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Thiamine Regulatory Mutants in *Escherichia coli**

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Mutants affecting regulation of thiamine biosynthesis from its pyrimidine and thiazole moieties were isolated from *Escherichia coli* K12 as resistant strains to growth inhibition by pyrithiamine, an antimetabolite of thiamine.

The mutants (PT-R1 and PT-R3) have 3-fold higher cellular thiamine content than the parent strain. Among four enzymes involved in thiamine synthesis from its pyrimidine and thiazole moieties, the mutants contain derepressed levels of hydroxyethylthiazole kinase [EC 2.7.1.50] and thiaminephosphate pyrophosphorylase [EC 2.5.1.3] and repressed levels of hydroxymethylpyrimidine kinase [EC 2.7.1.49] and phosphohydroxymethylpyrimidine kinase [EC 2.7.4.7]. The former two enzymes are not repressed by the end-product, thiamine, and the latter two enzymes can be derepressed by adenine that lowers the cellular thiamine content. Evidence is presented to indicate that these mutants are in the same class of mutation with only difference in the extent of the repressed level of phosphohydroxymethylpyrimidine kinase.

By conjugation tests the chromosomal position corresponding to the mutation in PT-R1 was found to link to the *arg* and *met* loci, and probably very close to the *thi* locus. The mutants might be similar to operator constitutive mutants studied in other systems.

Thiamine biosynthesis in *Escherichia coli* from its pyrimidine and thiazole moieties involves four enzymes (*1*), namely hydroxymethylpyrimidine kinase [I, EC 2.7.1.49], phosphohydroxymethylpyrimidine kinase [II, EC 2.7.4.7], hydroxyethylthiazole kinase [III, EC 2.7.1.50], and thiaminephosphate pyrophosphorylase [IV, EC 2.5.1.3], as shown in Fig. 1. The synthesis of these four enzymes in *E. coli* is regulated, mostly in parallel, by the end-product, thiamine (*2*). The levels of the four enzymes can vary from one, repressed level, to about 5—10 under conditions of thiamine limitation. Although it has not yet been determined whether genetic determinants of these enzymes are located in a cluster or in separate regions of the chromosome, it seems to be possible to assume that thiamine biosynthesis from hydroxymethylpyrimidine** and hydroxyethylthiazole is controlled by a similar regulatory mechanism to the operon model as proposed by Jacob and Monod (*3*).

There are several examples in which mutants selected as resistant strains to growth inhibition by antimetabolites are altered in their regulatory mechanisms, such as canavanine-resistant strains in arginine biosynthesis (*4*), 5-methyltryptophan-resistant strains in tryptophan biosynthesis (*5—7*), 2-thiazolealanine-resistant (*8*) and triazolealanin-resistant strains (*9*) in histidine biosynthesis.

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** The abbreviations used are;
Hydroxymethylpyrimidine: 2-Methyl-4-amino-5-hydroxymethylpyrimidine,
Hydroxyethylthiazole: 4-Methyl-5-hydroxyethylthiazole,
Pyrithiamine (PT): 1-[(4-amino-2-methyl-)5-pyrimidylmethyl]-2-methyl-3-(β-hydroxyethyl)pyridine,
pt^r and *pt*^{s}: Resistance and sensitivity to pyrithiamine,
sm^r and *sm*^{s}: Resistance and sensitivity to streptomycin.

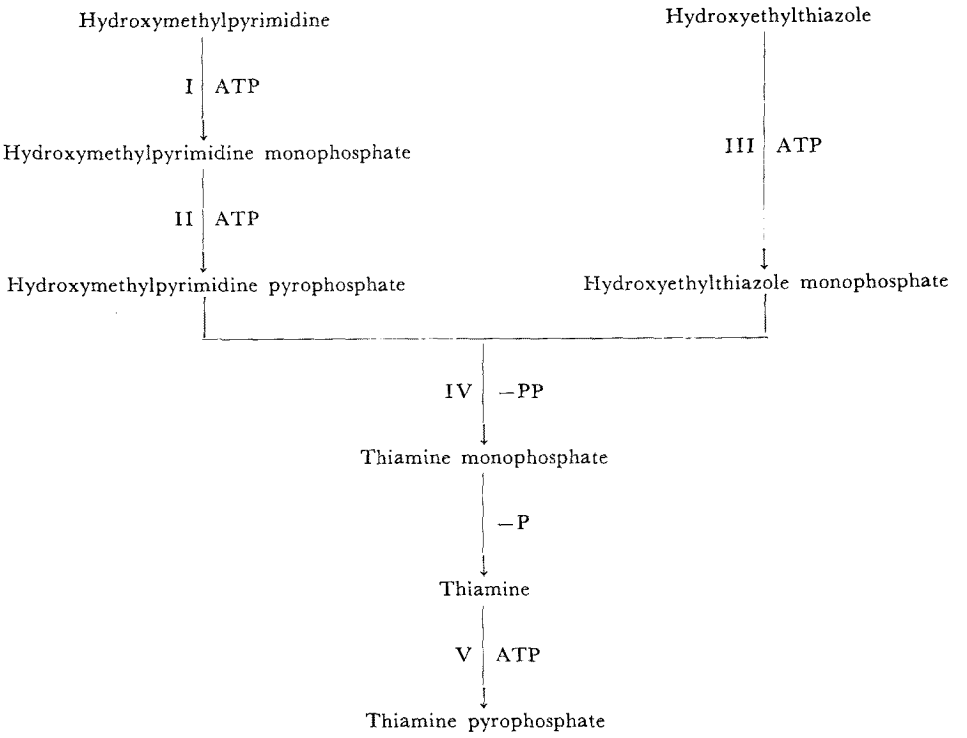

FIG. 1. The pathway of thiamine biosynthesis in *Escherichia coli*.
I: Hydroxymethylpyrimidine kinase, II: Phosphohydroxymethylpyrimidine kinase,
III: Hydroxyethylthiazole kinase, IV: Thiaminephosphate pyrophosphorylase,
V: Thiamine pyrophosphokinase.

No such mutant, however, has been reported on the biosynthesis of vitamins or coenzymes.

Thiamine regulatory mutants obtained have 3 times higher cellular thiamine content than the parent strain, and have derepressed levels of two enzymes (III and IV) among the four enzymes involved in thiamine synthesis. These two enzymes are not able to be repressed even in the presence of thiamine. The mutants were isolated by selection for resistance to growth inhibition by pyrithiamine, antimetabolite of thiamine (Fig. 2). This analogue, in which the thiazole ring of thiamine is replaced by the pyridine ring, has been shown to inhibit thiamine pyrophosphokinase [EC 2.7.6.2] in the membrane fractions of *E. coli* (*10*) as well as of yeast (*11*). The regulatory mutants, among the pyrithiamine-resistant strains, have a defective control mechanism for thiamine synthesis from hydroxymethylpyrimidine and hydroxyethylthiazole. Such mutants can increase cellular thiamine content, which is enough to overcome competition with the analogue, due to loss of repressibility of the derepressed two enzymes and thus escape growth inhibition. In this report are described some properties of the mutants and location of the mutation on *E. coli* chromosome.

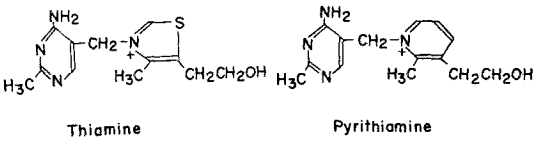

FIG. 2. The structure of pyrithiamine.

EXPERIMENTAL

Chemicals—Hydroxymethylpyrimidine, hydroxyethylthiazole and hydroxyethylthiazole monophosphate were kindly supplied by Dr. S. Yurugi of Takeda Chemical Industries, Osaka. Hydroxymethylpyrimidine monophosphate and its pyrophosphate used were the same as described previously (*2*). Disodium salt of ATP and pyrithiamine hydrobromide were purchased from Sigma. Takadiastase was purchased from Sankyo Co., Ltd., Tokyo. N-Methyl-N′-nitro-N-nitrosoguanidine was purchased from Aldrich Chemical Co. Acridine orange was the product of Merck. Adenine hydrochloride was the product of Kokoku Pulp Co., Ltd., Tokyo.

Media—The minimal salt liquid medium of DAVIS and MINGIOLI (*12*) was used with 0.2% glucose as carbon source and with 1.5% agar as solid medium. Brain heart infusion broth used as maximally supplemented medium was the product of Baltimore Biochemical Laboratory. The minimal eosin methylene blue medium without succinate and with 1% lactose (EM-lac) agar and the complete eosin methylene blue medium with 1% sugar (EMB-sugar) agar (*13*) were also used. Amino acids and thiamine supplements were added at a concentration of 50 μg/ml and 33.7 mμg/ml, respectively, unless otherwise noted.

Bacterial Strains—All strains used are derivatives of ***E. coli*** **K12.** **The following strains we**:e kindly supplied by Dr. Y. Hirota, Osaka University: AB261 (Hfr *met*$^-$), W4573 (F$^-$*ara*$^-$*xyl*$^-$*mtl*$^-$*mal*$^-$*gal*$^-$*lac*$^-$*sm*^{r}), and AB1450 (F$^-$*thi*$^-$*arg*$^-$*met*$^-$*ilv*$^-$*his*$^-$*xyl*$^-$*mtl*$^-$*mal*$^-$*gal*$^-$*lac*$^-$*sm*^{r}). Loci represented as *arg* and *met* in AB1450 are *argA*, *C*, *F*, *H* and *metB*, *F*, respectively (Y. Hirota, personal communication). Strain AB261 transfers the chromosome in the order *pro ara thi arg met ilv xyl mal sm his gal lac*. Strain AB14501 was selected as a thiamine-nonrequiring revertant from AB1450 (*14*). Only relevant markers are shown as genotype.

Isolation of Pyrithiamine-resistant Strains of E. coli K12—Mutants resistant to pyrithiamine, in which the regulatory mechanism of thiamine synthesis is altered, were isolated by two different procedures. Strain PT-R1 was obtained from *E. coli* K12 by a multi-step selection procedure (*15*, *16*). One drop of 50-fold diluted overnight culture of *E. coli* K12 in the minimal medium was inoculated in 5 ml of the same medium containing 10 μM pyrithiamine and allowed to stand at 37°C. The growth of bacteria in the presence of pyrithiamine required 12 more hours than the control to reach the stationary phase. One drop of 50-fold diluted cell culture thus obtained was inoculated and incubated in the presence of 1 mM pyrithiamine in the same manner as described above. The bacteria at the second incubation with a higher concentration of the analogue showed the normal growth curve. This procedure was repeated 10 times and finally PT-R1 strain was isolated as a single colony on minimal agar containing 1 mM pyrithiamine. Strain PT-R3 was isolated by a single-step selection procedure (*4*) of *E. coli* K12. Plating of 1×10^8 viable cells on minimal agar containing 0.1 mM pyrithiamine, followed by incubation for 2 days at 37°C, gave 118 survivals. Analysis of 15 colonies of the survivals selected at random revealed that 10 colonies had high cellular thiamine contents. One of these colonies was purified twice on the same agar as used for selection, and designated as PT-R3.

Strain PT-R1c used for mapping of the mutation is an Hfr strain carrying pyrithiamine resistance, which transfers the chromosome in the same order as AB261. This strain was obtained from the parent PT-R1 by the three-step procedure in essentially the same manner as described previously (*14*). The nitrosoguanidine treatment (*17*) converted PT-R1 (F$^+$*pt*^{r1}*lac*$^+$) to PT-R1a (F$^+$*pt*^{r1}lac$^-$), and the F$^+$ strain thus obtained was treated with acridine orange (*18*) to obtain the corresponding F$^-$ strain, PT-R1b (F$^-$*pt*^{r1}*lac*$^-$). Finally, PT-R1c was selected as recombinants in a cross of PT-R1b with AB261 on EM-lac agar medium. Strain PT-R1c was confirmed to be an Hfr strain by giving many recombinants of black color in a cross with W4573 on EMB-ara agar plate.

Genetic Procedures—Crosses between Hfr and F$^-$ strains were used for mapping of the mutation in the same manner as described previously (*14*). Donor bacteria, PT-R1c, and recipient bacteria, W4573 or AB14501, were grown overnight in 5 ml of brain heart infusion broth at 37°C. One ml each of the cultures was mixed together and then allowed to stand for 3 hr at 37°C, followed by centrifugation and washing with saline solution. The mating cells were resuspended in 3 ml of saline solution. Various dilutions of the mating cell suspension were plated on appropriate agar media containing selected markers and streptomycin (100 μg/ml) and incubated for 2 days at 37°C. Colonies (50 to 200/plate) appeared on those plates were twice purified on the same agar plates as used for selection of the recombinants. Overnight cultures of the recombinants in 5 ml of the minimal medium with appropriate supplements were streaked on appropriate EMB-sugar agar plate or minimal agar plate containing appropriate supplements and incubated overnight at 37°C and then unselected markers were scored. Pyrithiamine concentration used for test of the resistance in the recombinants was 0.1 mM in minimal agar with supplements. Appropriate controls for both donor and recipient bacteria were always run.

Preparation of Cell Suspensions and of Enzyme Extracts—Cells were grown in 400 ml of the minimal

medium in the presence or absence of supplements indicated in the text at 37°C with constant shaking and harvested at late exponential phase by centrifugation for 10 min at 6,000×*g*. Procedures for preparation of the washed-cell suspensions and of the enzyme extracts by sonic disruption were previously described (*2*). Preparation of the sonic membrane fractions used for assay of thiamine pyrophosphokinase was also described previously (*10*).

Assay Methods—The reaction mixtures and methods used for assay of activities of four enzymes involved in thiamine synthesis (*2, 14*) and used for assay of thiamine formation from the pyrimidine and thiazole moieties by washed-cell suspensions (*2*) were previously described. Thiamine pyrophosphokinase activity was determined manometrically (*10*).

RESULTS

Properties of PT-R1 and PT-R3 Mutants

The mutants isolated could grow in the presence of 10 mM pyrithiamine, while growth of the parent strain, *E. coli* K12, was inhibited by pyrithiamine at 10 μM when 5×10^5 cells were inoculated in 5 ml of the minimal medium and incubated for 20 hr at 37°C without shaking.

Cellular Thiamine Content—The resistant strains have an intracellular thiamine content about 3 times as high as that of the parent strain (Table I). However, no excretion of thiamine into the growth medium was detected in the resistant strains as well as in the wild parent strain.

Enzyme Activities Involved in Thiamine Synthesis—In order to know mechanisms to explain the *de novo* increase of cellular thiamine content in the resistant strains, four enzyme activities, which are involved in thiamine synthesis from hydroxymethylpyrimidine and hydroxyethylthiazole (see Fig. 1), were determined (Table II). Thiaminephosphate pyrophosphorylase and hydroxyethylthiazole kinase activities were 10- to 15-fold and 2-fold higher in the mutants than those in the parent strain, respectively. The increased activity of the former enzyme was equal to that in a mutant of *E. coli* W auxotrophic for hydroxyethylthiazole which was grown under thiamine limitation (*2*). In contrast, hydroxymethylpyrimidine kinase in both of the mutants and phosphohydroxymethylpyrimidine kinase in PT-R1 were too low to detect, whereas the latter enzyme in PT-R3 could be detected to be at a half level of the parent strain. Thiamine pyrophosphokinase, which is the target enzyme of pyrithia-

TABLE I

Cellular thiamine content.

All strains were grown on the minimal medium and washed-cell suspensions were prepared. An aliquot of the suspensions was added to 10 ml of 0.1 M acetate buffer, pH 4.5, and then heated for 15 min at 85°C to extract thiamine phosphates, followed by hydrolysis with 0.2% takadiastase for 30 min at 45°C. Thiamine thus obtained was measured by the thiochrome method.

Strain	Thiamine content (mμmole/mg dry weight)
E. coli K12	0.10
PT-R1	0.31
PT-R3	0.27

TABLE II

Enzyme levels involved in thiamine synthesis in pyrithiamine-resistant mutants.

Strain	Hydroxymethyl-pyrimidine kinase	Phosphohydroxy-methylpyrimi-dine kinase	Hydroxyethyl-thiazole kinase	Thiaminephos-phate pyrophos-phorylase	Thiamine pyrophospho-kinase
	(mμmole/mg protein/hr)				
E. coli K12	0.12	0.18	0.19	0.98	0.04
PT-R1	0	0	0.34	15.4	0.05
PT-R3	0	0.08	0.33	10.6	—

mine, showed practically the same activity in both of the membrane fractions of *E. coli* K12 and PT-R1 strain. A possibility that negligible activities of the two kinases are due to deletion is excluded by the facts that the mutants can grow in the minimal medium and that these two enzymes are able to be derepressed by adenine (Table IV).

Effect of Thiamine Added to the Growth Medium—Cellular thiamine content controls formations of the four enzymes involved in thiamine synthesis in *E. coli* (*2*); derepression was brought about by thiamine at 5 μM in the cell fluid (0.02 mμmole/mg dry weight), and complete repression was brought about at 35 μM (0.14 mμmole/mg dry weight). Thiamine content of the resistant strains was, as shown in Table I, about 0.31 mμmole/mg dry weight (75 μM in the cell fluid), which is the concentration far enough to cause complete repression of the thiamine synthesizing enzymes in *E. coli*. Nevertheless, derepressed levels of hydroxyethylthiazole kinase and thiaminephosphate pyrophosphorylase were detected in the both mutants, suggesting alterations of repressibility of these two enzymes by the end-product, thiamine. This was confirmed by the experiments (Table III) in which 1.0 μM thiamine in the growth medium did not cause reduction in activities of hydroxyethylthiazole kinase and thiaminephosphate pyrophosphorylase in the mutants, while 0.1 μM thiamine brought about complete repression of the four enzymes in the parent strain. A possibility that thiamine uptake by the mutants having high thiamine content was impaired was ruled out by the experiments in which the extent of thiamine uptake by washed-cell suspensions of the mutants incubated with ^{14}C-thiamine at 1 μM and 0.4% glucose was two third of that of the parent strain.

Effect of Adenine in the Growth Medium—Low activities of hydroxymethylpyrimidine kinase and phosphohydroxymethylpyrimidine kinase in the mutants may be explained by repression of their formations by the increased thiamine content. If this assumption is correct, formations of these two enzymes would be derepressed by reducing the enhanced thiamine concentration in the mutants. This reduction of the high thiamine content in the mutants could be brought about by growing the bacteria in the presence of adenine that causes derepression of thiamine synthesis in *E. coli* (*2*) as well as in *Salmonella* (*19*, *20*). As shown in Table IV, all four enzymes of the parent strain were derepressed

TABLE III

Effect of thiamine in the growth medium on enzyme activities involved in thiamine synthesis in pyrithiamine-resistant mutants.

All strains were grown on the minimal medium with or without thiamine as indicated.

Strain	Thiamine added (μM)	Hydroxymethylpyrimidine kinase	Phosphohydroxymethylpyrimidine kinase	Hydroxyethylthiazole kinase	Thiaminephosphate pyrophosphorylase
		(mμmole/mg protein/hr)			
E. coli K12	None	0.10	0.15	0.22	0.98
	0.1	0[1]	0[1]	0[1]	0.005
PT-R1	None	0	0	0.34	10.4
	1.0	0	0	0.38	10.7
PT-R3	None	0	0.07	0.33	11.3
	1.0	0	0	0.30	11.3

1) The levels of these enzymes were determined in the presence of the sonic extracts of PT-R1 strain to avoid a possibility that thiamine added to the growth medium represses only thiaminephosphate pyrophosphorylase.

TABLE IV

Effect of adenine added to the growth medium on thiamine synthesizing enzymes in pyrithiamine-resistant mutants.

All strains were grown in the minimal medium supplemented with (or without) 5 mM adenine. Doubling time of *E. coli* K12 and of the mutants was approximately 70 min and 50 min, respectively in the presence of adenine.

Strain	Adenine added (mM)	Hydroxymethylpyrimidine kinase	Phosphohydroxymethylpyrimidine kinase	Hydroxyethylthiazole kinase	Thiaminephosphate pyrophosphorylase
		(mμmole/mg protein/hr)			
E. coli K12	None	0.13	0.15	0.19	1.24
	5.0	0.31	0.38	0.54	9.92
PT-R1	None	0	0	0.39	10.0
	5.0	0.30	0.42	0.39	15.2
PT-R3	None	0	0.08	0.33	10.6
	5.0	0.42	0.57	0.60	11.0

TABLE V

Effect of adenine in the growth medium on thiamine synthesis by washed-cell suspensions of pyrithiamine-resistant strains.

All strains were grown in the minimal medium supplemented with adenine as indicated, and washed-cell suspensions were prepared. The reaction mixture contained: 0.05 M Tris-HCl, pH 7.5, 0.2% glucose, 0.9% NaCl, 10 μM hydroxymethylpyrimidine, 10 μM hydroxyethylthiazole, and cell suspensions (20 mg dry weight) in a total volume of 10 ml. After incubation for 30 min at 37°C with constant shaking, an aliquot was pipetted and amounts of thiamine formed were measured by the thiochrome method.

Adenine added (mM)	Thiamine formed (mμmole/mg dry weight/hr)		
	E. coli K12	PT-R1	PT-R3
None	0.46	0.06[1)]	0.06[1)]
0.5	1.81	0.33	2.06
1.0	2.12	0.39	2.37
2.0	——	0.56	2.53
5.0	2.82	2.66	2.74

1) These low activities of thiamine synthesis in the mutants are due to their repressed levels of hydroxymethylpyrimidine kinase and phosphohydroxymethylpyrimidine kinase as shown in Table II.

5- to 10-fold mostly in a coordinate manner by 5 mM adenine in the minimal medium. Hydroxymethylpyrimidine kinase and phosphohydroxymethylpyrimidine kinase of the mutants could also be derepressed to the same extent as above under the same conditions. Hydroxyethylthiazole kinase activity was slightly enhanced by adenine, whereas thiaminephosphate pyrophosphorylase activity remained constant in the mutants.

These results suggest that both strains of PT-R1 and PT-R3 belong to the same class of mutation affecting thiamine regulatory mechanism, except for more strongly repressed level of phosphohydroxymethylpyrimidine kinase in PT-R1 than in PT-R3. This suggestion was supported by the following experiment. When the washed suspensions of the cells grown in the presence of adenine were incubated with hydroxymethylpyrimidine

and hydroxyethylthiazole, it was found that PT-R1 required 10 times higher concentration of adenine than that for PT-R3 to cause derepression of thiamine synthesis (Table V).

This difference in the repressed level of phosphohydroxymethylpyrimidine kinase was probably resulted from difference in the isolation procedure, since PT-R1 strain was obtained by the multi-step procedure, while PT-R3 strain was obtained by the single-step isolation as described in "EXPERIMENTAL".

The results described led to an assumption that elevation of thiamine content in the mutants overcomes the inhibitory effect of pyrithiamine on thiamine pyrophosphokinase as results of derepression and loss of repressibility of hydroxyethylthiazole kinase and thiaminephosphate pyrophosphorylase. Thus, the mutants can escape from growth inhibition by the antimetabolite.

Mapping of the PT-R1 Gene

Because of no genetic marker on the chromosome of PT-R1 selected from the wild strain of *E. coli* K12, PT-R1 strain was converted to PT-R1c that is an Hfr strain. Strain PT-R1c was confirmed to have the same biochemical properties as the parent PT-R1 as described above.

In a preliminary experiment to determine the pt^{r1} locus, W4573 ($F^-ara^-xyl^-mtl^-mal^-sm^r gal^-lac^-pt^s$) was used as a partner in a cross with PT-R1c (Hfr $ara^+xyl^+mtl^+mal^+sm^s gal^+lac^+pt^{r1}$). The recombinants ara^+sm^r, xyl^+sm^r, and gal^+sm^r were selected and unselected markers were scored. The results indicate that the pt^{r1} locus is not linked to the *ara* gene, but is linked 22.0% to the *xyl* locus. In order to

TABLE VI

Linkage of pt^{r1} with arg and met in the cross between PT-R1c and AB14501[1].

Recombinants	*pt*	*arg*	*met*	*ilv*	*sm*	*his*	No. of recombinants[2]
arg^+sm^r	0	1	0	0	0	0	10 (10)
	1	1	0	0	0	0	31 (31)
	1	1	1	0	0	0	50 (50)
	0	1	1	0	0	0	8 (8)
	0	1	1	1	0	0	1 (1)
met^+sm^r	0	0	1	0	0	0	8 (8)
	1	0	1	0	0	0	11 (11)
	1	1	1	0	0	0	68 (68)
	0	1	1	0	0	0	4 (4)
	1	1	1	1	0	0	8 (8)
	1	0	1	1	0	0	1 (1)
$sm^r his^+$	0	0	0	0	0	1	49 (84.5)
	0	0	0	1	0	1	4 (6.9)
	1	0	0	0	0	1	2 (3.5)
	1	1	0	0	0	1	2 (3.5)
	1	1	1	0	0	1	1 (1.6)

1) PT-R1c: Hfr $pt^{r1}arg^+met^+ilv^+sm^s his^+$, 111111; AB14501: $F^-pt^s arg^-met^-ilv^-sm^r his^-$, 000000. The other markers in AB14501 that are not used as unselected markers in this experiment are not represented. The recombinants arg^+sm^r, met^+sm^r, and $sm^r his^+$ were selected on minimal agar supplemented with appropriate amino acid (50 μg/ml) and streptomycin (100 μg/ml). Other procedures are presented in the text. The alleles of markers derived from the Hfr parent are represented by 1, and those derived from the F^- parent, by 0.

2) Numbers in parentheses are percentage of the total.

determine the pt^{r1} locus more precisely, F^-pt^s strain carrying $arg^-met^-ilv^-sm^r his^-$ mutations (AB14501) was used in a cross with Hfr pt^{r1} (PT-R1c). The recombinants arg^+sm^r, met^+sm^r, and his^+sm^r were selected and unselected markers were scored. The pt^{r1} gene was detected to be very closely linked to the *arg* and *met* genes, which are located near the *thi* gene on the chromosome of AB14501, but not to the *his* gene (Table VI).

DISCUSSION

There are many types of biochemical mechanisms for drug resistance (*21*). Among those, the break or alteration in the regulation of enzyme synthesis seems to be the case of the present mutation in *E. coli* K12 resistant to pyrithiamine, although a possibility of alteration in the sensitivity of thiamine pyrophosphokinase to pyrithiamine is not completely excluded in this paper.

Biochemical properties of the mutants suggests that thiamine synthesizing system from hydroxymethylpyrimidine and hydroxyethylthiazole is composed of at least two operons; one with structural genes of hydroxymethylpyrimidine kinase and phosphohydroxymethylpyrimidine kinase and the other with those of hydroxyethylthiazole kinase and thiaminephosphate pyrophosphorylase.

As reported previously, a thiamine-auxotrophic mutant was isolated from *E. coli* K12, which lacks only thiaminephosphate pyrophosphorylase among these four enzymes, and this mutation was mapped on the chromosome closely linked to the *arg* and *met* genes (*14*). Results of preliminary mapping experiments with mutants auxotrophic for hydroxymethylpyrimidine and hydroxyethylthiazole suggest that these mutations on the chromosome are also linked to the *xyl* gene in a cross with W4573 (unpublished observation). It might be, therefore, possible to assume that genes of at least some enzymes other than the four enzymes involved in thiamine biosynthesis are located in a small region on the chromosome close to (or including) the *thi* gene in the standard chromosome map (*22*). The facts that the four enzymes are almost coordinately repressed by thiamine (*2*) and derepressed by adenine (Table IV) would support the idea that genes of these enzymes might be existent on the chromosome in a cluster as discussed in the other system (*23*).

No regulator gene mutant of thiamine biosynthesis has been reported, which corresponds to other systems such as arginine (*4*) and tryptophan biosynthesis (*5*) in *E. coli*. Therefore, it seems to be possible to explain the biochemical and genetic properties of thiamine regulatory mutants in two ways: (i) the strains are constitutive mutants of a regulator gene that governs an operon which involves structural genes of at least hydroxyethylthiazole kinase and thiaminephosphate pyrophosphorylase. This possibility suggests an existence of another regulator gene that controls a system which at least involves structural genes for hydroxymethylpyrimidine kinase and phosphohydroxymethylpyrimidine kinase; (ii) the strains are constitutive mutants of an operator gene of the operon which probably involves genes of hydroxyethylthiazole kinase and thiaminephosphate pyrophosphorylase. This is based on a possible existence of one regulator gene for a whole pathway of thiamine biosynthesis, and also of an intact operator gene which governs the operon composed of structural genes of hydroxymethylpyrimidine kinase and phosphohydroxymethylpyrimidine kinase. In order to decide exactly which possibility is correct to explain properties of the PT-R mutants described, further genetic evidence for thiamine synthesizing system should be accumulated. However, the following facts might favor to support the second possibility; (1) the pt^{r1} gene is mapped close to the *thi* gene probably including the structural gene of thiaminephosphate pyrophosphorylase in it, (2) regulator gene mutation of other systems is mapped on chromosome far from their structural genes or operons. PT-R1 and PT-R3 are therefore assumed to be similar to histidine regulatory mutants, *hisO*, obtained as triazolealanine-resistant strains (*9*).

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