



L-glutamine:D-fructose-6-phosphate Aminotransferase as a Key Protein Linked to Multidrug Resistance in *E. coli* KD43162

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Received: 21 May 2015 / Accepted: 27 May 2015 / Published Online: 30 September 2015
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Abstract A microarray study has been employed to understand changes of gene expression in *E. coli* KD43162 resistant to ampicillin, ampicillin-sulbactam, piperacillin, piperacillin-tazobactam, cefazolin, cefepime, aztreonam, imipenem, meropenem, gentamicin, tobramycin, ciprofloxacin, levofloxacin, moxifloxacin, fosfomicin, and trimethoprim-sulfamethoxazole except for amikacin using disk diffusion assay. Using Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and MALDI-TOF MS analyses, 36 kDa of outer membrane proteins (OMPs) was found to be deleted in the multidrug resistant *E. coli* KD 43162. Microarray analysis was used to determine up- and down-regulated genes in relation to multidrug resistant *E. coli* KD43162. Among the up-regulated

genes, these genes were corresponded to express the proteins as penicillin-binding proteins (PBPs), tartronate semialdehyde reductase, ethanolamine utilization protein, shikimate kinase I, allantoinase, predicted SAM-dependent methyltransferase, L-glutamine:D-fructose-6-phosphate aminotransferase (GFAT), phosphoglucosamine mutase, predicted *N*-acetylmannosamine kinase, and predicted *N*-acetylmannosamine-6-P epimerase. Up-regulation of PBPs, one of primary target sites of antibiotics, might be responsible for the multidrug resistance in *E. coli* with increasing amount of target sites. Up-regulation of GFAT enzyme may be related to the up-regulation of PBPs because GFAT produces *N*-acetylglucosamine, a precursor of peptidoglycans. One of GFAT inhibitors, azaserine, showed a potent inhibition on the growth of *E. coli* KD43162. In conclusion, up-regulation of PBPs and GFATs with the loss of 36 kDa OMP refers the multidrug resistance in *E. coli* KD 43162.

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Keywords *E. coli* · genomics · L-glutamine:D-fructose-6-phosphate aminotransferase · multidrug resistance

Introduction

Resistance to antimicrobial agents in clinical microorganisms has referred to the use of antimicrobials being in nothing but the name in treating infections. In recent being infected by microorganisms in hospitals or related institutions are widely accepted and reported wherever in the world including Korea (Lee et al., 2010; Lim et al., 2012). Additionally, some possibilities of the presence of antibiotic-resistant microorganisms in the stock products and in the feces of the livestock are cautiously reported (McEwen and Fedorka-Cray, 2002).

The mode of action of antibacterial agents contains inhibitory effect on cell wall synthesis, interference with protein synthesis, changes on nucleic acid synthesis, inhibitory effect on metabolic pathways, and finally disruption of membrane structure (Tenover,

2006). Therefore, resistance mechanisms to antimicrobial agents in clinical microbes are related to overcome the mode of action of antimicrobial agents and divided into four primary groups in the basis of their biochemical changes (Tenover, 2006). Firstly, microbes acquire resistant genes encoding β -lactamases to metabolize antibacterial agent before it shows biological activity (Park et al., 2013). Secondly, bacteria acquire efflux systems which can extrude the antimicrobial agents from the cell before they are reached to the target sites (Richmond et al., 2013). Finally, bacteria acquire genes that produce altered cell walls (Yuan et al., 2013) and down-regulate porin genes to make antimicrobials with limited accession to target sites (Lavigne et al., 2013).

Recently, many scientists have contributed to develop new antimicrobials with novel target sites to suppress microbes (Kurosu et al., 2013; Sommer et al., 2013; Tegos and Hamblin, 2013). In addition to development of new antimicrobials, it is also interested in understanding changes in whole gene or protein expression in antimicrobials-resistant clinical microbes because modification of protein expression related to antimicrobial resistance can induce to change gene or protein expression (Chopra et al., 2013; Cierniak et al., 2013; Farrugia et al., 2013). This change can be a clue for finding new target site to control multidrug resistant microbes.

Herein, a genomic study has been employed to understand changes of gene expression in *E. coli* KD43162 resistant to antimicrobials. Using MALDI-TOF MS analysis, outer membrane proteins (OMPs) have been determined in the microbe. Microarray analysis was used to determine up- and down-regulated genes in relation to multidrug resistant *E. coli* KD43162. Finally, a novel inhibitor (azaserine) on L-glutamine:D-fructose-6-phosphate amino-transferase activity (GFAT) was determined to its potent inhibitory activity on *E. coli* 43162 growth.

Materials and Methods

Microbes used in this study. *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were acquired from ATCC. *E. coli* KD43162 was found from a Korean patient. This strain was undertaken a susceptibility test and detection of beta-lactamase. The susceptibility test of selected antimicrobial agents were determined by disk diffusion method and Etest (AB bioMérieux, Sweden) (Thaller et al., 2011) using Mueller-Hinton agar (Oxoid, United Kingdom) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2010). *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were used as quality control. The imipenem-disk modified Hodge test and imipenem-EDTA+sodium mercaptoacetic

acid double-disk synergy test was performed as described previously (Lee et al., 2003; Lee et al., 2010; Lee et al., 2001) by using known metallo- β -lactamase (MBL)-producing isolates. The polymerase chain reaction conditions for detection of carbapenemases genes were used to amplify several beta-lactamases (*bla_{VIM}*, *bla_{IMB}*, *bla_{SIM}*, and *bla_{AIM}*) (Lee et al., 2005; Patzer et al., 2009). To detect or sequence *bla_{NDM}* alleles, the following primers were used: NDM-1-F, 5'-TCG CAC CGA ATG TCT GGC AGC A-3'; NDM-1-R, 5'-AAA GCG ATG TCG GTG CCG TCG A-3'. Primers *OmpF*-F (5'-GCAGTGGCAGGTGTCATAAA-3')/*OmpF*-R (5'-TCGGCATTAAACAAAGAGGTG-3') and *OmpC*-F (5'-GCAGGCCCTTTGTTCGATA-3')/*OmpC*-R (5'-GCCGACTGAT TAATGAGGGTTA-3') were used to amplify the *ompF* (1158 bp) and the *ompC* (1236 bp) genes, respectively, in the *E. coli* KD43162 and *E. coli* ATCC 25922 by the method previously reported (Oteo et al., 2008). With the respect of multidrug resistance in the *E. coli* KD43162, the strain was resistant to ampicillin, ampicillin-sulbactam, piperacillin, piperacillin-tazobactam, cefazolin, cefepime, aztreonam, imipenem, meropenem, gentamicin, tobramycin, ciprofloxacin, levofloxacin, moxifloxacin, fosfomycin, and trimethoprim-sulfamethoxazole except for amikacin using disk diffusion assay. Minimum inhibitory concentrations of imipenem and meropenem for the *E. coli* KD43162 were both 16 μ g/mL, respectively.

Sample preparation. Total RNA was extracted from the *E. coli* cell using the TRI REAGENT (MRC, OH) according to the manufacturer's instructions. Following homogenization, 1 mL of solution was transferred to a 1.5 mL Eppendorf tube and centrifuged at 12,000 g for 10 minutes at 4°C to remove insoluble material. The supernatant containing RNA was collected, mixed with 0.2 mL of chloroform, and centrifuged at 12,000 g for 15 min at 4°C. After RNA in the aqueous phase was transferred into a new tube, the RNA was precipitated by mixing 0.5 mL of isopropyl alcohol and recovered by centrifuging the tube at 12,000 g for 10 min at 4°C. The RNA pellet was washed briefly in 1 mL of 75% ethanol and centrifuged at 7,500 g for 5 minutes at 4°C. Finally, the total RNA pellet was dissolved in Nuclease-water, and its quality and quantity was assessed by Agilent bioanalyzer 2100 analysis (Agilent technologies, USA).

Microarray analysis. Each total RNA sample using 1 μ g made sense RNA after cDNA synthesis by reverse transcription to proceed after the in vitro transcription process. Then amplified using sense RNA in the process of cDNA synthesis by reverse transcription proceeds given to put the labeled-dCTP fluorescent incorporation labeled cDNA made. The Cy3-labeled cDNAs were resuspended in 50 μ L of hybridization solution (Agilent technologies). After labeled cDNAs were placed on Agilent *E. coli* 8 \times 15K array

Table 1 Identification of outer-membrane proteins by MALDI-TOF MS. Numbering in the table for proteins is equal to the numbering in Fig. 1

No.	Protein name	Accession no.	Score ($p=0.05$)	Species match	Gene ontology
1	Outer membrane C	AAN81212	100 (74)	<i>E. coli</i> CFT073	Ion transport
2	Outer membrane protein II	Q6WAH4_ECOLI	60 (58)	<i>E. coli</i>	Ion transport
3	DNA protection during starvation protein	DPS_ECO57	64 (58)	<i>E. coli</i> O157:H7	Cellular iron ion homeostasis

(Agilent technologies) and covered by a Gasket 8-plex slide (Agilent technologies). The slides were hybridized for 17 h at 65°C oven. The hybridized slides were washed in 2×SSC, 0.1% SDS for 2 min, 1×SSC for 3 min, and then 0.2×SSC for 2 min at room temperature. The slides were centrifuged at 3,000 rpm for 20 s to dry.

Data analysis. The arrays were analyzed using an Agilent scanner with associated software. Gene expression levels were calculated with Feature Extraction v10.7.3.1 (Agilent technologies). Relative signal intensities for each gene were generated using the Robust Multi-Array Average algorithm. The data were processed based on quantile normalization method using the GeneSpring GX 11.5.1 (Agilent technologies). This normalization method aims to make the distribution of intensities for each array in a set of arrays the same. The normalized, and log transformed intensity values were then analyzed using GeneSpring GX 11.5.1 (Agilent technologies). Fold change filters included the requirement that the genes be present in at least 200% of controls for up-regulated genes and lower than 50% of controls for down-regulated genes.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for OMP. SDS-PAGE was performed to determine alterations in outer membrane proteins, as described previously (Lee et al., 2007) but with some modification. Briefly, cells were disrupted by sonication and the supernatant was treated with 30% Sarkosyl (Sigma Chemical Co., USA) to solubilize the inner and outer membranes. Outer membranes were then sedimented by centrifugation at 45,000 g for 1 h at 4°C and outer membrane proteins were separated by SDS-PAGE on a Mini-PROTEAN 3 Cell apparatus (Bio-Rad, USA). The gels were stained with Coomassie Brilliant Blue and then de-stained.

Mass spectrometry. Digestion of selected gel portions was performed according to a previously reported method (Park et al., 2008). Protein spots were cut from the gels, and then the excised gels were washed 3 times for 20 min with 200 µL of 60% v/v 50 mM ammonium bicarbonate (pH 7.8) in 40% v/v acetonitrile at 37°C. The stained gels were dehydrated in a SpeedVac for 20 min. The remaining processes were exactly same from the method previously reported (Park et al., 2008).

Growth inhibition by addition of two GFAT inhibitors on multidrug resistant *E. coli* KD43162. Cell growth of *E. coli* KD43162 in the absence of cefoxitin and in the presence of cefoxitin was undertaken. Two GFAT inhibitors, azaserin (Aza) and 6-diazo-5-oxo-L-norleucine (Don), were added to the growth medium to validate their inhibitory effects on the GFAT activity, referring to inhibition on cell growth. Two inhibitors were added to the growth medium at the concentrations of 100 and 250 ppm. Control indicates no addition of GFAT inhibitors.

Statistical analysis. Statistical analysis was performed using the SPSS for Windows statistical package, version 10.0 (SPSS Inc., USA). Data are expressed as mean ± SD. The effects of drug treatments were evaluated statistically using the one-way analysis of variance (one-way ANOVA) followed by the Dunnett's post-hoc test to rectify for multiple comparison treatments. Statistical significance was set at $p < 0.05$.

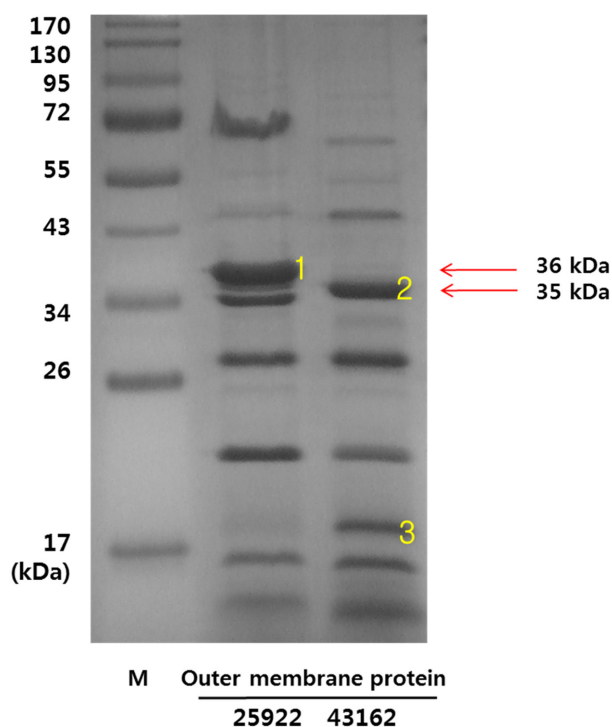


Fig. 1 Number of differentially expressed genes in *E. coli* 43162 after cefoxitin exposure. Number of significant up-and down-regulated genes (three samples *t*-tests, $p < 0.05$) in *E. coli* KD 43162 after exposure to cefoxitin in three different concentrations of cefoxitin. Values refer to the comparison with the respective control.

Results and Discussion

SDS-PAGE of outer membrane proteins and MALDI-TOF MS analysis.

The results of an imipenem-disk modified Hodge test and a double-disk synergy test using disks of imipenem and EDTA + sodium mercaptoacetic acid were negative, indicating production of a MBL, but bla_{IMP-1} , bla_{VIM-2} , bla_{SIM-1} , bla_{AIM-1} or bla_{NDM-1} -like, was not detectable by polymerase chain reaction. Lee et al. (2007) reported that CMY-2 or DHA-1 production combined with porin loss reduced carbapenem susceptibility in the *K. pneumonia* isolates. The OmpC gene was not detected by polymerase chain reaction from *E. coli* KD43162 but, OmpF gene was detectable. The loss of 36 and 35 kDa OMP were coffered to carbapenem resistant in isolates. Two major porins, OmpF, and OmpC, have been reported the loss or diminished expression of these two porins have been related to resistance to imipenem, meropenem, and cefoxitin in *E. coli* (Clarke et al., 2003; Latigue et al., 2007; Liu et al., 2008). The loss of a 36 kDa OMP conferred a high level of resistance to carbapenems upon the *E. coli* isolates (Liu et al., 2008). The OmpC was observed only in *E. coli* ATCC 25922, while an OmpF protein was observed in *E. coli* KD43162, as observed in Table 1 and Fig. 1, and in agreement with the our genetic study. OMP proteins were analyzed by MALDI-TOF MS as shown in Table 1.

Microarray analysis and validation. A gene list compiled on the

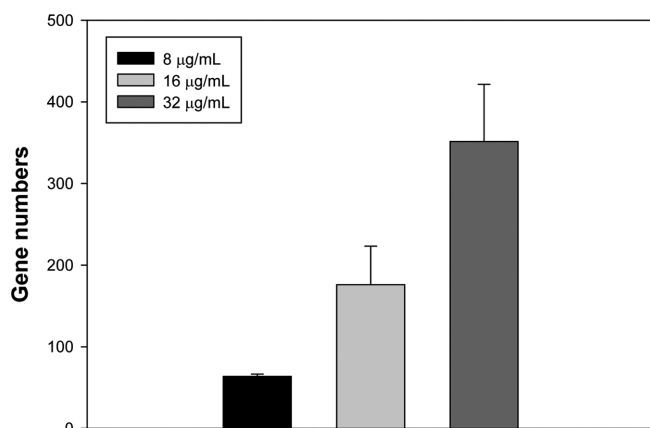


Fig. 2 Heat map and clustering analysis of cefoxitin samples. Heat map and hierarchical clustering of samples based on the differentially expressed genes (three samples *t*-test, $p < 0.05$) in *E. coli* KD 43162 when comparing: A, control; B, 8 µg/mL; C, 16 µg/mL; D, 32 µg/mL of cefoxitin.

basis of statistical analysis ($p < 0.05$). Using the criteria, 1198 genes were identified including non-changed genes. Number of significant up- and down-regulated genes in *E. coli* KD43162 after exposure to cefoxitin in three different concentrations was determined and increased with increasing concentration of cefoxitin treatment (Fig. 2). Based on the differentially expressed genes, a heat map and hierarchical clustering of samples was established in *E. coli* KD43162 with different concentrations of cefoxitin addition in the *E. coli* growth medium (Fig. 3).

Among them, genes at least 2-fold up-regulated in the resistant *E. coli* KD43162 were listed in Table 2. These genes were corresponded to express the proteins as penicillin-binding protein, tartronate semialdehyde reductase, ethanolamine utilization protein, shikimate kinase I, allantoinase, predicted SAM-dependent methyltransferase, L-glutamine:D-fructose-6-phosphate aminotransferase, phosphoglucosamine mutase, predicted *N*-acetylmannosamine kinase, and predicted *N*-acetylmannosamine-6-P epimerase. Peptidoglycans surround most bacteria, referring bacterial shape and protecting them against high osmotic pressure (Vollmer et al., 2008). They are net-like macromolecules and assembled by the membrane-bound peptidoglycans glycosyltransferases and transpeptidases. The penicillin-type antimicrobials

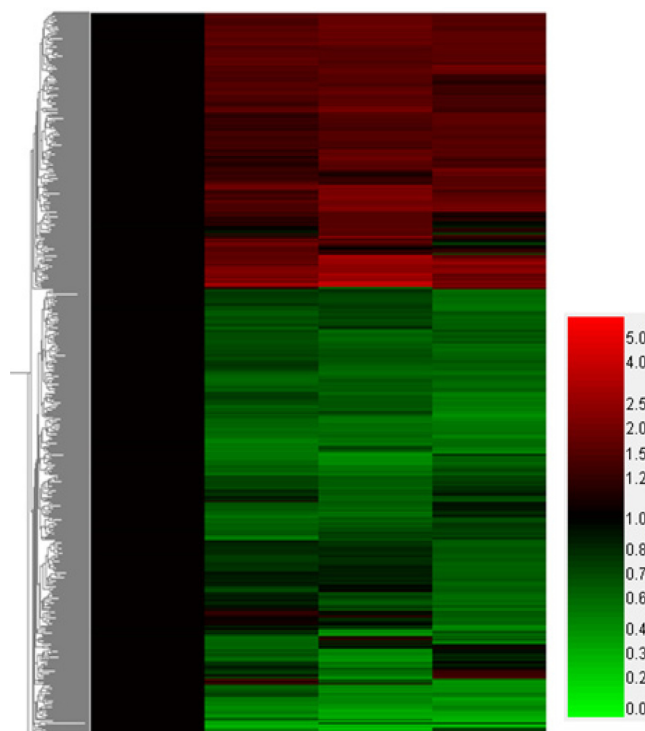


Fig. 3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis profiles of outer membrane protein. Lane M, marker; lane 25922, control *E. coli* ATCC 25922; lane 43162, *E. coli* KD 43162.

combine to the transpeptidases and inhibit the peptidoglycan formation (Derouaux et al., 2011). Bacterial peptidoglycan transpeptidases are known as penicillin-binding proteins (PBPs) and they are found to be up-regulated in the multidrug-resistant *E. coli* KD43162 in this study. It is an interesting result because the target site of antibiotics was up-regulated and this up-regulation might refer the bacteria to survive under the recommended treating doses of antibiotics. However, it is different result from the well-known resistance mechanism in relation to PBPs as the modification of the PBPs in the cell wall with low affinity for β -lactams (Zapun et al., 2008). This resistance mechanism is well-organized in the Gram-positive cocci, such as penicillin-resistant *Streptococcus pneumoniae* or the much-feared methicillin-resistant *Staphylococcus*

Table 2 List of representative genes up-regulated in *E. coli* KD 43162 after treatment of cefoxitin

No.	Gene Symbol	Gene name	Fold to control	Gene ontology
1	pbpC	penicillin-binding protein 1C	7.22 (± 0.86)	Peptidoglycan biosynthetic process
2	garR	tartronate semialdehyde reductase	5.66 (± 1.31)	Glyoxylate metabolic process
3	eutH	ethanolamine utilization protein	3.56 (± 0.55)	Ethanolamine catabolic process
4	aroK	shikimate kinase I	2.16 (± 0.23)	Aromatic amino acid family biosynthetic process
5	Z0666	allantoinase	4.89 (± 1.11)	Purine metabolism
6	yhiQ	predicted SAM-dependent methyltransferase	4.57 (± 1.61)	Methyltransferase
7	glmS	L-glutamine:D-fructose-6-phosphate aminotransferase	6.17 (± 3.38)	UDP-N-acetylglucosamine biosynthetic process
8	glmM	phosphoglucosamine mutase	2.12 (± 0.03)	UDP-N-acetylglucosamine biosynthetic process
9	nanK	predicted <i>N</i> -acetylmannosamine kinase	2.55 (± 0.54)	Kinase activity
10	nanE	predicted <i>N</i> -acetylmannosamine-6-P epimerase	3.31 (± 1.34)	Predicted

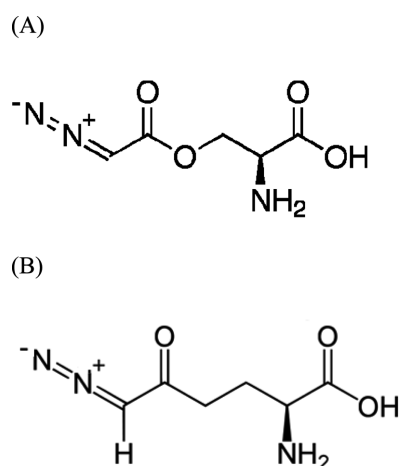


Fig. 4 Structures of (A) azaserine and (B) 6-diazo-5-oxo-L-norleucine (DON)

aureus (Zapun et al., 2008). Recently, this resistance mechanism is also reported in the Gram-negative rods (GNRs) as *Pseudomonas aeruginosa*, which it has not been thought to be important in GNRs (Moya et al., 2009). On the other hand, a recent study strongly showed a relationship between down-regulation of PBPs and alteration in PBPS for β -lactam resistance in *Acinetobacter baumannii* (Vashist et al., 2011). Therefore, our result differs from those two resistant mechanisms and it is first to report the up-regulation of PBPs in relation to antibiotic resistant mechanisms in *E. coli* KD43162.

Up-regulation of PBPs may lead over-production of precursors of peptidoglycans in the microbes. The precursors of peptidoglycans in microbes are *N*-acetylglucosamine (Glc-Nac) and *N*-acetylmuramic acid (MurNac)-peptide attached to the membrane-bound lipid synthesized inside the bacterial cell (Barreteau et al., 2008). In this study, the enzymes such as GFAT and phosphoglucosamine mutase were found to be up-regulated and they might be involved in the presumable over-production of Glc-Nac in the multidrug resistant *E. coli* KD43162. As PBPs play an important role in antibiotics target sites, inhibition of Glc-Nac production is a potential target site for novel anti-infective agents (Ramos-Aires et al., 2004). As up-regulation of *glmS* responsible for the expression of L-glutamine:D-fructose-6-phosphate aminotransferase was established in this study, an inhibitor on the enzyme activity might inhibit to form Glc-Nac, leading to kill the multidrug resistant *E. coli* KD43162 cells via inhibition of production of PBPs.

Two potent, specific inhibitors on GFAT activity, azaserine (Aza) and 6-diazo-5-oxo-L-norleucine (Don) (Fig. 4), were employed to confirm our suggestions on the control of the multidrug resistant *E. coli* KD 43162 by suppressing of PBPs through less production of Glc-Nac. Interestingly, Aza with the concentrations of 100 and 250 ppm completely suppressed the *E. coli* KD43162 growth, while Don did not show potent inhibitory effects on the microbial growth at the two concentrations with or without cefoxitin (Fig. 5). It might be resulted from the different affinity to the GFAT active site of *E. coli* KD43162, even if they are potent GFAT

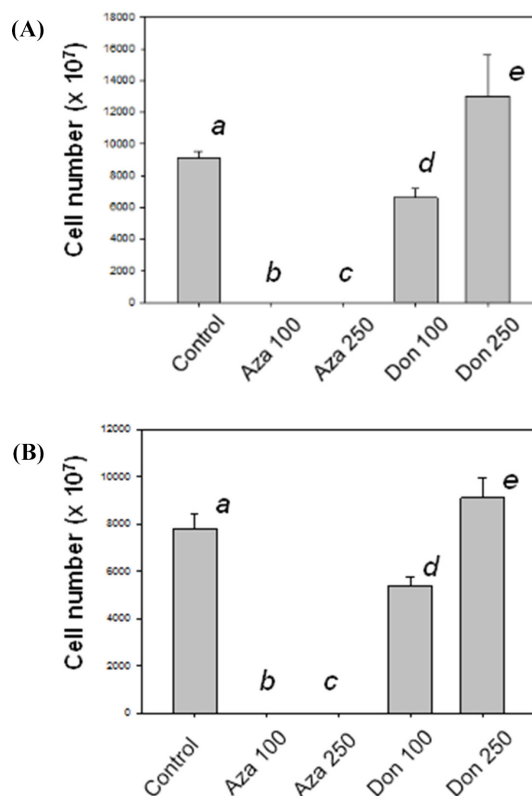


Fig. 5 Cell growth of *E. coli* KD43162 in the absence of cefoxitin (A) and in the presence of cefoxitin (B). Two inhibitors of L-glutamine:D-fructose-6-phosphate aminotransferase inhibitors, azaserine (Aza) and 6-diazo-5-oxo-L-norleucine (Don), were added to the growth medium at the concentrations of 100 or 250 ppm. Control indicates no addition of L-glutamine:D-fructose-6-phosphate aminotransferase inhibitors. Data followed by the same letter are not significantly different ($p < 0.05$).

inhibitors in other organisms (Dehennaut et al., 2007). Further studies will focus on the assessment of relationship between two inhibitors and the *E. coli* GFAT active sites and develop possible synthetic scheme to produce antibiotics to control multidrug resistant microbes via selective inhibition on microbial GFAT activity.

Other up-regulated genes are responsible for the expression of proteins involved in glyoxylate metabolic process, ethanolamine catabolic process, aromatic amino acid biosynthetic process, purine metabolism, and methyltransferase. They are possible target sites for antibiotics to suppress multidrug resistant *E. coli* KD43162. Therefore, up-regulation of PBPs and GFATs, and loss of 36 kDa OMP may confer the multidrug resistance in *E. coli* KD43162.

Acknowledgments This work was supported by Blue-Bio Industry Regional Innovation Center (RIC08-06-07) at Dongeui University as a RIC program under Ministry of Trade, Industry&Energy and Busan city.

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