

A Method for Selection of Mutations at the *tdk* Locus in *Escherichia coli*

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Mutants of *Escherichia coli* which are resistant to 5-fluorodeoxyuridine all have mutations which map at a single locus at 27.5 min on the genetic map of *E. coli*. Extracts prepared from each mutant were deficient in thymidine kinase activity measured in vitro. Simple selective conditions which allowed detection of one mutant in the presence of 10^7 wild-type bacteria were found. These results show that loss of thymidine kinase activity is the usual mechanism for 5-fluorodeoxyuridine resistance and that all such mutations occur at the locus previously designated *tdk*.

During the course of studies on expression of foreign genes encoding viral thymidine kinases (TKs), we required TK-deficient *Escherichia coli* strains with a number of different genetic backgrounds. The available, well-characterized allele, *tdk-1*, was obtained from the *E. coli* Genetic Stock Center in the originally described strain *E. coli* KY895 (5). For several reasons, this strain was unsuitable for our purposes. In order to move this allele into our required strains by P1 cotransduction with the adjacent *trp* marker, we first had to introduce the *trp* marker into those strains. Instead, we decided to construct a strain with a dominant selectable marker near the *tdk* mutation so that it could be more easily introduced into any desired strain by cotransduction for the dominant marker. In the course of these studies, we developed and evaluated a simple and reproducible selection method for *tdk* mutations.

Previous studies with both *E. coli* and *Salmonella typhimurium* suggested that it might be possible to select *tdk* mutations in any desired genetic background. While the original *tdk* mutation described by Hiraga et al. (5) was selected by resistance to [³H]thymidine suicide, Beck, Ingraham, Neuhaard, and Thomassen found that with *S. typhimurium*, *tdk* mutants could be isolated on the basis of their resistance to the pyrimidine analog fluorodeoxyuridine (FUdR) (2). They reasoned that when FUdR is phosphorylated by the TK to 5-fluorodeoxyuridine monophosphate, it will inhibit thymidylate synthetase, the final enzyme in the de novo synthetic pathway of thymidylate synthesis, and in the absence of a source of preformed thymidine, the cell will be deprived of TMP (Fig. 1). In their experiments, Beck et al. observed mutations to FUdR resistance only in cells with preexisting mutations in the gene for thymidine phosphorylase (*deoA*) (3). They suggested that without this block, the FUdR is broken down by the phosphorylase too rapidly to maintain a toxic intracellular concentration.

In *E. coli*, Drinkwater and Kleindinst (4) constructed an FUdR-resistant strain which was deficient in TK and uridine phosphorylase (*upp*). Presumably, the *upp* mutation also blocked the breakdown of the FUdR and helped to maintain the intracellular concentrations of the inhibitor.

Following these suggestions, we tested several concentrations of FUdR and found that we could reproducibly obtain

single-step resistance to 50 μ M FUdR in the presence of adenine, guanosine, cytidine, and uridine. All resistant mutants were deficient in TK activity, and all had mutations which mapped near 27.5 min on the *E. coli* genetic map (1). The FUdR resistance could be transduced with a high degree of efficiency, which suggested that it was determined by a single mutation, presumably in the *tdk* locus. The requirement for a second mutation in the *upp* or *deoA* gene appeared unnecessary under these conditions. One possible explanation for this situation is that the high concentration of uridine in the selective medium serves to inhibit the activity of thymidine pyrophosphorylase and thus obviate the need for the second mutation. We added sources of preformed bases to the medium in an attempt to avoid potential nucleotide pool imbalances which might occur as a result of the inhibition of thymidylate synthetase and disruption in the use of one-carbon fragments and pyrimidine precursors. We have not, however, tested whether any or all of these additions are essential.

First we isolated independent FUdR-resistant colonies as follows. *E. coli* C600 was streaked on Luria broth agar plates, and single colonies were inoculated into individual tubes containing Luria broth. After overnight growth, 50- μ l aliquots of each culture were spread on FUdR plates. These plates contained VB minimal medium (11); 1% glucose; 0.5% decolorized Casamino Acids; and 12.5 μ g of FUdR, 40 μ g of cytidine, 100 μ g of adenine, 100 μ g of uridine, and 40 μ g of guanosine per ml. Medium 56 (7) with these supplements was also used successfully. After overnight growth at 37°C, a few colonies were observed growing well against a light haze of confluent growth. One resistant colony was picked from each plate and streaked on FUdR plates. A single colony was isolated from each such plate and grown as representing an independent mutational event leading to FUdR resistance.

From 14 such resistant isolates, we prepared extracts for TK assay as follows. A 5-ml volume of an overnight culture was centrifuged, and the cell pellet was frozen, thawed, and then resuspended in 100 μ l of 50 mM EDTA–50 mM Tris HCl (pH 8)–10% glycerol–2 mg of lysozyme per ml. After 30 min at room temperature, the samples were frozen and thawed twice and 400 μ l of TK extract buffer (50 mM Tris HCl [pH 7.5], 200 μ M thymidine, 10% glycerol) was added. The samples were mixed, and 10 μ l of 10% Triton X-100 was added. After centrifugation at 10,000 \times g for 2 min to remove

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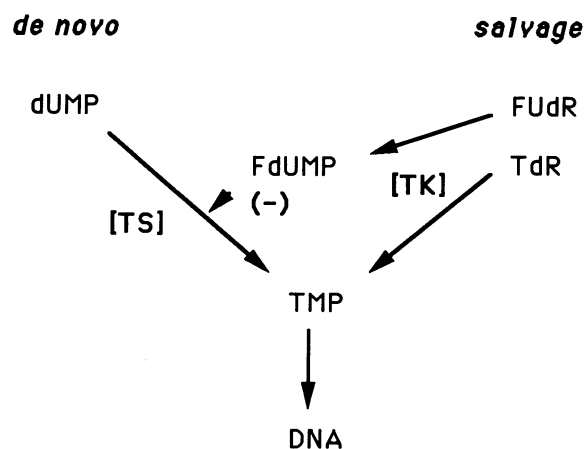


FIG. 1. Outline of pyrimidine metabolism in *E. coli*. In the *de novo* pathway, thymidylate synthetase (TS) converts deoxyuridine monophosphate (dUMP) to TMP, while in the salvage pathway, TK catalyzes the phosphorylation of thymidine (TdR) to form TMP. FdUMP is also a substrate for TK and is converted into 5-fluoro-deoxyuridine monophosphate (FdUMP), a noncompetitive inhibitor of TS. The presence of excess uridine and cytosine prevents FdUMP and its cleavage product, fluorouracil, from affecting ribonucleotide metabolism.

cellular debris, the supernatants were assayed for TK activity as previously described (8).

Two extracts made from two FdUMP-sensitive isolates of *E. coli* C600 were used as TK-positive controls, and TK extract buffer alone was assayed to provide a background value. The two TK-positive extracts produced 2.5 and 3.5 pmol of TMP per h. The 14 extracts from FdUMP-resistant strains produced between 0 and 0.26 pmol of TMP per h, with a mean of 0.12 pmol of TMP per h. Thus, all 14 strains appeared to be deficient in TK activity.

We next asked if all such FdUMP resistance mutations mapped at the expected locus for the *tdk* gene. We reasoned that if a wild-type *tdk* near map position 27.5 min were introduced by cotransduction with a dominant selectable marker nearby, the recipients should become FdUMP sensitive with a high frequency. We prepared a P1virS phage lysate on *E. coli* 12168 (MG1655 *zch-506::Tn10*) (9). This strain is prototrophic and FdUMP sensitive and carries a Tn10 transposon at 27.25 min which renders the strain resistant to tetracycline. We infected each of the 14 FdUMP-resistant strains of *E. coli* C600 with this P1 stock at a multiplicity of infection of about 0.2 and selected for tetracycline-resistant colonies. These transductants were restreaked on Luria broth agar with tetracycline (25 μ g/ml) and then replica plated on minimal medium plates with nucleosides, glucose, and Casamino Acids with or without FdUMP. For each transduction, between 80 and 100% (mean, 94%) of the tetracycline-resistant transductants had also acquired the FdUMP-sensitive, wild-type marker. This strong linkage between the unselected FdUMP sensitivity and the selected tetracycline resistance suggests that at least one requirement for FdUMP resistance maps near 27.25 min.

In order to determine if the locus near 27.25 min was sufficient to carry all the genetic information needed for FdUMP resistance, we prepared a P1virS-transducing lysate from a strain of *E. coli* 12169 selected for resistance to FdUMP. The recipient in this transduction was *E. coli* CT2004 (FdUMP sensitive, tetracycline sensitive, *trp*). We then tested

TABLE 1. Strains used in this study

<i>E. coli</i> strain	Genotype	Source
C600	K-12 F ⁻ <i>thi-1 thr-1 leuB6 lacY1 tonA21 supE44</i>	P. M. Glazer
KY895	K-12 F ⁻ <i>tdk-1 ilv</i>	W. D. Rupp
CT2004	K-12 Hfr(PO1) <i>lacZ125 trpA49 NaI^r</i>	K. B. Low
12169	K-12 F ⁻ <i>zch-506::Tn10</i>	K. B. Low
BL21 (DE3)	B F ⁻ <i>hsdS gal λ(DE3)</i>	F. W. Studier

for the cotransduction of FdUMP resistance with the tetracycline resistance. The cotransduction was 94% (72 of 77 isolates), in agreement with the individual transductions of the FdUMP sensitivity and tetracycline resistance described above. Cotransduction between tetracycline resistance and the nearby *trp* locus at 28 min was 65% (90 of 138 isolates) in two experiments. The strong linkage (94% cotransduction) between FdUMP resistance and the tetracycline resistance marker at 27.25 min suggests that a single locus in this strain is responsible for the FdUMP resistance. The slightly lower frequency of linkage of the Tn10 to *trp* (65% cotransduction) is consistent with the published map order: Tn10-*tdk-trp* (6).

The experiments in which we selected FdUMP-resistant mutants of *E. coli* 12168, a derivative of *E. coli* AB1157, suggested that this selection is not peculiar to *E. coli* C600 or strains with its particular genetic makeup but that it is useful for other *E. coli* K-12 strains. In other experiments with an *E. coli* B strain [BL21 (DE3)] (10), we found that all of eight FdUMP-resistant mutants lacked TK activity in cell extracts. For strains which can be grown on simple glucose-Casamino Acid media, selection for FdUMP resistance appears to be a generally applicable procedure to obtain single-step TK-deficient mutants. The strains used in this study are listed in Table 1.

The strong linkage of the FdUMP resistance (*tdk*) marker with the transposon *zch-506::Tn10* makes this drug resistance marker useful for strain constructions with various alleles of the *tdk* gene. Further, direct isolation of mutants deficient in TK activity by selection with 50 μ M FdUMP provides an easy and reproducible alternate route to introducing *tdk* mutants into specific genetic backgrounds in *E. coli*.

The efficiency of FdUMP as a selective agent in recombination and mutagenesis studies was tested by asking if rare FdUMP-resistant mutants could be detected in the presence of FdUMP-sensitive, wild-type bacteria. We made artificial mixtures of sensitive and resistant bacteria and plated different numbers of bacteria on FdUMP-containing medium (medium 56 with nucleosides, glucose, Casamino Acids, and 50 μ M FdUMP). Resistant colonies were detectable with 100% recovery and no background in the presence of 1.5×10^7 sensitive bacteria per plate spread on 100-mm-diameter plates. If 10-fold more sensitive bacteria were plated, however, there was background growth of the sensitive bacteria sufficient to obscure the resistant colonies.

The experiments reported here show that selection for FdUMP resistance reproducibly gives only mutants with mutations at the *tdk* locus at 27.5 min on the *E. coli* genetic map and that such selection allows recovery or isolation of rare mutants from vast excess numbers of wild-type bacteria.

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