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Genetic Evidence for a Repressor of Synthesis of Cytosine Deaminase and Purine Biosynthesis Enzymes in *Escherichia coli*

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Addition of purines to the growth medium of *Escherichia coli* represses synthesis of cytosine deaminase (*codA*) and enzymes of purine de novo synthesis. After Tn*10* mutagenesis, mutants displaying derepressed levels of cytosine deaminase in the presence of hypoxanthine were isolated. One of these had simultaneously acquired resistance to the hypoxanthine analog 6-mercaptopurine. The mutation *purR6*::Tn*10* was shown to affect de novo synthesis of the purine enzymes glutamine phosphoribosylpyrophosphate amidotransferase (*purF*) and phosphoribosyl glycinamide synthetase (*purD*). The mutation was mapped by P1 transduction at 36 min on the *E. coli* linkage map. A plasmid containing the *purR* region was obtained by complementation of the *purR6*::Tn*10* mutation. By comparing the restriction maps of the cloned fragment and the *E. coli* chromosome, the *purR* gene was found to be located very close to the *lpp* gene (36.3 min).

Addition of purine bases to the growth medium of *Escherichia coli* and *Salmonella typhimurium* stops purine de novo synthesis. The structural genes encoding the 13 enzymes of the purine de novo synthesis pathway are organized in nine genetically unlinked transcriptional units. Expression of these genes is negatively regulated by purines (13). A number of mutants defective in this repression have previously been isolated in both *E. coli* (7) and *S. typhimurium* (2, 16). Several of these mutants were found to be resistant to the toxic hypoxanthine analog 6-mercaptopurine (2, 7); none was characterized genetically.

Cytosine deaminase encoded by *codA* is a pyrimidine salvage enzyme. It catalyzes the deamination of cytosine to uracil and enables the cell to utilize cytosine for pyrimidine nucleotide synthesis. The regulation of cytosine deaminase synthesis is very complex. Synthesis is derepressed under pyrimidine and nitrogen limitation and is repressed by exogenous purines (13). In this report, we describe the properties of a *purR*::Tn*10* mutant which was obtained as a mutant defective in purine repression of cytosine deaminase synthesis. The mutation also affects the regulation of *purF* and *purD* expression and confers resistance to 6-mercaptopurine.

Recently, *S. typhimurium* mutants displaying constitutive high expression of purine biosynthesis genes have been isolated and genetically characterized (T. Guo-Min, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, H-163, p. 172).

MATERIALS AND METHODS

Bacterial strains. The *E. coli* K-12 strains used are listed in Table 1.

Media and growth conditions. The growth medium was AB medium (4) containing glucose (0.2%) and thiamine (1 μg/ml); solid medium also contained 1.5% agar. Unless otherwise stated, supplements were added at the following final concentrations: hypoxanthine, 30 μg/ml; 2-amino-4-hydroxypyrimidine (isocytosine), 10 μg/ml; 5-fluorocytosine, 20 μg/ml; 6-mercaptopurine, 150 μg/ml; Casamino Acids, 0.2%; ampicillin, 50 μg/ml; tetracycline, 10 μg/ml; and pyridoxal hydrochloride, 1 μg/ml. Luria broth (11) was used as complex medium.

Enzyme assays. Cells were grown exponentially for several generations and harvested at a density of about 5×10^8 cells per ml. After being washed in 0.9% NaCl, cells were frozen at −20°C. Crude cellular extracts for enzyme assays were prepared by sonic disruption of cells suspended in 30 mM phosphate buffer (pH 7.1) containing 1 mM EDTA and 1 mM dithiothreitol. The activities of glutamine phosphoribosylpyrophosphate (PRPP) amidotransferase and phosphoribosyl glycinamide (GAR) synthetase were determined as described by Saxild and Nygaard (15). Cytosine deaminase activity was determined by measuring the amount of uracil formed per minute in a reaction mixture containing 5.0 mM [^{14}C]cytosine (0.45 Ci/mol), 50 mM Tris hydrochloride (pH 7.8), and cellular extract (0.5 mg of protein per ml). At intervals during incubation at 37°C, 5-μl portions were withdrawn and applied to cellulose thin-layer chromatograms in premarked spots which contained 4 μg of cytosine and 4 μg of uracil as markers. The chromatograms were developed in 1-butanol–water (86:14). The separated uracil and cytosine spots were marked under UV light, cut out, and counted in a Packard liquid scintillation analyzer. Enzyme

TABLE 1. Bacterial strains used

Strain	Genotype or phenotype	Source or reference
AT3196	*pdxH15*(Am) *relA1 spoT1 thi-1*	Strain CGSC 4553
KL226	*HfrC rel-1 tonA22* T2^r	8
MC1061	*araD139* Δ(*ara leu*)*7697* Δ(*lac cod*)*74 galU hsdR rpsL*	12
S343	*aroD argEH manA gal lac mlt*	14
SØ106	*thi lacZ rpsL pyrF30*	Laboratory collection
SØ5044	*thi lacZ rpsL*	SØ106 by P1(wild type)
SØ5046	*thi lacZ rpsL pyrF30 purR6*::Tn*10*	This work
SØ5052	*thi lacZ rpsL purR6*::Tn*10*	SØ5044 by P1(SØ5046)
SØ5066	*galU hsdR rpsL purR6*::Tn*10*	From MC1061
SØ5068	*aroD argEH manA gal lac mlt purR6*::Tn*10*	S343 by P1(SØ5046)
SØ5069	*pdxH15*(Am) *relA1 spoT1 thi-1 purR6*::Tn*10*	AT3196 by P1(SØ5046)

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TABLE 2. Effect of *purR6*::Tn*10* on enzyme activities

Relevant genotype[a]	Supplement	FC sensitivity[b]	Enzyme activity (U/mg of protein)			Hypoxanthine utilization[c]
			Cytosine deaminase	PRPP amidotransferase	GAR synthetase	
purR$^+$	None	S	105	54	36	3.0
purR$^+$	Hx[d]	R	44	6	5	
purR6::Tn*10*	None	S	98	86	100	3.3
purR6::Tn*10*	Hx	S	110	55	54	

[a] The strains were SØ5044 (*purR*$^+$) and SØ5052 (*purR6*::Tn*10*).
[b] Sensitivity to 5-fluorocytosine (FC) at 20 μg/ml (S, sensitive; R, resistant).
[c] Expressed as nanomoles of hypoxanthine incorporated into acid-insoluble material per minute per milligram of protein.
[d] Hx, Hypoxanthine (50 μg/ml).

activities are given in units; 1 U is the amount of enzyme that produces 1 nmol of product per min at 37°C. Hypoxanthine utilization was determined in exponentially growing cells at a density of 5×10^8 cells per ml. Cells were washed and suspended in fresh AB glucose medium containing 6 μM [^{14}C]hypoxanthine (1 Ci/mol). Portions were removed after 1 and 2 min, precipitated in 5% trichloroacetic acid, and filtered on glass filters. The filters were washed, dried, and counted. Protein determinations were performed as described by Lowry et al. (9).

Genetic techniques. Tn*10* mutagenesis was performed as described by Kleckner et al. (5) with lambda NK370. Infection of SØ106 with 2×10^{11} PFU of the bacteriophage at a multiplicity of infection of about 1 resulted in 12,000 tetracycline-resistant colonies. Selection for *purR*::Tn*10* mutants was done by replication to glucose minimal plates containing Casamino Acids, isocytosine, hypoxanthine, and tetracycline. P1-mediated generalized transduction was performed as described by Miller (11). Supplements for *aroD* mutants were tryptophane (20 μg/ml), phenylalanine (20 μg/ml), tyrosine (20 μg/ml), *p*-aminobenzoic acid (1 μg/ml), *p*-hydroxybenzoic acid (0.05 μg/ml), and 3,4-dihydroxybenzoic acid (0.5 μg/ml).

DNA techniques. The methods for plasmid isolation, restriction endonuclease digestion and ligation of DNA fragments, and transformation of plasmid DNA have been described by Maniatis et al. (10).

RESULTS AND DISCUSSION

Selection and characterization of *purR6*::Tn*10*. Pyrimidine-requiring mutants of *E. coli* can utilize cytosine as the sole pyrimidine source provided that they contain a functional cytosine deaminase (13). The cytosine analog isocytosine, which is a poor substrate of cytosine deaminase, is deaminated to uracil and may serve as a pyrimidine source in cells containing derepressed levels of cytosine deaminase. Thus, a pyrimidine-requiring mutant grows well on isocytosine in glucose minimal medium, whereas it cannot grow on isocytosine if the medium is supplemented with hypoxanthine because of the repressing effect of this compound on cytosine deaminase synthesis.

The pyrimidine-requiring strain SØ106 was mutagenized with Tn*10* (see Materials and Methods), and tetracycline-resistant mutants capable of growth on isocytosine in the presence of hypoxanthine were selected. Among the transposition events, 14 resulted in mutants with the desired phenotype. On further characterization, one of these (SØ5046) was found to be resistant to 6-mercaptopurine. A P1 lysate prepared on SØ5046 was used to transduce SØ106 to tetracycline resistance. Of 200 transductants, all grew on isocytosine plus hypoxanthine and were 6-mercaptopurine resistant, indicating that these phenotypes were caused by the Tn*10* insertion.

The *purR6*::Tn*10* mutation resulted in derepressed levels of cytosine deaminase under purine-repressing conditions (Table 2). The resistance to 6-mercaptopurine suggested that the mutation also affects regulation of purine nucleotide synthesis. To confirm this, the levels of two enzymes of the purine de novo synthetic pathway, PRPP amidotransferase and GAR synthetase, were determined. The genes encoding these two enzymes, *purF* and *purD*, belong to two different transcriptional units. The *purR6*::Tn*10* mutation rendered synthesis of both enzymes virtually insensitive to purine repression (Table 2).

The lack of purine repression observed in the *purR6*::Tn*10* mutant may be due to inactivation of a regulatory protein (i.e., a repressor) or may be the result of inactivation of an enzyme involved in hypoxanthine utilization. Hypoxanthine utilization, measured as hypoxanthine incorporated into acid-insoluble material, was determined for both wild-type and mutant cells. Hypoxanthine utilization was the same in both strains (Table 2), suggesting that the *purR* gene encodes a repressor protein.

TABLE 3. Genetic mapping of *purR6*::Tn*10* by P1-mediated transduction

Donor strain (genotype)	Recipient strain (genotype)	Selected marker[a]	Unselected markers			No. of transductants
SØ5068 (*manA aroD purR6*::Tn*10*)	AT3196 (*pdxH*)	*purR6*::Tn*10*	*manA*	*pdxH*	*aroD*	
			+	−	+	48
			+	−	−	8
			+	+	+	70
			−	+	+	69
S343 (*manA aroD*)	SØ5069 (*pdxH purR6*::Tn*10*)	*pdx*$^+$	*manA*	*purR*	*aroD*	
			+	−	+	34
			+	+	+	14
			−	−	+	25
			−	−	−	1
			−	+	+	26

[a] *purR6*::Tn*10* transductants were selected on glucose minimal plates containing Casamino Acids, tetracycline, pyridoxal hydrochloride, and *aroD* requirements (see Materials and Methods); *pdx*$^+$ transductants were selected on glucose minimal medium containing *aroD* requirements. The *pdx*$^+$ transductants were purified on the same medium before further tests were performed. + and −, Wild-type and mutant alleles, respectively.

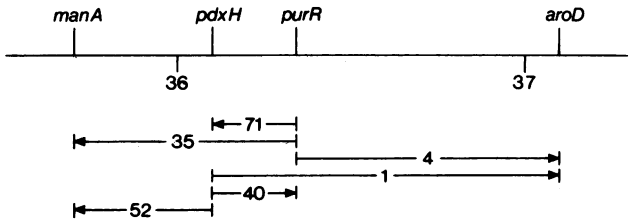

FIG. 1. Genetic map of the region around 36 min on the *E. coli* linkage map (1). Cotransduction frequencies are based on data in Table 3. Arrows point toward the unselected markers.

Genetic mapping of *purR6*::Tn*10*. Preliminary conjugation experiments between an HfrH *purR6*::Tn*10* donor strain and various recipient strains indicated a map position around 35 min on the *E. coli* linkage map (data not shown). Transductional mapping of the *purR6*::Tn*10* mutation relative to *manA*, *pdxH*, and *aroD* showed the *purR* locus to be located at 36.3 min on the linkage map (Table 3 and Fig. 1).

S. typhimurium mutants defective in transcriptional regulation of the purine biosynthesis pathway have recently been isolated. The mutations affect a single locus, *purK*, which was mapped near 30 min on the *S. typhimurium* chromosome (Guo-Min, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988). The genetic data suggest that the *purK* locus in *S. typhimurium* is homologous to the *purR* locus in *E. coli*.

Molecular cloning of the wild-type *purR* gene. 5-Fluorocytosine is toxic to pyrimidine-prototrophic strains of *E. coli* because of its conversion to 5-fluorouracil catalyzed by cytosine deaminase (13). Exogenous hypoxanthine reverses this toxicity because it represses the synthesis of cytosine deaminase. Pyrimidine-prototrophic strains carrying the *purR6*::Tn*10* mutation contain constitutive levels of cytosine deaminase and are sensitive to 5-fluorocytosine in the presence of hypoxanthine. Thus, provided that the *purR* gene encodes a *trans*-acting, dominant element required for purine repression of cytosine deaminase synthesis, a plasmid carrying the *purR* gene will confer resistance to 5-fluorocytosine in the presence of hypoxanthine when introduced into a *purR6*::Tn*10* strain.

A mixture of *E. coli* chromosomal DNA and pBR322 (3) was digested with *Eco*RI, ligated, and transformed into SØ5066. Transformants were selected on glucose minimal plates containing 5-fluorocytosine, hypoxanthine, and ampicillin. Plasmid DNA from two of the transformants yielded 5-fluorocytosine-resistant colonies upon retransformation of SØ5066. Both plasmids (pMK150 and pMK151) contained a 9.5-kilobase *Eco*RI fragment inserted in the *Eco*RI site of pBR322.

pMK150 complemented the *purR6*::Tn*10* mutation by restoring purine repression of synthesis of cytosine deaminase, PRPP amidotransferase, and GAR synthetase (Table 4). Thus, *purR* encodes a *trans*-acting regulatory element, probably a repressor, involved in the control of both cytosine utilization and purine nucleotide synthesis in *E. coli*.

TABLE 4. Effect of pMK150 on enzyme levels in a *purR6*::Tn*10* mutant[a]

Plasmid	Enzyme activity (U/mg of protein)		
	Cytosine deaminase	PRPP amidotransferase	GAR synthetase
None	100	45	55
pMK150	54	3	0.2

[a] Strain SØ5052 was used in these experiments. Cells were cultured in AB glucose minimal medium containing hypoxanthine (50 μg/ml).

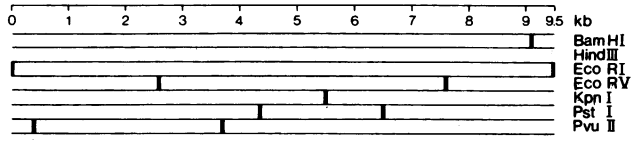

FIG. 2. Restriction map of the 9.5-kilobase (kb) DNA fragment in pMK180 which harbors the *purR* gene.

A restriction map of the 9.5-kilobase *Eco*RI insert in pMK150 is shown in Fig. 2. It confirms the chromosomal location of the *purR* gene as determined genetically, since the same restriction pattern is found around kilobase 1760 on the *E. coli* restriction map between the *uidR* gene (35.9 min) and the *lpp* gene (36.3 min) (6).

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