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Note

Fosmidomycin Resistance in Adenylate Cyclase Deficient (*cya*) Mutants of *Escherichia coli*

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Adenylate cyclase deficient (*cya*) mutants of *E. coli* K-12 were found to be resistant to fosmidomycin, a specific inhibitor of the non-mevalonate pathway, just like to fosfomycin. *E. coli glpT* mutants were resistant to fosfomycin and also to fosmidomycin. This fact shows that fosmidomycin was transported inside *via* the glycerol-3-phosphate transporter, GlpT. DNA micro-array analysis showed that the transcription of *glpT* and other genes concerning glycerol utilization were highly dependent on the presence of cAMP.

Key words: fosmidomycin; cAMP; *Escherichia coli*; DNA micro-array

Fosmidomycin (FR-31564) is an antibiotic that has a C-P bond and is effective on various Gram-positive and Gram-negative bacteria (Fig. 1).[1] The target of it, however, had long been unknown. Recently Seto and his group found that 1-deoxy-D-xylulose 5-phosphate (DXP) reductoisomerase in the non-mevalonate pathway is the target of fosmidomycin.[2] The antibiotic is thought to inhibit the enzyme as a structural analogue. The non-mevalonate pathway was firstly found by Rohmer *et al.* in *Zymomonas mobilis*.[3,4] Now it is known that non-mevalonate pathway is widely distributed in bacteria including *Escherichia coli*, *Salmonella typhimurium*, *Bacillus subtilis*, and *Clostridium acetobutyricum* as an alternative to the mevalonate pathway. The first enzyme in the pathway is DXP synthetase, which catalyzes the formation of DXP from pyruvate and D-glyceraldehyde-3-phosphate.[5] The second enzyme, DXP reductoisomerase, catalyzes NADPH-dependent reaction from DXP to 2-*C*-methyl-D-erythritol 4-phosphate, a preceding metabolite to isopentenyl diphosphate.[6,7]

Fosfomycin (phosphonomycin) is another well-known antibiotic which has a C-P bond (Fig. 1).[8,9] Its target is known as the enzyme forming UDP-*N*-acetylmuramic acid from UDP-*N*-acetyl glucosamine and phosphoenolpyruvic acid.[10] Thus fosfomycin is an antibiotic that inhibits bacterial peptidoglycan synthesis. It has been known that *cya* or *crp* mutants of *Salmonella typhimurium* are resistant to fosfomycin.[11] *cya* and *crp* genes encode adenylate cyclase and cAMP receptor protein, respectively. Fosfomycin resistance in the bacterium is, therefore, dependent on the lack of cAMP-function.

From the structural similarity of fosmidomycin to fosfomycin in relation to the C-P bond region, we examined fosmidomycin resistance in *cya* mutants of *E. coli*. *E. coli* K-12 derivative HT28 strain (*Δcya*::Km) and its parent W3110 (F^-, *thy*, λ) were kindly provided by H. Aiba.[12] Another *E. coli* K-12-derived *cya* mutant, CA8306 (*Δcya*, *relA*) and its parents CA8000 (HfrH, *relA*, *thi*, λ) were kindly provided some years ago by J. Beckwith.[13,14] The minimum inhibitory concentration (MIC) was measured by an agar plate-paper disc method using paper discs (8 mm in diameter, Advantec, Tokyo, Japan) and nutrient agar plates. Nutrient agar was the product of Eiken Chemical Co. (Tokyo, Japan). Fosfomycin and cAMP were purchased from Wako Pure Chemical Co. (Osaka, Japan). Fosmidomycin was supplied from Fujisawa Pharmaceutical Industry (Osaka, Japan). As shown in Table I both *Δcya*

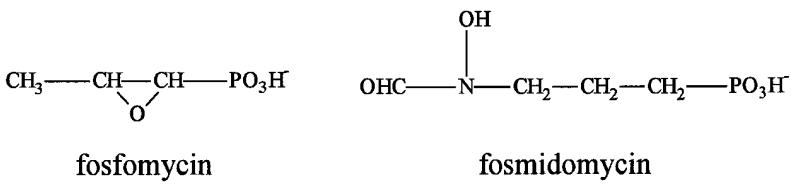

Fig. 1. Structures of Fosfomycin and Fosmidomycin.

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mutants, HT28 and CA8306 were resistant to fosmidomycin as well as to fosfomycin but sensitive if they were cultivated in the presence of 1 mM cAMP. It is known that fosfomycin is transported into *E. coli* cells *via* the glycerol-3-phosphate transporter (GlpT) and in part *via* the hexose phosphate transporter.[10,15] The expression of *glpT* is known to be dependent on the presence of cAMP.[11] From the study on cross-resistance to fosfomycin and fosmidomycin, both antibiotics were suggested to be transported inside *via* GlpT in *Pseudomonas aeruginosa*.[16] We tested fosmidomycin resistance in two mutants of *E. coli* K-12 derived *glpT* mutans, ME7760 and ME8261. The mutants were obtained from the National Institute of Genetics (Mishima, Japan). Both *glpT* mutants were resistant to fosmidomycin and fosfomycin (Table 1). This fact shows that fosmidomycin was transported inside *via* the glycerol-3-phosphate transporter, GlpT.

Table 1. Minimum Inhibitory Concentrations of Fosfomycin and Fosmidomycin to *E. coli* K-12 Strain and the Derived Mutants

Strain	Genotype	cAMP (1 mM)	MIC (μg/ml)	
			Fosfomycin	Fosmidomycin
W3100		−	500	15
HT28	*Δcya*	−	4,000	125
		+	250	15
CA8000	*relA*	−	250	7
CA8306	*relA*, *Δcya*	−	4,000	125
		+	250	7
ME7760	*glpT*	−	4,000	1,000
ME8261	*glpT*	−	2,000	4,000

We examined the cAMP-dependence of *glpT* gene expression by using an *E. coli* K-12 DNA micro-array (Takara Bio Inc., Otsu, Japan). *E. coli* strains to be tested were grown aerobically to the mid-exponential phase in L-tubes containing 10 ml of LB broth. Total RNA of *E. coli* cells (ca. 5×10^8) was prepared using a Rneasy Mini Kit (Qiagen K. K., Tokyo, Japan) according to its protocol with RNAprotect Bacteria Reagent (Qiagen K. K.) as a stabilizer of RNA. The RNA was routinely purified. The purified RNA samples should be above 1.85 in A_{260}/A_{280}. The

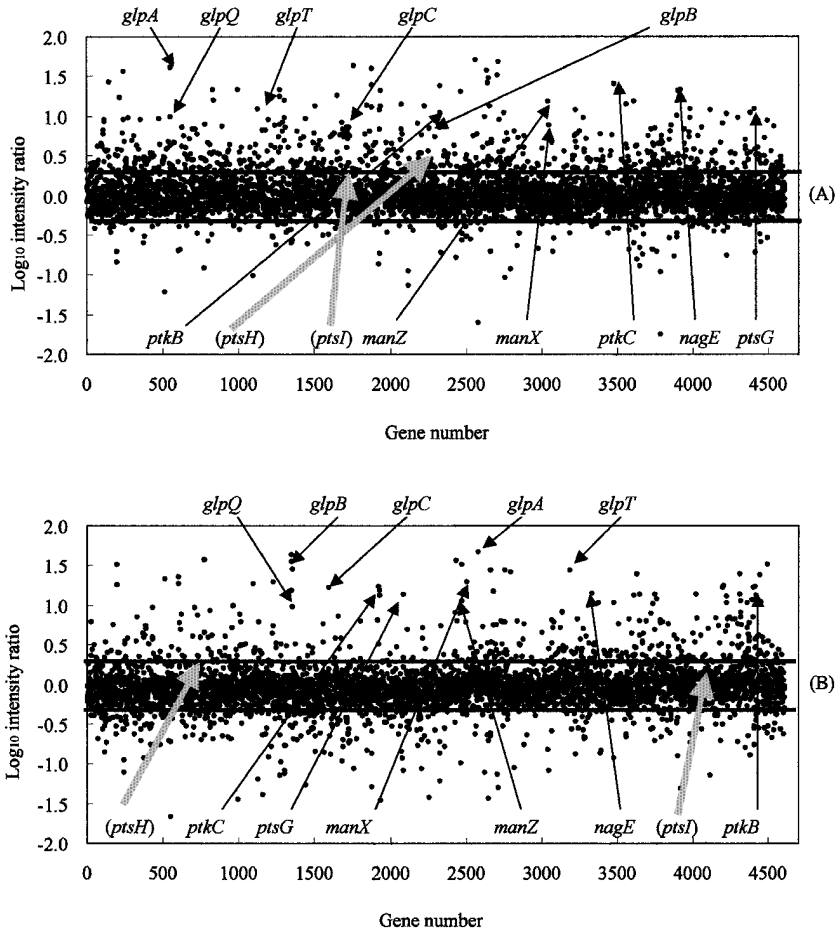

Fig. 2. DNA micro-array Analysis of Ratio in the Gene Expression of *E. coli* W3110/HT28 (A) and CA8000/CA8306 (B).

All log 10 intensity ratios of the gene of *E. coli* W3110/HT28 are represented in the scatter plot, with each circle indicating the log 10 expression ratio of an individual gene. Genes more highly expressed in W3110 (CA8000) are shown above the centerline, while those below the centerline are more highly expressed in HT28 (CA8306). Lines indicate the standard of the significant expression (2.0 times). Gene numbers in (A) and (B) are different.

reverse transcriptase reaction was done with a RNA Fluorescence Labeling Core Kit (M-MLV Version) (Takara Bio Inc.) with the mixed primer and Fluoro-Link Cy3- and Cy5-dUTP, according to the protocol of the manufacturer. Fluorescence-labeled cDNAs were purified, precipitated with ethanol, and dissolved in a hybridization buffer (6×SSC, 0.2% sodium dodecyl sulfate, 5×Denhardt's solution, and 0.1 mg/ml denatured salmon sperm DNA). The hybridization was done with an IntelliGene DNA Chip (Takara Bio Inc.) and Spaced Cover Glass (Takara Bio Inc.) according to the protocol at 65°C for 16 hours. The micro-array was routinely rinsed. The scanning of the DNA micro-arrays was done at DNA Chip Research (Yokohama, Japan) with CRBIOIIe (Hitachi Software Engineering Co., Ltd., Yokohama Japan). DNA-microarray experiments were done twice, and the results were reproducible.

As shown in Fig. 2, *glpT* and other glycerol metabolism-related genes, *glpQ*, *glpA*, *glpB*, and *glpC* had a high gene expression ratio in both parent/*Δcya* mutant sets. The *glpABC* operon, at 50.7 min in the *E. coli* K-12 genetic map, encodes the three subunits of glycerol-3-phosphate dehydrogenase (EC 1.1.99.5) and its transcription direction is clockwise to the map.[17] The *glpTQ* operon, at 50.6 min in the genetic map, encodes glycerol-3-phosphate permease and glycerol-3-phosphate diesterase, respectively. The transcription direction is opposite to that of the *glpABC* operon.[17] Both operons have cAMP receptor protein (CRP)-binding boxes in their 5′-control regions.[18,19] These situations well explain the coordinated high expression of the genes in each operon. Both operons are under the control of GlpR, the repressor for the *glp* regulon. To our surprise, the *glpTQ* operon and *glpABC* operon were within the highest 100 group among ca. 4500 genes in gene-expression ratio between the wild strain (parent) and *Δcya* mutant in the DNA mirco-array assay (Fig. 2).

The high gene expression ratio, in the top 100 class, of PTS sugar enzyme II genes was also remarkable. Their gene names are cited in the lower region of either Fig. 2A or Fig. 2B. *manX*, *manZ*, and *nagE* encode Enzyme II A (Man), Enzyme II B (Man) and Enzyme II (GlcNAc), respectively. And *ptkB*, *ptkC*, and *ptsG* encode Enzyme II (galactitol), Enzyme II C (galactitol), and Enzyme II (Glc), respectively. The high gene expression ratio of these genes makes a sharp contrast to the low response ratio of the *ptsHIcrr* operon, which has a CRP binding site in the 5′-control region. *ptsH* and *ptsI* genes, shown in the parenthesis in the lower region of either Fig. 2A or Fig. 2B, encode HPr and Enzyme I, respectively. HPr and Enzyme I are the common components for various sugar-specific Enzyme IIs in the phosphotransferase system. To our best knowledge this is an early attempt on the DNA micro-array analysis concerning the effects of cAMP on an *E. coli* gene expression profile.

In conclusion *E. coli* K-12 *Δcya* mutants were resistant to fosmidomycin. The antibiotic was shown to be transported inside *via* the glycerol-3-phosphate transporter, GlpT. The expression of *glpT* and other genes concerning glycerol utilization was highly dependent on the presence of cAMP.

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