JOURNAL OF BACTERIOLOGY, Mar. 1969, p. 1431-1436
Vol. 97, No. 3
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Isolation and Mapping of Polynucleotide Phosphorylase Mutants of *Escherichia coli*

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Received for publication 27 November 1968

Three strains of *Escherichia coli* with altered polynucleotide phosphorylase, Q7, Q13, and Q27, were isolated by screening clones from heavily mutagenized cultures for low levels of the enzyme. The three mutations were found to cotransduce with *argG* and *asp*, and the *pnp* locus which they define was mapped with respect to these loci. An explanation for the nonreciprocal cotransduction frequencies observed with *asp* is provided by the demonstration of an unlinked *asp*-suppressing locus.

Polynucleotide phosphorylase (PNPase) catalyzes the phosphorolysis of ribonucleic acid (RNA) to nucleoside diphosphates, the synthesis of polyribonucleotides from nucleoside diphosphates, and an exchange reaction between the β-phosphate of the diphosphate and inorganic phosphate (4, 7). The in vitro synthesis of polynucleotides by use of PNPase has been of great importance in studies on the genetic code (11). The physiological function of the enzyme is unknown. Random ordering of nucleotides in the polymerization reaction and the relatively weak affinity of PNPase for diphosphates make it unlikely that polymerization is significant in vivo. Because of the energy conservation of its phosphorolysis reaction and the implication of diphosphates as the breakdown products of messenger RNA (mRNA), PNPase is a good candidate for involvement in mRNA breakdown (15). To pursue this problem further, a search for PNPase mutants of *Escherichia coli* was undertaken. Three strains with altered PNPase were isolated by screening 950 heavily mutagenized clones for their PNPase level.

In achieving a mutation frequency of 3 in 950 for PNPase, many other mutations were induced in each strain. To characterize the PNPase mutations and look for possible physiological effects, it was desirable to separate the PNPase lesions from the others present in the original isolates. Transduction by phage P1 provides a means to transfer each of the PNPase lesions into a genetically characterized strain and thus to construct a family of strains which differ only in a small region surrounding the PNPase locus. Since PNPase is a nonselectable trait, it was first necessary to know its location on the chromosome so that it might be transferred by cotransduction with a neighboring selectable marker. This paper describes the isolation of the three PNPase mutants and the mapping of the *pnp* locus. In the accompanying paper (13) the mutations are characterized in nearly isogenic strains.

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MATERIALS AND METHODS

Parental strain. The parental strain A19 is a ribonuclease I$^-$, Met$^-$, HfrH, λ lysogen with an unidentified growth requirement. It was isolated by Gesteland (3) by nitrosoguanadine mutagenesis of AB301 (Met$^-$, λ lysogen, HfrH). The unidentified defect is a trace requirement, since its removal by transduction results in colonies which support a substantial surrounding halo of growth of A19 (14).

Bacterial growth. Bacteria were grown in yeast-tryptone (YT) broth containing 5 g of NaCl, 8 g of tryptone (Difco), and 5 g of yeast extract per liter. Mineral medium for selection plates contained (per liter): 5 g of NaCl, 1 g of NH_4Cl, 0.25 g of $MgSO_4$, 0.15 g of K_2HPO_4, 2 g of glucose, 75 ml of 1 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.6 at 37 C, and a trace of thiamine. Amino acids other than arginine and aspartate were added by spreading 0.1 ml of 1% L-amino acid per plate. L-Aspartate (250 mg/liter) and L-arginine (15 mg/liter) were added to plates before pouring. Materials used as carbon sources were added to a final concentration of 20 mM.

Mutagenesis. Bacterial cultures were grown over-

night in YT at 37 C, and 10-ml amounts were centrifuged. The cells were washed once and resuspended in 0.5 ml of 0.2 M sodium acetate buffer (*p*H 5.0). A 0.1-ml amount of nitrosoguanadine (4 mg/ml) was added, and the mixture was incubated at 37 C without shaking for 3 hr. Bacteria were then diluted 1:50 into fresh YT, separated into 50 tubes, shaken overnight at 25 C to allow segregation, and plated.

Screening for PNPase mutants. Following mutagenesis and segregation, individual colonies were spotted onto duplicate YT plates and were grown overnight at 30 C. From one plate, each spot was suspended in 150 μliters of 0.02 M Tris (*p*H 8.0) plus 10^{-4} M ethylenediaminetetraacetate (EDTA), with one drop of toluene per tube. The tubes were incubated for 60 min at 43 C, and the contents were then assayed for PNPase by inorganic ^{32}P-adenosine diphosphate (ADP) exchange as follows. A 300-μliter amount of solution was added to the cell suspension to achieve a final concentration of 100 mM Tris (*p*H 8.0), 2.5 mM ADP, and 2.5 mM sodium phosphate containing 10,000 to 100,000 counts/min of $^{32}P_i$ (carrier-free, Tracerlab, Waltham, Mass.), 5 mM $MgCl_2$, and 0.5 mM EDTA. The reaction mixture was incubated for 120 min at 43 C and the reaction was stopped by adding 0.7 ml of cold 7% perchloric acid containing 20 mg of Norit. After 10 min at 0 C, the suspension was filtered on a glass-fiber filter. The filter was washed with 18 ml of water containing dissolved inorganic phosphate and was then glued Norit-side down to a planchet and counted. With this procedure, 100 clones/day could be screened conveniently.

PNPase assays in cell extracts. Cultures were harvested in late log phase (optical density of 2.0 to 2.5 in YT at 37 C). Cells were washed in 0.02 M Tris (*p*H 7.8), pelleted, frozen and ground with 2.5 g of alumina per g of frozen cells. Alumina and and debris were removed by centrifugation, and the supernatant fluid was dialyzed two times against 500 volumes of 0.02 M Tris (*p*H 7.8). Extracts contained 15 to 20 mg of protein/ml (biuret), and 5-μliter amounts of extract were used in 100-μliter reaction mixtures.

The exchange reaction mixture was at the concentration of the screening assay. The phosphorolysis reaction was the same except that ADP was replaced by 0.5 mg of polyadenylic acid (Miles Laboratories, Elkhart, Ind.) per ml, and the sodium phosphate concentration was 10 mM. The polymerization reaction mixture was 75 mM Tris (*p*H 8.0), 5 mM $MgCl_2$, and 5 mM ADP containing 20,000 counts/min of ^{14}C-ADP (50 mc/mmole; Schwartz BioResearch, Inc., Orangeburg, N.Y.). The exchange and phosphorolysis reactions were stopped after 10 min at 37 C by adding 0.5 ml of cold 5% perchloric acid containing 20 mg of Norit; the Norit was filtered, washed, and counted as described above. The polymerization reaction was stopped after 10 min by adding 0.5 ml of cold 5% perchloric acid. The mixture stood for 10 min at 0 C and was then filtered on DA filters (Millipore Corp., Bedford, Mass.), washed three times with 2.5 ml of 1% cold perchloric acid, dried, and counted on planchets.

Scoring the pnp alleles. PNPase can be conveniently assayed in toluene-treated cells by either the exchange or the polymerization reaction; polymerization was routinely used in scoring. To distinguish either *pnp-7* or *pnp-13* (the defects originally isolated in Q7 and Q13, respectively) from the wild-type allele, colonies were spotted on YT plates, grown overnight at 37 C, suspended in 100 μliters of 0.02 M Tris (*p*H 8.0), and made porous by incubating at 37 C for 10 min with 10 μliters of toluene. Samples (50 μliters) were assayed in 100-μliter reaction mixtures, at the conditions of the polymerization assay in cell extracts. In a typical scoring, 50 *pnp-7* or *pnp-13* clones incorporated 10 to 30 counts/min, and 50 wild-type clones incorporated 110 to 170 counts/min. To distinguish *pnp-27* from the wild-type allele, colonies were grown overnight at 43 C in YT liquid containing 0.2% glucose. A 300-μliter amount was centrifuged and the cells were washed once, suspended in 50 μliters of 0.02 M Tris (*p*H 8.0), and assayed as above.

The rationale for these assays will be apparent when the characterization of the altered PNPases is presented (13). Briefly, the *pnp-7* enzyme has undetectable polymerization activity and the *pnp-13* enzyme is virtually inactive because Mn^{++} is not present. The *pnp-27* enzyme is temperature-sensitive and in nongrowing cells is inactivated at 43 C if conditions are acidic. Overnight cultures grown on YT plus glucose reach *p*H 6 or less and inactivate the *pnp-27* enzyme but not the wild type.

Bacterial crosses. Log-phase cells grown at 37 C in YT plus 0.2% glucose were mixed to final concentrations of 5×10^7 males per ml plus 5×10^8 females per ml, and were incubated at 37 C with large surface exposure but without shaking. For interrupted mating, this mixture was diluted 1:100 after 4 min into fresh YT plus 0.2% glucose and incubated as above. At appropriate times, 1-ml samples were removed and vortexed for 60 sec at top speed on a Super-Mixer no. 1290 (Lab-line Instruments, Melrose Park, Ill.); 0.1-ml portions were then plated.

P1 Transduction. For preparation of P1 stocks, 2×10^5 P1 grown on AB301, the parent strain of A19, were added to 2×10^8 bacteria in YT plus 10 mM Ca^{++}, incubated for 15 min at 37 C, and plated onto YT plates from 2.5 ml of YT-soft agar. Plates and soft agar contained 10 mM Ca^{++}. After 6 hr at 37 C, the plates were scraped, the soft agar was removed by centrifugation, and the supernatant liquid was chloroformed. Yields were 4×10^9 to 8×10^{10} phage per plate, titered on *Shigella*. For transduction 2×10^8 recipient bacteria were mixed with 10^8 P1 in 0.2 ml of YT plus 10 mM Ca^{++}, incubated at 37 C for 10 min, and spread onto selection plates.

RESULTS

Isolation of PNPase mutants. Since no physiological function for PNPase was known, no selection procedure for mutants was possible. A rapid, specific assay for PNPase on a small quantity of cells was required to enable the screening of a large number of mutagenized

strains. In the screening procedure devised, individual clones were suspended in a small volume of buffer, made porous by toluene, and assayed for PNPase level (*see* Materials and Methods).

Three strains exhibiting low $^{32}P_i$-ADP exchange activity in toluenized cells were detected. Q7 and Q13 were isolated in Walter Gilbert's laboratory on the same day by the author and Diane Vargo. Several months later, Dr. Gilbert resumed the mutant search, using a screening pool enriched with temperature-sensitive strains obtained by pretesting a larger mutagenized stock for clones which grew on YT at 25 C but not at 43 C. Q27 was one of these temperature-sensitive strains. To confirm that the three strains were mutant in PNPase, Q7, Q13, and Q27 were assayed for PNPase under standard conditions by polymerization, phosphorolysis, and exchange assays in cell extracts. Q7 and Q13 showed <10% wild-type activity, and Q27 could be heat-inactivated to this level in 10 min at 48 C (the wild-type enzyme was stable at 58 C). Experiments in which mutant and wild-type extracts were mixed showed that inhibitors were not the cause of the reduced activities. The lesions are designated *pnp-7*, *pnp-13*, and *pnp-27*, respectively.

The PNPase mutants have been used in other laboratories for studies on RNA breakdown (1, 8–10) and to provide conditions of reduced nuclease activity (5, 16, 17). In addition, Thang, Thang, and Grunberg-Manago (19) and Hsieh and Buchanan (6) have reported on the PNPase in Q13. My characterization of the *pnp-7*, *pnp-13*, and *pnp-27* mutations is presented in the accompanying paper (13).

Phenotypic properties of the mutant strains. Additional mutations were apparent in these strains. Q7 reverted to ribonuclease I^+ and lost the A19 trace requirement. Q13 retains the A19 defects and in addition requires tyrosine. Kivity-Vogel and Elson (8) reported that its β-galactosidase is unstable. Natori and Mizuno (9) reported growth of Q13 on a glucose-mineral medium supplemented by methionine, tyrosine, leucine, and tryptophan, but in my medium no combination of amino acids without a trace addition of yeast extract supported growth. Q27 grew in liquid YT at 43 C but not on YT plates at 43 C. By transduction, the temperature sensitivity of the strain was shown to be unrelated to the *pnp-27* defect (13). Q27 has lost the Hfr properties of A19; it does not donate markers in conjugation and is not sensitive to R17 male-specific phage.

An artifact involving the exchange reaction of Q13 was observed. Although dialyzed Q13 extracts catalyze ADP-stimulated $^{32}P_i$ incorporation into Norit-adsorbing material at <10% of the wild-type level, undialyzed Q13 extracts catalyze incorporation at 100% of the wild-type level. More than 95% of the Q13 activity which disappears upon dialysis reappears with the addition of boiled cell extract to the reaction mixture. In contrast, extracts from the parental strain A19 lose no activity upon exhaustive dialysis, and neither Q7 nor A19 gain any activity from boiled cell extract. The active component was purified from boiled extract by adsorption to and desorption from Norit and by fractionation on a Biogel P2 and a diethylaminoethyl (DEAE) cellulose column. Following electrophoresis and measurement of its absorption spectrum, the component was tentatively identified as coenzyme A. Chromatographically pure coenzyme A (PL Biochemicals, Milwaukee, Wis.) was run on a DEAE column, and the major peak, which contained 80% of the ultraviolet-absorbing material and eluted at the position of the component purified from cell extract, was found to be highly active. Although boiled cell extract stimulated only Q13 extracts, purified coenzyme A stimulated Q7 and A19 extracts as well. The radioactive product of the reaction stimulated by either coenzyme A or boiled extract was identified as adenosine triphosphate [by Dowex-1 column chromatography and isopropanol-1% ammonium sulfate (2:1) paper chromatography]. The observed reactions are thus similar to those of the succinyl coenzyme A synthase system (12), and a mutation involving one of these enzymes, unrelated to PNPase, may have occurred in Q13.

Mapping of the pnp locus near argG and asp. Preliminary crosses of Q7 with a multiply marked F^- strain showed a high linkage of *pnp-7* with *argG* (Fig. 1). [When Q7 or Q13 was used as Hfr donor in conjugation, it was difficult to transfer *pnp* or earlier loci by selecting on markers other than *argG*. This difficulty is not due to zygotic induction of λ, and is a property of the parental line of the PNPase mutants (14).] Transducing AB1302 (*argG*, etc.; see Table 1 for description of strains) to Arg^+ by P1(Q7) (P1 phage grown on bacterial strain Q7) and scoring for *pnp-7* confirmed this linkage, showing a cotransduction frequency of 75 to 80% for *argG* and *pnp-7*. Since *asp* was known from mating experiments to map near *argG* (R. Lavellé, *personal communication*), PA3403 (*asp*, etc.) was transduced to Asp^+ by P1(Q7). A cotransduction frequency of 30 to 35% was obtained for *asp* and *pnp-7*. Transducing with P1(Q13) and P1(Q27), these same approximate linkages of *pnp* to *argG* and *asp* were obtained. The three mutations were assumed to map at a single structural gene for PNPase, which was designated *pnp*.

Ordering of *asp*, *argG*, and *pnp*. Two positions of *pnp* are consistent with the transduction data: between *asp* and *argG* or on the side of *argG*, opposite from *asp*. To order uniquely the three genes, an *asp argG* strain was constructed by crossing AT12-55 (Hfr AB312, *argG*, etc.) with PA3403L (*asp ilv*, etc.), selecting for Ilv^+, and scoring the recombinants for Asp and Arg. Of the 440 recombinants, 6 were Asp^- Arg^-, and one such strain, AA1, was selected for further use. AA1 was transduced with P1(Q13), either selecting for Asp^+ and scoring for *pnp* and *argG* or selecting for Arg^+ and scoring for *pnp*. The results from 200 transductants (Table 2 and Fig. 2) uniquely determine the order *asp argG pnp*. (AA1 is more likely to receive $argG^+$ alone than *pnp-13* alone in Asp^+ selection, showing that *argG* is the nearer locus to *asp*.)

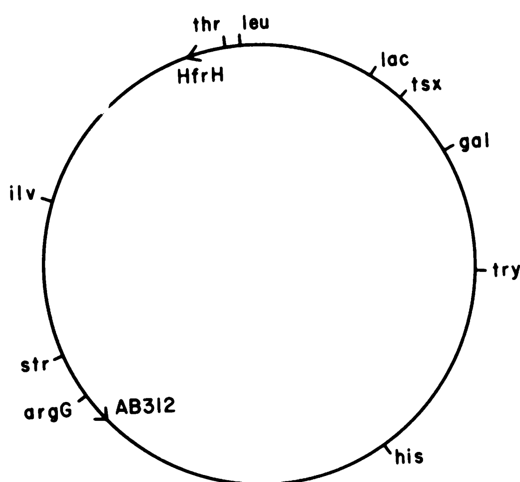

FIG. 1. *Genetic map of the E. coli chromosome [from Taylor and Trotter (18)]; thr and argG are transferred early by HfrH and AB312, respectively.*

Scoring for *asp* was unreliable, because the Asp^- colonies grew more poorly than the Asp^+ at all concentrations of aspartate tried, and often were lost. This did not affect the above cotransduction frequencies, however, since linkage of *pnp* and *argG* determined separately for 50 Asp^+ and 50 Asp^- transductants was 76 and 78%, respectively. High concentrations of aspartate partially satisfy the arginine requirement in some *argG* strains. At 2 mM exogenous aspartate, the *asp* requirement is satisfied whereas the *argG* requirements remain strict. (The *argG* enzyme catalyzes the synthesis of argininosuccinate from aspartate and citrulline, and increased aspartate concentrations presumably enable the mutant enzyme to function partially.)

Ordering of asp, argG, pnp, and str. To verify the orientation of *asp argG pnp* on the chromosome, *asp* and *argG* were ordered with respect to *str* by crossing AT12-55 (*argG* Sm^R $T6^S$, etc.) × PA3403L (*asp ilv* Sm^S $T6^R$, etc.), selecting for Ilv^+ Asp^+ Arg^+ recombinants, counterselecting

TABLE 1. *Strains used*[a]

Strain	Sex	Markers	Source
A19	HfrH	RNAse I^- Met^-, unidentified trace requirement, (λ)	Gesteland
Q7	HfrH	*pnp-7* Met^- (λ)	Mutagenesis of A19
Q13	HfrH	*pnp-13* RNAse I^- Met^- Tyr^-, unidentified trace requirement, (λ)	Mutagenesis of A19
Q27	?	*pnp-27* RNAse I^-, temperature-sensitive growth (*see text*), (λ)	Mutagenesis of A19
AB301	HfrH	Met^- (λ)	Adelberg
AB1302	F^-	*thr leu argG thi lac gal mal xyl mtl* Sm^R	Gorini
PA3403	F^-	*his asp lac gal mal xyl mtl* Sm^R $T6^R$	Lavallé
AB684	Hfr AB312	*ilv* Arg^- Pur^- *xyl*	Adelberg
PA3403L	F^-	*ilv asp* Pur^- *lac gal xyl* $T6^R$	AB684 × PA3403
AT12-55	Hfr AB312	*thr leu argG thi* Sm^R	Lavallé
AA1	F^-	*argG asp* Pur^- *lac gal xyl* Sm^R $T6^R$	AT12-55 × PA3403L
AT2446	HfrH		Taylor

[a] Abbreviations: *arg, asp, his, ilv, leu, met,* Pur^-, *thi, thr,* and Tyr^- denote inability to grow without arginine, aspartate, histidine, isoleucine and valine, leucine, methionine, adenosine (purine), thiamine, threonine, and tyrosine, respectively; *lac, gal, mal, xyl,* and *mtl* denote inability to ferment lactose, galactose, maltose, xylose, and mannitol, respectively; Sm^R and $T6^R$ denote resistance to streptomycin and phage T6, respectively; all strains not marked Sm^R are streptomycin-sensitive; (λ) denotes strains lysogenic for λ; RNAse I^- indicates strains which lack ribonuclease I.

TABLE 2. *Three-point mapping of asp, argG, and pnp by P1(Q13) transduction of AA1 (Asp⁻ Arg⁻)*

Selected marker	Scored markers	Percentage of scored markers	
Asp^+	Arg^+ *pnp-13*	30	100
Asp^+	Arg^+ pnp^+	11	
Asp^+	Arg^- *pnp-13*	2	
Asp^+	Arg^- pnp^+	57	
Arg^+	*pnp-13*	77	100
Arg^+	pnp^+	23	

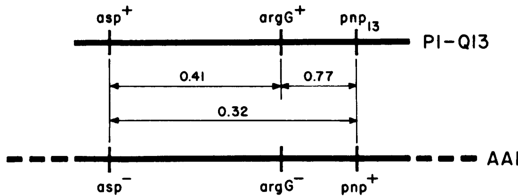

FIG. 2. *Cotransduction frequencies of asp, argG, and pnp.*

with T6, and scoring for Sm (Fig. 3). Of 240 recombinants scored, 71% were Sm^R, indicating that *asp* rather than *argG* was nearer to *str* and thus supporting the order *str asp argG pnp*. This is in agreement with the orientation of *asp argG* determined by R. Lavelle (*personal communication*).

Explanation for inconsistencies observed in transduction of asp. The high linkage (41%) observed between *asp* and *argG* in transduction of AA1 (*asp argG*, etc.) to Asp^+ Arg^+ is not demonstrated in all transductions. For example, P1 grown on PA3403, a strain with the identical *asp* lesion as AA1, does not donate *asp* (<0.2%) in the transduction of AB1302 (*argG*, etc.) or of AT12-55 (*argG*, etc.) to Arg^+. In transducing PA3403 (*asp*, etc.) to Asp^+, P1(AT12-55) donates *argG* with 30% frequency, whereas P1(AB1302) does not donate *argG* (<0.2%). (Conjugation experiments confirm that the Arg^- defects of AT12-55 and AB1302 map at *argG*.)

These observations can be explained if AB1302 and AT12-55 contain a gene unlinked to *argG* which suppresses the donated *asp* mutation. To see whether such a gene existed, I looked for an Hfr strain which could convert AA1 (*asp argG*, etc.) to Asp^+ by transferring a region of the chromosome unlinked to *argG*. HfrH strain AT2446 was found to do so. In an interrupted mating of AT2446 × AA1, the Asp^+ phenotype is transferred about 20 min after *lac*, unlinked to $argG^+$ transfer. In addition, P1(AT2446) cotransduces *argG* and *asp*.

Table 3 shows the aspartate genotypes of relevant strains if an *asp* and an *asp*-suppressing locus are assumed. For Asp^- strains, both alleles are negative. For Asp^+ strains, the *asp* genotype linked to *argG* is determined by whether the strain transfers its *argG* allele in transduction of AA1 or PA3403 to Asp^+. The *asp*-suppressing genotype is determined for AB1302 and AT12-55 by their inability to express *asp* from PA3403 in Arg^+-selected transduction, and for HfrH strains AT2446 and A19 by their respective ability and inability to transfer in conjugation with AA1 an Asp^+ function unlinked to *argG*. This work does not distinguish whether this gene is involved in aspartate metabolism or is a nonsense or missense suppressor. Since neither A19 (the parental strain of Q7, Q13, and Q27) nor AA1 suppresses *asp*, the cotransduction frequencies obtained for *asp*, *argG*, and *pnp* are unaffected.

DISCUSSION

To isolate PNPase mutants without knowing a physiological function for the enzyme, an assay was devised which could measure the PNPase level of 100 clones/day, by using patches of cells grown overnight. Since heavily mutagenized strains were used, the numbers of cells in each patch which grew up overnight fluctuated. To eliminate low activity levels due to reduced cell quantity the assays were run considerably longer than the time normally required for the ADP-$^{32}P_i$ exchange reaction to approach equilibrium. This might have masked certain mutants with reduced PNPase activity by allowing them a longer effective reaction time. Nevertheless, three mutants were isolated from 950 strains tested.

If PNPase were an essential enzyme, cells devoid of it could be isolated only as conditional lethals. To detect mutants with temperature-sensitive PNPase, suspended cells were preincubated at 43 C for 1 hr during toluene treatment

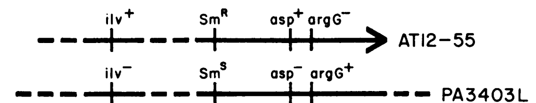

FIG. 3. *Three-point cross ordering str asp argG. (Sm is the phenotypic designation for the str gene.)*

TABLE 3. *Inferred aspartate genotypes*

Strain	*asp* locus linked to *argG*	*asp*-suppressing locus unlinked to *argG*	Aspartate phenotype
AB1302	−	+	+
AT12-55	+	+	+
PA3403	−	−	−
AA1	−	−	−
A19	+	−	+
AB2446	+	+	+

and then were assayed at 43 C. In addition, during part of the mutant search the screening pool was enriched with temperature-sensitive strains. In this way, Q27 was isolated.

Nitrosoguanidine has recently been reported to mutagenize preferentially the growing zone of the *E. coli* chromosome (2). In nitrosoguanidine mutagenesis of stationary-phase cells, Botstein (Cold Spring Harbor Symp. Quant. Biol., *in press*) found that mutations cluster exclusively in one-third of the chromosome near the region where replication starts. The observation that *pnp* maps in the middle of that region is suggested as an explanation for the fortuitously high frequency of PNPase mutations which I obtained.

The Q13 coenzyme A artifact indicates the difficulty of attempting to study a mutation in a heavily mutagenized strain containing other known and unknown lesions. Characterization of the PNPase mutations in nearly isogenic strains is presented in the accompanying paper (13).

ACKNOWLEDGMENTS

I am grateful for the guidance of Walter Gilbert throughout this work.

This investigation was supported in part by Public Health Service grant GM 09541 from the National Institute of General Medical Sciences. The author was supported by a National Science Foundation pre-doctoral fellowship.

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