JOURNAL OF BACTERIOLOGY, Dec. 1984, p. 1101–1104
0021-9193/84/121101-04$02.00/0

Vol. 160, No. 3

Purine-Mediated Growth Inhibition Caused by a *pyrE* Mutation in *Escherichia coli* K-12

MAKOTO SHIMOSAKA,* YASUKI FUKUDA, KOUSAKU MURATA, AND AKIRA KIMURA

Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan

Received 6 April 1984/Accepted 10 September 1984

A purine-sensitive phenotype results from a previously described mutation in the structural gene (*pyrE*) for orotate phosphoribosyltransferase (OPT) in *Escherichia coli* K-12. OPT from both the mutant and the wild-type was partially inhibited by adenine and adenosine, although other purine derivatives were not effective for this inhibition. The K_m values of the mutant OPT were 580 and 760 μM for orotate and 5′-phosphoribosyl-1′-pyrophosphate (PRib-PP), respectively, whereas the corresponding values for the wild-type OPT were 40 and 60 μM. The intracellular level of PRib-PP was decreased to less than 15% of the normal level when purine derivatives were added to exponentially growing cultures of both the parent and mutant strains. However, this decrease of the PRib-PP level was not found in strains derived from the mutant, in which the purine-sensitive phenotype was suppressed by a secondary mutation. The purine-sensitive phenotype was caused by retardation of the pyrimidine de novo pathway, when the intracellular level of PRib-PP was diminished by exogenously supplied purine derivatives.

A purine-sensitive mutant (designated PS100) was isolated from *Escherichia coli* K-12 derivative strain C600 (15). The mutant PS100 had the following characteristics. (i) Cell growth in Davis-Mingioli minimal medium (7) was slower when compared with growth of parent strain C600. (ii) This slow cell growth was stimulated by the addition of uridine. (iii) Cell growth was completely inhibited by the addition of purine bases, nucleosides, and nucleotides. (iv) This inhibitory effect of purine derivatives was reversed by the simultaneous addition of pyrimidine derivatives. (v) Orotic acid accumulated in the culture fluid. Genetic analysis revealed that all of these characteristics were governed by a single mutation located in the structural gene (*pyrE*) for orotate phosphoribosyltransferase (OPT), an enzyme of the pyrimidine pathway de novo. The specific activity of OPT in the mutant PS100 decreased to 7% of that in the parent C600 (15).

Growth inhibition of *E. coli* by adenine has been described (8–10, 12). Recently, Levine and Taylor (12) reported that adenine exerted its toxic effects by depleting guanine nucleotide pools. On the other hand, Hosono and Kuno (10) reported that adenine inhibited the growth of *E. coli* W3110 and that this inhibition could be ascribed to inhibition of the de novo synthesis of pyrimidine nucleotides. Bagnara and Finch (2, 3) indicated that the addition of adenine decreased the intracellular concentration of 5′-phosphoribosyl-1′-pyrophosphate (PRib-PP) and pyrimidine nucleotides, and they postulated that this decrease in the PRib-PP concentration resulted in a decrease in the rate of pyrimidine de novo synthesis at the OPT step. Growth characteristics of the mutant PS100 were similar to those reported by the above workers, although growth inhibition was drastic and caused by all purine derivatives other than adenine. The mutation in the structural gene for OPT (*pyrE*) was postulated to play a crucial role in the phenotype of this purine sensitivity. In this paper, we examine the properties of OPT from the mutant PS100 in comparison with the wild-type enzyme from C600 and suggest a possible mechanism for purine sensitivity.

* Corresponding author.

MATERIALS AND METHODS

Strains and media. The strains used in this study are all derivatives of *E. coli* K-12 and are listed in Table 1. Davis-Mingioli minimal medium (7) [0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.01%$(NH_4)_2SO_4$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.2% glucose (citrate free)] supplemented with 10 μg of leucine per ml, 50 μg of threonine per ml, and 1 μg of thiamine per ml was used. Mutation of the *E. coli* strain was carried out by the method of Adelberg et al. (1), with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

Preparation of OPT. The preparation of the crude extract was performed at 0 to 4°C by a previously published procedure (15). The crude extract was 35% saturated with solid ammonium sulfate. After stirring for 20 min, the mixture was centrifuged for 10 min at 10,000 × *g*. Solid ammonium sulfate was added to the supernatant to obtain 60% saturation. After stirring for 20 min, the mixture was centrifuged for 10 min at 10,000 × *g*. The precipitate was dissolved in a small volume of 5 mM Tris-hydrochloride buffer (pH 7.6). This extract was dialyzed against the same buffer overnight and stored at −20°C.

Enzyme assay. OPT was assayed by the method of Brown and O'Sullivan (4), with some modifications. To estimate the effect of PRib-PP on the velocity, we carried out the reaction at 37°C for 10 min in a 0.5-ml mixture containing 50 mM Tris-hydrochloride (pH 8.0), 0.2 mM (2.5 μCi/μmol) [*carboxyl*-^{14}C]orotic acid, 1 mM $MgCl_2$, 0.05 to 2.0 mM PRib-PP, 0.1 U of yeast orotidine-5′-monophosphate decarboxylase, and crude extract (ca. 0.5 mg of protein). For crude extract from the mutant PS100, 1.2 mM (1.0 μCi/μmol) orotate was used. To estimate the effect of orotate concentration on the velocity, we used 0.05 to 1.2 mM (0.5 μCi/μmol) orotic acid. Also, concentrations of PRib-PP were fixed at 0.4 mM for the crude extract from the parent C600 and at 1.6 mM for that from the mutant PS100. The reaction was initiated by adding the crude extract and terminated with 0.8 ml of 4% perchloric acid. The radioactive CO_2 evolved was absorbed to β-phenylethylamine in an apparatus devised by Iwai and Taguchi (11). Velocity was indicated as radioactivity (counts per minute) of CO_2 evolved per minute per milligram of protein.

TABLE 1. *E. coli* strains used

Strain	Genotype (or phenotype)	Source or prepn
C600	F⁻ *leu thr thi lacY supE tonA* r_K^- m_K^-	K-12 derivative
PS100	Same as C600 except for *pyrE*	15
PS101	Derived from PS100; resistant to all purine derivatives	See text
PS102	Derived from PS100; resistant to guanine and guanosine	See text

Protein was determined by the method of Lowry et al. (13).

Determination of the intracellular content of PRib-PP. PRib-PP was extracted from exponentially growing *E. coli* cells by the method of Burton (5), with some modifications. Cell culture (300 μl) was mixed with 7.5 μl of 2 M Tris-hydrochloride (pH 7.8)–80 mM EDTA. The mixture was boiled for 30 s and immediately cooled at 0°C in ice water. This extract was used for determination of PRib-PP. The reaction mixture (40 μl) contained 130 mM Tris-hydrochloride (pH 7.6), 0.3 mM (125 nCi) [*carboxyl*-^{14}C]orotic acid, 10 mM $MgCl_2$, 4.3 mM IMP, and 0.05 U of the mixed enzymes of OPT and orotidine-5′-phosphate decarboxylase. To this reaction mixture (40 μl), 100 μl of PRib-PP solution was added, and the mixture was incubated for 1 h at 37°C. The reaction was terminated with 0.4 ml of 4% perchloric acid. The radioactive CO_2 evolved was absorbed to β-phenylethylamine in an apparatus devised by Iwai and Taguchi (11) and was counted in 7 ml of scintillation solution containing 12 g of 2,5-diphenyloxazole (PPO) and 0.3 g of 1,4-bis-(5-phenyloxazolyl)benzene (POPOP) per liter of toluene. Intracellular PRib-PP content was expressed as the amount of PRib-PP (nanomoles) per milligram (dry weight) of cells.

Chemicals. Purine and pyrimidine compounds were purchased from Kojin Co., Ltd., Tokyo, Japan. [*Carboxyl*-^{14}C]orotic acid was purchased from New England Nuclear Corp., Boston, Mass. Mixed enzymes of OPT and orotidine-5′-phosphate decarboxylase were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Inhibition of OPT activity by adenine and adenosine. In *E. coli*, exogenously added purine bases and purine nucleosides are used for synthesis of purine nucleotides by the purine salvage pathway. The addition of purine bases or purine nucleosides to the culture increases intracellular levels of purine nucleotides (3, 10). To determine whether direct inhibition of OPT activity of the mutant PS100 by some purine nucleotides might block the pyrimidine pathway de novo, we obtained the enzyme OPT from both C600 and PS100 by ammonium sulfate precipitation, as described above. The activity was assayed in the presence of various purine bases, nucleosides, and nucleotides, which were added to the assay mixture at a concentration of 50 μM. The OPT from both C600 and PS100 was partially inhibited to the same degree by adenine (50% inhibition) and adenosine (30% inhibition) (Fig. 1). However, other purine compounds tested, such as AMP, ADP, ATP, hypoxanthine, inosine, IMP, guanine, guanosine, and GMP, were not effective for this inhibition (data not shown).

K_m values of OPT for orotate and PRib-PP. K_m values of OPT were determined for the two substrates orotate and PRib-PP. The K_m values of OPT from C600 were 40 μM for orotate and 60 μM for PRib-PP, whereas the K_m values were

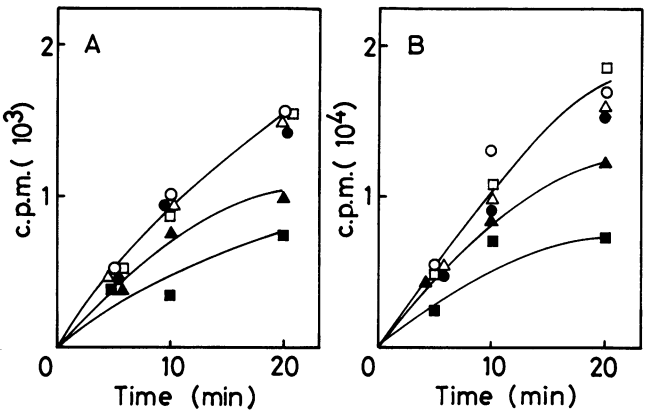

FIG. 1. Inhibition of OPT activity by adenine and adenosine. OPT activity was assayed for strains PS100(A) and C600(B), with no addition (○) and with the addition of adenine (■), adenosine (▲), AMP (□), ADP (△), and ATP (●). All adenine derivatives were added at a concentration of 50 μM. The radioactivity of CO_2 evolved in the reaction was measured.

580 μM for orotate and 760 μM for PRib-PP for OPT from the mutant PS100 (Fig. 2). Judging from the increased K_m value for PRib-PP (13-fold), OPT activity in PS100 was strongly influenced by the intracellular level of PRib-PP. The intracellular PRib-PP concentration and its change caused by exogenous purine compounds were examined.

Change in intracellular PRib-PP level by the addition of various purine compounds. Bagnara and Finch (2, 3) reported that exogenously added purine bases or purine nucleosides decreased the intracellular level of PRib-PP in *E. coli*. This decrease of PRib-PP was believed to be caused by the function of purine phosphoribosyltransferase in the purine salvage pathway. We checked intracellular PRib-PP content

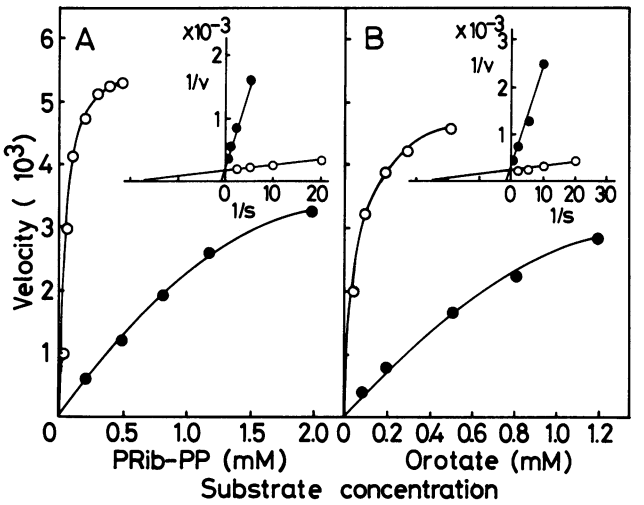

FIG. 2. Effect of substrate concentration on the velocity of OPT reaction. Crude preparations of the OPT from both the parent strain C600 (○), and the mutant PS100 (●) were assayed as a function of (A) PRib-PP or (B) orotate concentration. Velocity was indicated as radioactivity (counts per minute) of $^{14}CO_2$ evolved per minute per milligram of protein. The conditions used for enzyme assay are described in the text. (A) The fixed concentrations of orotate were 0.2 mM for C600 and 1.2 mM for PS100. (B) The fixed concentrations of PRib-PP were 0.4 mM for C600 and 1.6 mM for PS100. Values are graphed in the form of Lineweaver-Burk plots (inset).

in both the mutant PS100 and the parent strain C600 after various purine derivatives were added to the culture. Cells were grown in Davis-Mingioli minimal medium (7) to the early logarithmic phase, and adenine (100 μM) was added to the medium. Intracellular PRib-PP content was determined at appropriate times as described above. PRib-PP content decreased to less than 10% of the level of the normal state (ca. 1 μmol/g [dry weight]) immediately after the addition of purine derivatives, and this decreased level remained for at least 150 min (data not shown). No significant difference was observed in the pattern of PRib-PP decrease between strains C600 and PS100. Changes in PRib-PP content in strain PS100 were examined at 10 and 90 min after the addition of other purine compounds to a concentration of 20 μM (Table 2). All purine compounds tested, such as adenosine, hypoxanthine, inosine, guanine, and guanosine, decreased the PRib-PP content in PS100. The PRib-PP content was less than 15% of the normal level 90 min after the addition of purine compounds (Table 2).

All purine compounds decreased the intracellular level of PRib-PP, at a concentration of 20 μM, which is close to the lower limit required for inhibition of cell growth. Purine-resistant derivatives were isolated from the mutant PS100, and the relationship between purine sensitivity and the change in PRib-PP level was examined.

Purine-resistant strains isolated from the purine-sensitive mutant PS100. Purine-resistant colonies were selected from the mutant PS100 after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Selection was done on minimal agar plates containing one of three purine nucleosides (adenosine, inosine, or guanosine) at a concentration of 100 μM. Large colonies which appeared on these plates were purified and checked as to their sensitivity to various purine compounds such as adenine, adenosine, hypoxanthine, inosine, guanine, and guanosine. The existence of the original mutation in the *pyrE* gene was also checked by assaying OPT activity in crude extract.

Most of the isolates were resistant to all purine compounds tested, and the K_m values for OPT were similar to those obtained for the wild-type enzyme. These isolates presumably arose from reversion in the mutated *pyrE* gene. However, two other types of isolate were obtained. A representative of the first type of isolate (designated PS101) was selected for resistance to adenosine. Although PS101 was resistant to all other purine compounds tested, the mutation in *pyrE* remained (data not shown). That is, OPT from PS101 showed the increased K_m values in a similar manner to that from PS100. The second type of isolate (representated by PS102) was selected for resistance to guanosine. Strain PS102 was resistant to both guanine and guanosine but was still sensitive to adenine, adenosine, hypoxanthine, and inosine (data not shown). Since OPT from PS102 showed K_m values similar to those of PS100, the mutation in the *pyrE* gene remained.

TABLE 2. Change of intracellular PRib-PP content after the addition of various purine derivatives[a]

Purine derivative added	PRib-PP content[b] after:	
	10 min	90 min
None	100	100
Adenine	7	1
Adenosine	7	1
Hypoxanthine	32	4
Inosine	23	10
Guanine	5	14
Guanosine	25	15

[a] Cells of PS100 were cultured to the early logarithmic phase, and purine derivatives (20 μM) were added at time zero.

[b] Relative values as compared with the value with no addition are shown. The absolute value of PRib-PP content with no addition was about 1 μmol/g (dry weight).

The intracellular PRib-PP content was determined for both PS101 and PS102 after one of three purine bases (adenine, hypoxanthine, or guanine) was added to the culture at a concentration of 20 μM (Table 3). The original purine-sensitive mutant, PS100, showed a decreased level of PRib-PP (14% of the normal level) 90 min after the addition of all purine bases. For mutant PS101, the PRib-PP levels were 22, 56, and 93% of the normal levels for adenine, hypoxanthine, and guanine, respectively. In the case of PS102, adenine and hypoxanthine caused a decrease in PRib-PP similar to that observed for PS100. However, the addition of guanine did not lead to a decrease in PRib-PP as was observed for PS100.

DISCUSSION

To elucidate the mechanism of purine-sensitivity caused by the single mutation in the structural gene for OPT (*pyrE*), we examined some properties of OPT from the purine-sensitive mutant PS100 and compared these properties with those of the parent C600. OPT catalyzes the reaction (orotate + PRib-PP → orotidine 5'-monophosphate + PP_i) in the pyrimidine de novo pathway, so repression or inhibition of OPT results in a requirement for pyrimidine compounds (not orotate) for growth. Since growth inhibition in PS100 by purine derivatives could be reversed by pyrimidine derivatives, it was assumed that the addition of purine derivatives caused a block in the pyrimidine de novo pathway because of some changes in OPT reactivity.

First, OPT of the mutant PS100 was checked for inhibition by purine derivatives. If that was the case, the higher intracellular level of purine nucleotides might have blocked the pyrimidine de novo pathway in PS100, when purine compounds were added exogenously. However, our results show that the OPT from strains C600 and PS100 was partially inhibited by only adenine and adenosine (Fig. 1). The possibility that this inhibition of OPT stopped the growth of PS100 was ruled out for the following reasons. (i) Exogenously added adenine or adenosine was readily converted to adenine nucleotides, so the increase in the level of intracellular adenine or adenosine, if it occurred, was very small. (ii) Other purine compounds tested, which could inhibit cell growth of PS100, did not inhibit OPT activity. (iii) The minimum concentration of adenine or adenosine required for growth inhibition of PS100 was very low as compared with that required for OPT inhibition. Cell growth was inhibited by 0.6 μM adenosine, whereas this concentration did not inhibit OPT significantly in the in vitro enzyme assay (data not shown).

On the other hand, the enzyme OPT from the mutant PS100 showed increased K_m values (15-fold for orotate and 13-fold for PRib-PP) as compared with the wild-type enzyme (Fig. 2). It was revealed that the apparent decrease in OPT specific activity in the mutant (to 7% of that in the parent strain) assayed under normal conditions (15) was caused by the increased K_m values of the mutant enzyme OPT. The K_m values obtained may not have been certain values, since crude extract was used for the enzyme assay. Recently this enzyme was purified from an *E. coli* strain carrying a multicopy plasmid containing the *pyrE* gene, by Poulsen et al. (14), but the K_m values were not reported. However, the

K_m values for the parent C600 (40 μM for orotate and 60 μM for PRib-PP) were comparable to those reported for OPT purified from *Saccharomyces cerevisiae* (33 μM for orotate and 62 μM for PRib-PP) (16).

PRib-PP is an important intermediate involved in many biosynthetic pathways such as biosynthesis of purine nucleotides, pyrimidine nucleotides, NAD^+, $NADP^+$, histidine, and tryptophan. PRib-PP is also consumed by purine and pyrimidine salvage pathways when purine or pyrimidine derivatives are added exogenously. OPT is one reaction step requiring PRib-PP as a substrate in the pyrimidine de novo pathway, so the 13-fold increase in K_m value for PRib-PP might stress the OPT reaction. Bagnara and Finch (2, 3) reported that the PRib-PP pool immediately fell to low levels when purine base or purine nucleosides were added to exponentially growing cultures of *E. coli*. Our experiments verified that the decrease in PRib-PP level was caused by exogenous purine bases or nucleosides, all of which could inhibit the growth of PS100. However, no differences were found in the pattern of PRib-PP decrease between the mutant PS100 and the parent C600 (Table 2). Furthermore, Bagnara and Finch (3) reported that the addition of adenine to *E. coli* cultures caused a temporary drop in the pools of UTP and CTP. They inferred that this decreased level of pyrimidine nucleotides resulted from retardation of pyrimidine de novo synthesis by the very low PRib-PP level (3, 6). Judging from these data, the decreased level of PRib-PP caused by exogenous purine compounds probably drastically retarded the pyrimidine de novo synthesis for the mutant PS100, since the K_m value of OPT for PRib-PP was 13-fold greater than the normal value. We have previously reported that the growth of mutant PS100 was slower than that of parent strain C600 and that this slow growth was stimulated by the addition of uridine to the culture (15). This suggests that the normal level of intracellular PRib-PP in the exponentially growing cells was not enough to support the pyrimidine de novo synthesis in PS100. Christopherson and Finch (6) stated that the rapid decrease in the concentration of PRib-PP (from 500 to 20 μM) would lead to an inhibition of the OPT reaction even for wild-type *E. coli*, since the K_m of OPT for PRib-PP was 97 μM.

The notion that the low level of PRib-PP retarded the pyrimidine de novo pathway and resulted in growth inhibition by purine derivatives in the mutant PS100 was also supported by acquisition of the purine-resistant derivative having a secondary mutation. PS101 (resistant to all purine bases and nucleosides) and PS102 (resistant to only guanine and guanosine) were obtained from PS100. Both strains showed a small decrease in PRib-PP when purine compounds, unable to inhibit growth, were added to the culture (Table 3). This secondary mutation may lead to one of the following conditions: (i) a deficiency in the transport system of purine bases or nucleosides. (ii) a deficiency in the enzyme purine phosphoribosyltransferase, (iii) activation or derepression of the PRib-PP synthesizing enzyme, phosphoribosylpyrophosphate synthetase. These possibilities are currently being examined in our laboratory.

The phenotype of purine sensitivity of mutant PS100 is closely related to the function of the purine salvage pathway and the intracellular level of PRib-PP. As was observed for PS101 and PS102, there is a possibility that a mutation in the genes of the purine salvage pathway or PRib-PP metabolizing pathway could be obtained easily from PS100 by a positive selection for purine resistance. The *pyrE* mutation of the mutant PS100 is therefore expected to become a useful tool for the study of cellular metabolism of the important intermediate, PRib-PP.

TABLE 3. Change of PRib-PP content after the addition of various purine bases[a]

Purine base added	PRib-PP content[b] for strain:		
	PS100	PS101	PS102
None	100	100	100
Adenine	1	22	1
Hypoxanthine	4	56	1
Guanine	14	93	120

[a] Purine bases (20 μM) were added in early logarithmic phase.
[b] PRib-PP content was determined 90 min after the addition of the purine base. Relative values as compared with the value with no addition are shown. The absolute value of PRib-PP content with no addition was about 1 μmol/g (dry weight) for all three strains.

LITERATURE CITED

1. **Adelberg, E. A., M. Mandel, and G. C. C. Chen.** 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. **Bagnara, A. S., and L. R. Finch.** 1973. Relationships between intracellular contents of nucleotides and 5-phosphoribosyl 1-pyrophosphate in *Escherichia coli*. Eur. J. Biochem. **36**:422–427.
3. **Bagnara, A. S., and L. R. Finch.** 1974. The effects of bases and nucleosides on the intracellular contents of nucleotides and 5-phosphoribosyl 1-pyrophosphate in *Escherichia coli*. Eur. J. Biochem. **41**:421–430.
4. **Brown, G. K., and W. J. O'Sullivan.** 1977. Subunit structure of the orotate phosphoribosyltransferase-orotidylate decarboxylase complex from human erythrocytes. Biochemistry. **16**:3235–3242.
5. **Burton, K.** 1983. Transport of nucleic acid bases into *Escherichia coli*. J. Gen. Microbiol. **129**:3505–3513.
6. **Christopherson, R. I., and L. R. Finch.** 1978. Response of the pyrimidine pathway of *Escherichia coli* K12 to exogenous adenine and uracil. Eur. J. Biochem. **90**:347–358.
7. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B12. J. Bacteriol. **60**:17–28.
8. **Hill, D. L., and R. F. Pittillo.** 1973. Use of *Escherichia coli* mutants to evaluate purines, purine nucleosides, and analogues. Antimicrob. Agents Chemother. **4**:125–132.
9. **Hoshino, J., S. Izumi, and T. Sato.** 1965. Growth inhibition and its reversion by nucleic acid derivatives in *Escherichia coli*. J. Gen. Appl. Microbiol. **11**:161–173.
10. **Hosono, R., and S. Kuno.** 1974. Mechanism of inhibition of bacterial growth by adenine. J. Biochem. **75**:215–220.
11. **Iwai, K., and H. Taguchi.** 1973. Distribution of quinolinate phosphoribosyltransferase in animals, plants and microorganisms. J. Nutr. Sci. Vitaminol. **19**:491–499.
12. **Levine, R. A., and M. W. Taylor.** 1982. Mechanism of adenine toxicity in *Escherichia coli*. J. Bacteriol. **149**:923–930.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **Poulsen, P., K. F. Jensen, P. V. Hansen, P. Carlsson, and L. G. Lundberg.** 1983. Nucleotide sequence of the *Escherichia coli pyrE* gene and of the DNA in front of the protein-coding region. Eur. J. Biochem. **135**:223–229.
15. **Shimosaka, M., Y. Fukuda, K. Murata, and A. Kimura.** 1984. Growth inhibition by purine derivatives and its reversal by pyrimidine derivatives in a mutant of *Escherichia coli* K12. Agric. Biol. Chem. **48**:1303–1310.
16. **Umezu, K., T. Amaya, A. Yoshimoto, and K. Tomita.** 1971. Purification and properties of orotidine-5'-phosphate pyrophosphorylase and orotidine-5'-phosphate decarboxylase from bakers' yeast. J. Biochem. **70**:249–262.