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

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AbstractA 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, gentamicin, tobramycin, ciprofloxacin, levofloxacin, moxifloxacin, fosfomycin, 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 KD43162. 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), phosphoglucomutase, 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.

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,
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 undertook 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-resistant KD43162 was used as a novel strain. The strain was resistant to ampicillin, amoxicillin-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 µg/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 1ug 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 mL of hybridization solution (Agilent technologies). After labeled cDNAs were placed on Agilent E. coli 8x15K array

| No. | Protein name | Accession no. | Score (p =0.05) | Species match | Gene ontology |
|-----|--------------|---------------|----------------|--------------|---------------|
| 1   | Outer membrane C | AAN81212 | 100 (74) | E. coli CFT073 | Ion transport |
| 2   | Outer membrane protein II | Q6WAH4_ECOLI | 60 (58) | E. coli | Ion transport |
| 3   | DNA protection during starvation protein | DPS_ECO57 | 64 (58) | E. coli O157:H7 | Cellular iron ion homeostasis |
multidrug resistant
Growth inhibition by addition of two GFAT inhibitors on
previously reported (Park et al., 2008).

The remaining processes were exactly same from the method
(SDS-PAGE) for OMP.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
genes and lower than 50% of controls for down-regulated genes.

The genes be present in at least 200% of controls for up-regulated
technologies). Fold change filters included the requirement that
were then analyzed using 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).

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 \textit{E. coli} KD43162. Cell growth of \textit{E. coli}
KD43162 in the absence of cefoxitin and in the presence of
cefoxitin was undertaken. Two GFAT inhibitors, azaserin (Aza)
and 6-diazoo-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 \( p < 0.05 \).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Number of differentially expressed genes in \textit{E. coli} 43162 after
cefoxin exposure. Number of significant up- and down-regulated genes
(three samples t-tests, \( p < 0.05 \)) in \textit{E. coli} KD 43162 after exposure to
cefoxitin in three different concentrations of cefoxitin. Values refer to the
comparison with the respective control.}
\end{figure}
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 \textit{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 \textit{E. coli} KD43162 with different concentrations of cefoxitin addition in the \textit{E. coli} growth medium (Fig. 3).

Among them, genes at least 2-fold up-regulated in the resistant \textit{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, phosphoglucomamine 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 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 \textit{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 $\beta$-lactams (Zapun et al., 2008). This resistance mechanism is well-organized in the Gram-positive cocci, such as penicillin-resistant \textit{Streptococcus pneumonia} or the much-feared methicillin-resistant \textit{Staphylococcus aureus}.

\begin{table}[h]
\centering
\begin{tabular}{lll}
\hline
No. & Gene Symbol & Gene name & Fold to control & Gene ontology \\
\hline
1 & \textit{pbpC} & penicillin-binding protein 1C & 7.22 (±0.86) & Peptidoglycan biosynthetic process \\
2 & \textit{garR} & tartronate semialdehyde reductase & 5.66 (±1.31) & Glyoxylate metabolic process \\
3 & \textit{eutH} & ethanolamine utilization protein & 3.56 (±0.55) & Ethanolamine catabolic process \\
4 & \textit{aroK} & shikimate kinase I & 2.16 (±0.23) & Aromatic amino acid family biosynthetic process \\
5 & \textit{Z0666} & allantoinase & 4.89 (±1.11) & Purine metabolism \\
6 & \textit{yhiQ} & predicted SAM-dependent methyltransferase & 4.57 (±1.61) & Methyltransferase \\
7 & \textit{glmS} & L-glutamine:D-fructose-6-phosphate aminotransferase & 6.17 (±3.38) & UDP-N-acetylglicosamine biosynthetic process \\
8 & \textit{glmM} & phosphoglucomamine mutase & 2.12 (±0.03) & UDP-N-acetylglicosamine biosynthetic process \\
9 & \textit{nanK} & predicted N-acetylmannosamine kinase & 2.55 (±0.54) & Kinase activity \\
10 & \textit{nanE} & predicted N-acetylmannosamine-6-P epimerase & 3.31 (±1.34) & Predicted \\
\hline
\end{tabular}
\caption{List of representative genes up-regulated in \textit{E. coli} KD 43162 after treatment of cefoxitin.}
\end{table}
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 KD43162 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 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.

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

![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).]
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