TWO KINDS OF MUTANTS DEFECTIVE IN MULTIPLE CARBOHYDRATE UTILIZATION ISOLATED FROM *IN VITRO* FOSFOMYCIN-RESISTANT STRAINS OF *ESCHERICHIA COLI* K-12

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Two types of *in vitro* fosfomycin-resistant mutants defective in multiple carbohydrate utilization were selected from *Escherichia coli* strain K-12. One mutant, FR182, was defective in phosphoenolpyruvate: sugar phosphotransferase system and the ability to form adenosine 3′, 5′-cyclic monophosphate (cAMP) was lowered. Another mutant, FR190, was defective in cAMP formation.

Restoration by cAMP of fosfomycin (FOM) sensitivity coupled with recovery of utilization of many carbohydrates including *sn*-glycerol-3-phosphate (G-3-P) was observed in both of the resistant mutants. FOM was not taken up by these resistant strains but, in the cells cultured in the presence of cAMP, accumulation of FOM was equivalent to that of the sensitive parent strain. Decreased uptake of G-3-P was also restored in both of the resistant strains cultured in the presence of cAMP. These results indicate that the resistance to FOM in these mutants is due to impairment of G-3-P transport system, one of the pathways for uptake of FOM. They were sensitized to FOM by D-glucose-6-phosphate because of the induction of hexose phosphate transport system, another uptake pathway.

Fosfomycin (FOM) is known to kill bacteria by covalently binding with phosphoenolpyruvate (PEP): uridine diphospho-N-acetylglucosamine enolpyruvyl transferase, an enzyme forming UDP-N-acetylmuramic acid in the biosynthesis of cell wall peptidoglycan[1]. The antibiotic is taken up by cells to exert its bactericidal effect using two independent active transport systems, hexose phosphate transport system (designated genetically *uhp*)[1,2] and *sn*-glycerol-3-phosphate (G-3-P) transport system (*glp*T)[1,3].

We previously isolated, from FOM-resistant mutants of *Escherichia coli* strain B (*E. coli* B), a strain which exhibited several phenotypic changes characteristic for mutants of PEP: sugar phosphotransferase system (PTS[4])[5]. This mutant, strain FR90, was not studied extensively.

In this communication, we wish to report the isolation and identification of two types of *in vitro* fosfomycin-resistant mutants of *E. coli* strain K-12. One mutant was proved to be defective in PTS activity and another mutant was defective in the formation of adenosine 3′,5′-cyclic monophosphate (cAMP). In *Salmonella typhimurium*, it was reported that the former type was selected by FOM, but the reason of its resistance of FOM was not fully documented[6]. The mechanism of resistance to FOM in our two mutants will be discussed.

Materials and Methods

Media: Nutrient broth was Bacto-nutrient broth (Difco) containing NaCl at 0.2% and solidified by addition of agar (Difco) at 1.5% (nutrient agar). Minimal medium was K10 medium[7]. K10 medium used in this work contained; Na_2HPO_4, 46 mM; NaH_2PO_4, 23 mM; $(NH_4)_2SO_4$, 8 mM; $MgSO_4$, 0.4 mM; KCl, 10 mM; $FeSO_4$, 6 μM. (Thiamine was omitted from the original K10 medium.) Carbon

source was used at 0.4%. Amino acid at 20 μg/ml or thiamine hydrochloride at 5 μg/ml was added when required. K10 plates were prepared by the addition of purified agar (Oxoid Ltd., London) at 1%. Eosine Methylene Blue agar without lactose (EMB agar base, BBL), supplemented to 1% with a selected carbon source, was used for indicator plates.

Strains: *E. coli* K–12 strains used in these experiments are listed in Table 1.

Isolation of FOM-resistant Strains: In this work, cells were grown at 37°C. Strain CSH2, aerated in nutrient broth until the stationary growth phase, was inoculated into fresh nutrient broth and aerated again. The stationary phase culture was diluted with nutrient broth and spread on nutrient agar plates containing FOM at 200 μg/ml. After plates had been incubated for 2~3 days, the colonies were isolated on EMB plates containing glucose or lactose. After incubation overnight, fermentors or nonfermentors were purified on nutrient agar plates and stocked on nutrient agar slants.

Growth: The strains on the slants were cultivated in nutrient broth without shaking (preculture). For strain YE100, single colony was inoculated for preculture because of its frequent reversion. These precultures were usually inoculated into nutrient broth and shaken for aeration. Inoculum size was 5% for the determination of PTS activity or uptake experiments and 10% for measurement of cAMP formation. During the log phase growth on nutrient broth, doubling times for strains CHS2 and FR182 were 40~45 minutes and 50~55 minutes, respectively. Both strains entered the stationary growth phase at 5~7 hours after inoculation at 1% (see Fig. 1). In contrast, strain FR190 grew similarly to FR182 in an early phase (phase I) but then its growth rate decreased rapidly (phase II, see Fig. 1). The growth of FR190, which was indistinguishable whether at log phase or stationary phase, ceased at 16~17 hours after inoculation at 10%.

Determination of Sensitivity to FOM: Sensitivity to FOM was determined by two methods. i) One loopful of preculture was streaked on nutrient agar plates containing several concentrations of the antibiotic, and the growth was observed for 2 days (streak test). ii) Each strain was aerated in nutrient broth after inoculation at 1%. At the log growth phase, the antibiotic was added to the culture and the growing process or the lysis was observed (lysis test). The lysis test was used as a method for determination in a short period in which the effect of the antibiotic on a large part of the population could be examined without interference of a very few resistant cells. For this observation, the automatic growth recorder, Ohtake Bio Scanner OT–BS–12, was used.

Carbohydrate Utilization: One loopful of the preculture was streaked on minimal agar plates containing one of the desired carbohydrates and the growth was observed for 2 days. For examination of the effect of cAMP on the utilization, the precultured cells were washed with minimal medium (K10) once by centrifugation at 10,000×*g* for 5 minutes at 30°C and resuspended in the medium at a density of 0.65 at 550 nm and 0.5 mm slit width with a Hitachi spectrophotometer 139 (0.65 A_{550} unit per ml). The cell suspension was inoculated at 2% in liquid minimal medium containing one of several carbo-

Table 1. *E. coli* K–12 strains used

Strain	Genotype	Fosfomycin-sensitivity	Source[a]
CSH2	F⁻ *met pro*	sensitive	YOSHIKAWA
FR182	F⁻ *met pro*	resistant	CSH2
FR190	F⁻ *met pro*	resistant	CSH2
1100	Hfr *thi*–1 *rel*–1 λ⁻	sensitive	NAKAZAWA[8]
1103[b]	Hfr *thi*–1 *rel*–1 λ⁻ *pts* I	resistant	NAKAZAWA[8]
HfrR1	Hfr *met*B1 *rel*–1	sensitive	YOKOTA
YE100[c]	Hfr *met*B1 *rel*–1 *cya*	resistant	YOKOTA[9]

[a] Authors thank Dr. M. YOSHIKAWA of Institute of Medical Science, Tokyo University, Dr. T. YOKOTA of Juntendo University, and Dr. J. NAKAZAWA of Chiba University for the gift of bacterial strains.

[b] Strain 1103 was originally isolated as a mutant defective in enzyme I of PTS from strain 1100 by FOX and WILSON[8].

[c] Isolated as a mutant defective in adenylate cyclase from HfrR1.

hydrates. Time course of growth was recorded and carbohydrate utilization was determined.

PTS Activity: Each 100 ml of the early stationary phase culture of CSH2 or FR182, or mid phase II culture of FR190, was harvested by centrifugation at 4°C. Then the cells were washed two times by centrifugation in 10 ml of 50 mM Tris-HCl (pH 7.5) and resuspended in the same buffer at a density of 10 A_{550} unit per ml, which corresponded to 13~16 mg of wet cell weight per ml for the strains examined.

PTS activity was measured in toluene-treated cells[10] and the assay procedure was essentially that of FOX and WILSON[8]. A mixture of 100 μl of toluene-treated cell suspension and 300 μl of preincubated (30°C, 2 minutes) reaction mixture, containing 1 mM $MgCl_2$, 20 μl; 0.5 M Tris-HCl (pH 7.5), 40 μl; 20 mM dithiothreitol, 20 μl; 0.2 M KF, 20 μl; 1 mM methyl α-D-[U-^{14}C]glucopyranoside, 40 μl; 0.1 M PEP, 20 μl; and water, was incubated for 30 minutes at 30°C. The reaction was terminated by the addition of 10 μl of 40 mM disodium ethylenediaminetetraacetate (EDTA) and by rapid cooling in an ice bath. Then the mixture was centrifuged at 20,000$\times g$ for 10 minutes at 4°C, and 100 μl of this supernatant was applied to a column (1$\times$2 cm) of Dowex 1 X2 treated with formic acid and washed with water. The column was washed with 10 ml of water and eluted with 0.5 M ammonium formate - 0.2 N formic acid (8 ml). An aliquot of the eluate was assayed for radioactivity by a scintillation counter.

Cyclic AMP: For determination of cellular or extracellular cAMP, an aliquot (5 ml) of the culture, incubated for 1~2 hours after the end of cell growth, was filtered on Millipore membranes (HAWP, 0.45 μm pore size, 25 mm diameter). Samples were prepared as described by PETERKOFSKY and GAZDAR[11] with some modifications. The cells on the filter were washed with 5 ml of 10 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and 0.5 mM $MgCl_2$ (washing solution II), and immersed in 2 ml of 0.2 N formic acid. The cell suspension was heated in a boiling water bath. Then the Millipore filter was removed and the sample was lyophilized. For determination of extracellular cAMP, 1 ml of the culture filtrate from the Millipore filter was added to 0.4 N formic acid (1 ml), boiled, and lyophilized, as was done to the trapped cells. These dried samples were dissolved in 1 ml of 50 mM Tris-HCl (pH 7.5) containing 4 mM EDTA and centrifuged at 3,000 rpm for 10 minutes. The supernatant was used for cAMP determination by the cAMP assay kit (The Radiochemical Centre, Amersham, England).

Uptake of FOM and G-3-P: The mid log phase culture (100 ml) of CSH2 or FR182, or the early phase II culture (100 ml) of FR190 was harvested by centrifugation, the cells were washed twice by centrifugation in 10 ml of 50 mM Tris-HCl (pH 7.5) containing 0.1 mg of chloramphenicol per ml (washing solution I), and resuspended in the washing solution I at the cell density of 10 A_{550} units per ml, corresponding to 14~19 mg wet cell weight per ml. Incubation mixture contained; 1 mg/ml of chloramphenicol, 50 μl; 0.5 M Tris-HCl (pH 7.5), 50 μl; 2.5 mM [1,2-^{3}H]FOM or 250 μM *sn*-[U-^{14}C]glycerol-3-phosphate, 100 μl; and water, 300 μl. The mixture and the cell suspension were preincubated separately at 30°C for 2 minutes. The reaction was started by addition of the cell suspension (0.5 ml) to the mixture and incubated at 30°C. Then 100 μl of samples were collected on Millipore filters (HAWP) premoistened with the washing solution II, and washed rapidly with 5 ml of the washing solution II. The filters with cells were dried, placed in scintillation vials, and solubilized by methylcellosolve. The radioactivity was counted after the addition of toluene-based scintillation cocktail.

Reagents: cAMP, D-glucose-6-phosphate, and PEP were mono-, di-, and tri-sodium salts, respectively. G-3-P was used as disodium DL-α-glycerophosphate hexahydrate (α-GP). The concentration of G-3-P was expressed as an L-isomer. Methyl α-D-[U-^{14}C]glucopyranoside (α-MG) and disodium *sn*-[U-^{14}C]-glycerol-3-phosphate were from New England Nuclear Corp. Specific activity of α-MG was lowered to 10 mCi/mmol by the addition of unlabeled α-MG and that of G-3-P was lowered to 20 mCi/mmol by the addition of α-GP. Disodium fosfomycin (FOM) and phenethylammonium [1,2-^{3}H]fosfomycin monohydrate (4.04 mCi/mmol) were the gift from Merck Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A.

Results

Isolation of FOM-resistant Strains

FOM-resistant mutants were isolated from *E. coli* strain CSH2 on the nutrient agar plates contain-

ing 200 μg/ml of FOM at a frequency of 1.2×10^{-6}. Mutants were tested for fermentation of glucose and lactose. In 438 strains tested, 23 strains did not ferment the two sugars and 8 strains fermented only glucose. One of each strain was selected from both types of mutants for further identification of the mutants; strain FR182 not fermenting glucose and lactose, and strain FR190 fermenting glucose but not lactose.

The sensitivity of the parent and two resistant strains to FOM is shown in Table 2. The sensitivity of FR182 and FR190 to FOM was estimated to be 250~1,000 times lower than that of the parent strain CSH2 in these determinations, except that of minimal concentration of FOM for no growth. Minimal concentration for no growth was high in CSH2. This phenomenon was due to dotted colonies or pale growth on the streaked plates containing concentrations over maximal concentration for growth.

The abilities to utilize carbohydrate were further tested as shown in Table 3. Both of FOM-resistant strains and the sensitive parent strain could utilize G-6-P, but G-3-P, mannitol, glycerol, and lactose were not utilized by the mutants. Glucose was utilized by mutant FR190 but not by mutant FR182. Glucose and mannitol are known as PTS sugars[12,13], and the utilization of G-3-P, glycerol, or lactose is under the effect of catabolite repression[14].

Defect of PTS Activity in Mutant FR182

Two different mechanisms could be assumed for simultaneous loss of ability in these mutants for utilization of variety of sugars as described in the preceding section; defect in PTS activity and defect in cAMP formation. Activity of PTS in the toluene-treated cells of resistant strain was therefore assayed.

Table 2. Sensitivity of FOM-sensitive parent and two resistant strains to FOM
Concentrations of FOM were serial two-fold dilutions. For the lysis test, serial four-fold dilutions of FOM were used below 100 μg/ml.

	Streak test		Lysis test	
E. coli strain	Maximal concentration of FOM for growth[a] (μg/ml)	Minimal concentration of FOM for no growth[b] (μg/ml)	Maximal concentration of FOM for no lysis[c] (μg/ml)	Minimal concentration of FOM for lysis[d] (μg/ml)
CSH2 (Parent)	0.39	100	0.098	0.39
FR182	200	>400	100	200
FR190	100	400	100	200

[a] Maximal concentration of FOM for exhibiting growth on plate same as on control plate (no antibiotic).
[b] Minimal concentration of FOM for exhibiting no growth or formation of below 20 colonies on plate.
[c] Maximal concentration of FOM for no lysis even though growth was repressed.
[d] Minimal concentration of FOM for clear lysis.

Table 3. Carbohydrate utilization of the parent and the resistant strains

E. coli strain	G-6-P	G-3-P	Glucose	Mannitol	Glycerol	Lactose
CSH2 (Parent)	+	+	+	+	+	+
FR182	+	±	±	±	−	±
FR190	+	±	+	±	−	±

One loopful of the preculture was streaked on minimal agar plates containing one of the indicated carbohydrates at 0.4%. The plate was incubated and scored for growth. +, growth after 1 day; ±, growth or pale growth after 2 days; −, no growth after 2 days.

Table 4. PTS activity of toluene-treated cells in FOM-sensitive parent and resistant strains

E. coli strain	Incubation condition		
	Complete	Minus PEP	Difference
CSH2 (Parent)	0.41	0.01	0.40
FR182	0.001	0.002	—
FR190	0.78	0.02	0.76

The values are expressed as nmol phosphorylated methyl α-D-glucopyranoside per min per mg of wet cells. For experimental detail see Materials and Methods.

Table 5. Cellular and extracellular cAMP concentrations

E. coli strain	Cellular[a]	Extracellular[b]
CSH2 (Parent)	32	3.6
FR182	< 0.01	0.09
FR190	< 0.01	<0.01

[a] nmoles of cAMP per g of wet cells.
[b] μmoles of cAMP per liter of growth medium.

Table 4 shows that almost complete loss of PTS activity occurred in FR182, whereas the activity was present in FR190.

Lowered Level of cAMP in FR182 and FR190

Both resistant strains, FR182 and FR190, had extremely low levels of formation of cAMP as shown in Table 5. Concentration of cAMP in growth medium was low in the mutant FR182 and undetectable in FR190. The concentration of cAMP was also extremely low in the cells of both resistants.

Relation of FOM-resistance to the Defect in PTS Activity or to Lowered Level of cAMP in Resistant Strains

Experiments described in the preceding sections suggest that the FOM-resistance is related to the defect in PTS activity and/or to the lowered level of cAMP. This assumption could, however, be confirmed by experiments using mutants isolated by selection for defective PTS or defective cAMP formation. Strain 1103[8] is defective in enzyme I of PTS and strain YE100[9] is defective in adenylate cyclase. Both strains, as shown in Table 6, were resistant to FOM, whereas parent strains of each of the mutants were sensitive to this antibiotic. High value of minimal concentration of FOM for no growth in 1100 or HfrR1 was due to dotted colonies or pale growth on the plates containing concentrations of FOM over maximal concentration for growth, as in the case of CSH2 (Table 2).

Table 6. Sensitivity of 1103 (*pts* I) and YE100 (*cya*) to FOM

E. coli strain	Streak test		Lysis test	
	Maximal concentration of FOM for growth (μg/ml)	Minimal concentration of FOM for no growth (μg/ml)	Maximal concentration of FOM for no lysis (μg/ml)	Minimal concentration of FOM for lysis (μg/ml)
1100	0.39	50	0.098	1.56
1103	100	400	100	400
HfrR1	0.78	100	0.098	0.39
YE100	200	>400	200	>400

Experiments were performed as in Table 2.

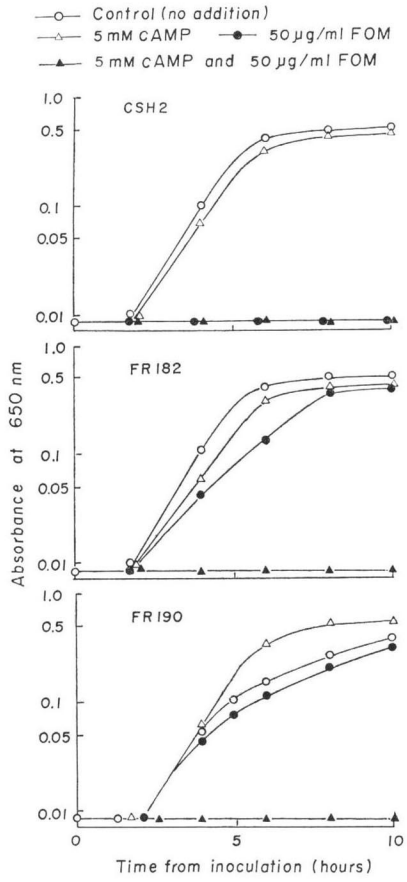
Simultaneous Restoration by cAMP of FOM-sensitivity Coupled with Recovery of Multiple Carbohydrate Utilizability

As presented in Fig. 1, growth of two resistant strains, FR182 and FR190, in nutrient broth was almost unaffected by the addition of FOM (50 μg/ml). The growth of both resistant strains was significantly inhibited when cAMP was added to the culture in addition to FOM. In FR190, addition

of cAMP alone enhanced the growth. No significant effect by cAMP was observed in FOM-sensitive parent strain CSH2. The same results were also obtained with two other strains, 1103 and YE100 (results not shown). The sensitivity to FOM was restored in these resistant strains and growth of YE100, a *cya* mutant, was stimulated in the presence of cAMP alone.

The restoration by cAMP of sensitivity to FOM of these resistant mutants was associated with a recovery of ability of these mutants to utilize multiple carbohydrates. Utilization of carbohydrate in the FOM-sensitive parent strain CSH2 and in the resistant strains, FR182 and FR190, was determined for a short period in the presence and absence of 5 mM cAMP (Table 7). The resistant strain FR182 could not utilize G-3-P, glucose, mannitol, glycerol, or lactose, but utilized G-6-P. In the presence of cAMP, utilization of G-3-P, glycerol, and lactose was recovered in FR182, whereas utilization of glucose and mannitol was not. These results were compatible to those obtained with a *pts* I mutant, strain 1103, except that this strain utilized glucose in the presence of cAMP. The resistant strain FR190 did not utilize G-3-P, mannitol, glycerol and lactose. In the presence of cAMP, all of the above-mentioned carbohydrates tested were utilized by this strain. These results were compatible to those obtained with YE100 (*cya*), except that the utilization of glucose in YE100 was negative in this determination.

Fig. 1. Recovery of sensitivity to FOM by cAMP. Preculture was inoculated at 1% into nutrient broth containing 5 mM cAMP, 50 μg/ml FOM, or 5 mM cAMP and 50 μg/ml FOM. Growth was followed by use of an automatic growth recorder.



All of the above-mentioned strains did not utilize G-3-P, but grew on G-6-P and were sensitized to FOM by G-6-P as shown in Table 8.

Uptake of FOM and G-3-P, and Effect of cAMP

Fig. 2 shows the uptake of FOM by the cells of sensitive parent strain CSH2. In resistant strains, FR182 and FR190, no accumulation of FOM was observed in the experimental conditions used (Fig. 2A). In the cells cultured in the presence of 5 mM cAMP, accumulation of FOM was as great in the resistant strain as in the sensitive parent (Fig. 2B).

Similarly, uptake experiments of G-3-P were carried out and the results are shown in Fig. 3. The cells of the sensitive parent grown in nutrient broth rapidly took up G-3-P, but uptakes by cells of the resistant strains were slow (about 1/20 of the initial velocity in the parent cells) (Fig. 3A). By cultivation in the presence of cAMP, the rates of uptake of G-3-P by both of the resistant strains

Table 7. Recovery of utilization of carbohydrate by cAMP addition

E. coli strain	Addition	G-6-P	G-3-P	Glucose	Mannitol	Glycerol	Lactose
CSH2	none	+	+	+	+	+	+
	cAMP	+	+	+	+	+	+
FR182	none	+	−	−	−	−	−
	cAMP	+	+	−	−	+	+
FR190	none	+	−	+	−	−	−
	cAMP	+	+	+	+	+	+
1100	none	+	+	+	+	+	+
	cAMP	+	+	+	+	+	+
1103	none	+	−	−	−	−	−
	cAMP	+	+	+	−	+	+
HfrR1	none	+	+	+	+	+	+
	cAMP	+	+	+	+	+	+
YE100	none	+	−	−	−	−	−
	cAMP	+	+	+	+	+	+

Washed cells were inoculated into a liquid medium and scored for growth by using an automatic growth recorder. Carbon source and cAMP were supplemented to one test medium at 0.4% and 5 mM, respectively. +, beginning of growth was observed within 10 hours after growth of each parent strain had been initiated in each growth medium (no cAMP); −, growth was not observed within the period.

increased to the value of the parent cells (Fig. 3B). Time course of G-3-P uptake was compatible with that in the experiments in which the reaction mixture was supplemented with glycerol at 2.5 mM of final concentration for confirming the uptake of G-3-P without hydrolysis by non-specific alkaline phosphatase[15].

Table 8. Sensitization to FOM by G-6-P

E. coli strain	Control	G-6-P	FOM	G-6-P + FOM
CSH2	+	+	−	−
FR182	+	+	+	−
FR190	+	+	+	−
1100	+	+	−	−
1103	+	+	+	−
HfrR1	+	+	−	−
YE100	+	+	+	−

Each preculture of the strains was inoculated at 1% into nutrient broth (control) containing 50 μg/ml G-6-P, 50 μg/ml FOM, or 50 μg/ml G-6-P and 50 μg/ml FOM. Growth was observed by using an automatic growth recorder. +, growth similar to that of control was observed; −, growth was delayed over 10 hours compared with control growth.

Discussion

A mutant, FR182, defective in PTS, and a mutant, FR190, defective in cAMP formation were selected from FOM-resistant strains. Furthermore, 1103 (*pts* I) and YE100 (*cya*) were resistant to FOM (Table 6). In *Salmonella typhimurium*, it was reported that the mutants defective in the gene regions containing *pts* I were selected by FOM and mutants in which some or all of the *pts* I gene was deleted were resistant to FOM[6]. Several characteristics of their strains, sensitization to FOM by cAMP and G-6-P and the recovery of carbohydrate utilization by cAMP, were the same as those of FR90 in our previous observations[5] and FR182 in this study.

In *E. coli*, enzyme I of PTS is related to the activation of adenylate cyclase[16,17]. The decrease in cAMP formation in FR182 is expected to arise from lowering of adenylate cyclase activity due to loss of the PTS component. FR190 shows a decrease in cAMP formation. In 1103, the cellular cAMP was lower than that of the parent, 1100[16], which was sensitive to FOM. The amount of cellular cAMP in YE100 (*cya*) is thought to be lowered significantly. As a conclusion, it is suggested that lowering of cellular cAMP is related to the resistance to FOM.

Fig. 2. Uptake of FOM by cells grown on nutrient broth (A) and cells grown on nutrient broth supplemented with 5 mM cAMP (B).

The assay was conducted as described under Materials and Methods. In FR190, because the cells cultured in the presence of cAMP were not fully sedimented by the usual centrifugation, the sedimented cells (90 to 95% of the total cells) were used for the assay.

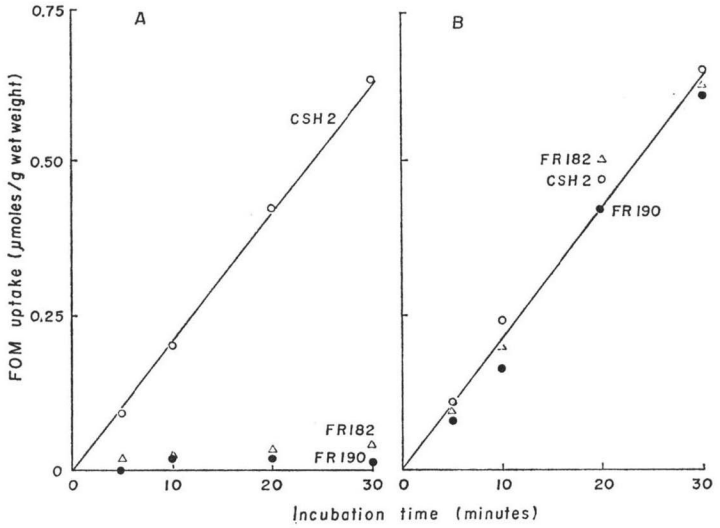
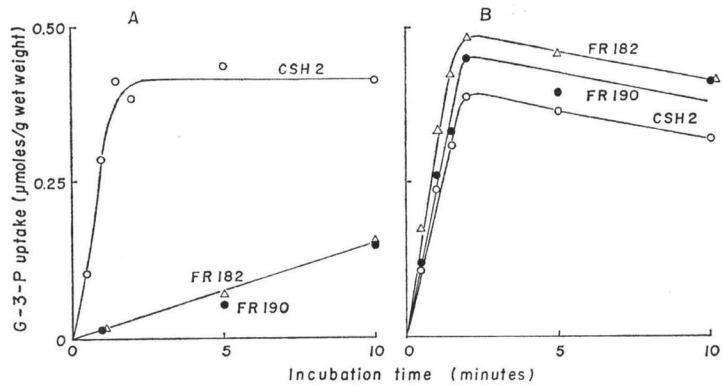


Fig. 3. Uptake of G-3-P by cells grown on nutrient broth (A) and cells grown on nutrient broth supplemented with 5 mM cAMP (B).

The assay was conducted as described under Materials and Methods. For FR190 cultured in the presence of cAMP, see the legend to Fig. 2.



FOM is taken up by the cells *via* the G–3–P transport system[1,3] and the hexose phosphate transport system[1,2]. The latter transport system requires induction by extracellular but not intracellular G–6–P[18,19]. G–6–P was utilized by FR182, FR190, 1103 or YE100 (Table 7) and they became sensitive to FOM by G–6–P addition (Table 8). Therefore, in these strains, the hexose phosphate transport system is active for FOM. On the other hand, the resistant strains used in this work had decreased G–3–P utilization in common. Hence it is suggested that these strains are resistant to FOM because their G–3–P transport system is not functioning.

The G–3–P transport system is under the regulation of *glp* regulon which is inducible by G–3–P[20,21]. Catabolite repression in the transport system was reported[20] and this repression was overcome by

cAMP[14,22]. From this point of view, induction of the transport system is thought to require both G-3-P as the inducer and cAMP. In a FOM-sensitive strain cultured in nutrient broth, transport site for FOM is thought to be that of G-3-P. Therefore, the system may not be strictly inducible (constitutive in part) or may be induced by cellular G-3-P, in this growth condition. It was confirmed that G-3-P transport system became functional by growing CSH2 in nutrient broth (Fig. 3A).

In uptake experiments, FR182 or FR190 did not take up FOM at all within 30 minutes (Fig. 2A). Simultaneously, the uptake of G-3-P was lowered, but the uptake was observed (Fig. 3A). Since the initial velocity in the uptake of FOM was lowered significantly compared with that of G-3-P in spite of the higher concentration of FOM in CSH2, it is anticipated that the affinity of FOM to G-3-P transport site is significantly low in contrast with that of G-3-P. Incomplete loss of utilization of G-3-P in the resistant strains (Table 3, reference 6) may reflect this leaky lowered uptake and this possibility is under examination. Sensitization by cAMP (Fig. 1) could be explained by the recovery of the uptake of FOM and G-3-P by cAMP (Fig. 2B, Fig. 3B).

In summary, it is suggested that the newly isolated mutant strains FR182 and FR190 in this work are resistant to FOM because of their incapability to take up the antibiotic *via* the G-3-P transport system by a reduction in the amount of their cellular cAMP, and they are different from the previously isolated resistant strain which was reported as *glp* T by KAHAN *et al.*[1]

Finally, FOM-resistant strains defective in the utilization of many carbohydrates have not been isolated *in vivo*, perhaps due to loss of pathogenicity.

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