA levels in *K. pneumoniae* STU1, which probably explains the decreased MrkA protein expression which finally inhibits the biofilm formation. However, this phenotype was not found in every *K. pneumoniae* isolates from Tzu Chi Hospital, Hualien–Taiwan isolated from different patients. From three *K. pneumoniae* isolates tried, we found that the high glucose concentration did not affect the protein expression of MrkA from isolates with code *Kp* 045045 and *Kp* 046614. We then thought to analyze the sequences of *etcA*, *etcB*, and *etcC* since the overexpression of EtcA, EtcB, and EtcC increased MrkA expression levels, adhesion ability, and biofilm formation of STU1.

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