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Genetic Separation of Hypoxanthine and Guanine-Xanthine Phosphoribosyltransferase Activities by Deletion Mutations in *Salmonella typhimurium*

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Certain *proAB* deletion mutants of *Salmonella typhimurium* were found to be simultaneously deleted in a gene required for the utilization of guanine and xanthine (designated *gxu*). These mutants were resistant to 8-azaguanine and when carrying an additional *pur* mutation were unable to use guanine or xanthine as a purine source. The defect was correlated with deficiencies in the uptake and phosphoribosyltransferase activities for guanine and xanthine. Hypoxanthine and adenine activities were unaltered. The deficiency was restored to normal by transduction to *pro*$^+$ and in F′ merodiploids.

Purine phosphoribosyltransferases are used to convert purine bases to their respective ribonucleotides. It has been generally accepted that hypoxanthine and guanine share a single common transferase (inosine monophosphate: pyrophosphate phosphoribosyl transferase, EC 2.4.2.8). The evidence for this comes from studies with highly purified preparations obtained from mammalian tissues (see reviews 24 and 26) and from yeast cells (23). Xanthine appears to be another, but much weaker, substrate for the mammalian enzyme (17, 20) but not for that of yeast (23). Mutational alterations of the enzyme with concomitant loss of activity for all substrates have been found in cultured mammalian cells (4, 6, 24, 26) and in human mutants (27).

In bacterial systems, the evidence for a single hypoxanthine-guanine phosphoribosyl transferase is less rigorous, more confusing, and often conflicting. Concomitant loss of both activities (5, 6, 14), as well as their separation (9, 14, 19), has been obtained by mutational events selected on the basis of resistance to inhibition by purine analogues. In our own earlier studies with such resistant mutants of *Salmonella* (14), several patterns were revealed which included either concomitant loss of both activities or differential loss of one or the other.

Interpretations as to the multiplicity of the enzymes, and the genes that control them, were complicated by the observation that genetic changes could create missense modifications of a single enzyme with alterations in range of substrate specificities (1, 15, 16). Unequivocal genetic separation of this class of enzymes would therefore require the complete absence of a gene product as might be obtained with nonsense or deletion mutations. In this paper we describe the discovery of such a mutation, in *Salmonella*, that removes phosphoribosyltransferase activity for guanine and xanthine without affecting hypoxanthine activity. The responsible gene, designated *gxu* (guanine-xanthine utilization), is located close enough to the *pro* (proline) region on the chromosome that it is simultaneously deleted in certain *pro* deletion mutants. In a concurrent independent study, Chou and Martin (8) have found another mutation in *Salmonella* that specifically affects hypoxanthine activity.

MATERIALS AND METHODS

Growth measurements. The minimal salts medium used was medium E described by Vogel and Bonner (29) with glucose (0.2%) as the carbon source. Supplements were added as indicated. Qualitative growth responses were scored on minimal agar plates containing the various supplements. Quantitative turbidity measurements were made in liquid media with a Klett-Summerson photocolorimeter using the green filter (no. 54). Where growth is recorded as

absorbance at 540 nm, turbidity was measured in a Zeiss spectrophotometer. Incubation was usually carried out at 37 C on a roller drum. Measurement of growth inhibition by 8-azaguanine required a small initial inoculum of about 10^3 bacteria per ml for reproducible and consistent results. This was obtained by inoculating 5 ml of media with 0.1 ml of a 10^{-4} dilution of an overnight broth culture.

Bacterial strains. The auxotrophic mutants (*pur* and *pro*) were derived from *Salmonella typhimurium*, strains LT-2 or LT-7, and were obtained from the Demerec collection through the courtesy of the late M. Demerec or K. Sanderson. Strain *proB25* F' *pro-lac* was obtained from A. Newton, and the episome was transferred to strain SL751 (*proA46 purC7 purI590 ilvA405 rha461 flaA56 strA*) by selecting for Pro$^+$ and streptomycin resistance. This then served as the standard F' donor for other *pro* recipients. The F_{13} episome carrying the *lac-purE* region was transferred directly from *Escherichia coli* strain W3747 (obtained from A. Garen) to strain *purE66 proAB47* by selecting for Pur$^+$.

Chemicals. The tetrasodium salt of 5-phosphoribosyl-1-pyrophosphate was purchased from Sigma Chemical Co. (St. Louis, Mo.), and 8-azaguanine (2-amino, 6-oxy, 8-azapurine) was from Calbiochem (Los Angeles, California). Hypoxanthine-8-^{14}C (3.07 mCi/mmole) and guanine-8-^{14}C (54 mCi/mmole) were obtained from New England Nuclear (Boston, Mass.); xanthine-2-^{14}C (48 mCi/mmole) was from Schwartz-Mann (Orangeburg, N.Y.), and adenine-8-^{14}C (17.9 mCi/mmole) was from Calbiochem. The scintillation fluid used in the Packard Tri-Carb scintillation counter consisted of the Packard fluor, dimethyl POPOP (1,4-bis-2-[5-phenyloxazolyl] benzene; 100 mg) and (2,5-diphenyloxazole; 4 g) dissolved in 1,000 ml of toluene (Baker analytical grade).

Preparation of extracts. Cultures were grown in 100 ml of E medium with appropriate supplements in 500-ml flasks with aeration by shaking in a rotary shaker water bath. Cells were harvested by centrifugation, washed three times (30 ml per wash) with 0.03 M sodium phosphate buffer (*p*H 7.4), and suspended in 1.0 ml of fresh buffer containing 2-mercaptoethanol (2 mM). The cell suspension was ruptured by sonic treatment consisting of four bursts of 20 sec each with intermittent cooling periods. The crude extract was clarified by centrifugation at 29,000 $\times$ *g* for 1 hr, and all dilutions of the extract were made in the disruption medium.

Enzyme assays. Guanosine monophosphate (GMP) reductase (reduced nicotinamide adenine dinucleotide phosphate [NADPH]:GMP oxidoreductase; EC 1.6.6.9) was assayed by the spectrophotometric method previously described (2) using continuous recording at 340 nm.

Purine phosphoribosyltransferase activities were assayed in a mixture volume of 0.5 ml containing 0.05 μmole (1 μCi/μmole) of ^{14}C-labeled purine, 0.5 μmole of tetrasodium 5-phosphoribosyl-1-pyrophosphate, 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (*p*H 8.0), 0.01 M magnesium sulfate, and 0.1 to 0.3 mg of protein of the cell-free extract. The reaction mixture was incubated at 37 C in a stationary water bath for 5 min, and the reaction was terminated in a boiling-water bath for 2 min. Protein was removed by centrifugation, and 25 μliters of the supernatant fluid was applied to a thin-layer cellulose chromatogram sheet (Eastman no. 6065 with fluorescent indicator). The appropriate unlabeled purine ribonucleotide was added at the point of each sample application (5 μg per marker), and the sheets were developed in 5% potassium phosphate-isoamyl alcohol (1:1) until the solvent front reached approximately 1.5 inches (3.81 cm) from the top of the sheet. Nucleotides were identified by ultraviolet absorption, cut from the sheet, immersed in 10 ml of scintillation fluid, and counted. At the same time, 25 μliters of unchromatographed mixture was also counted, and the ratio of radioactivity of the isolated nucleotide and unchromatographed sample was used to calculate specific activity expressed as nanomoles of nucleotide formed per minute per milligram of protein.

Protein concentrations were determined by the Lowry method (21) with bovine serum albumin as the standard.

Incorporation of radioactive purines. An overnight culture was diluted 1/20 in fresh minimal-glucose E medium containing nutritional supplements as required and incubated until logarithmic phase of growth was reached. At this point, the culture was diluted to an absorbancy of 0.1 at 540 nm with fresh medium and ^{14}C-purines were added (20–34 μg/ml). Two-milliliter samples were collected immediately and at various times by filtration through a 25-mm membrane filter (0.45 μm; Millipore Corp.). When the absorbancy reached 0.5, 1-ml samples were taken and filtered. Each filter was immediately washed with 40 ml of glucose-free E medium containing 100 μg of the appropriate unlabeled purine per ml. Filters were dried, immersed in 10 ml of scintillation fluid, and counted.

RESULTS

Description of the nutritional phenotype. Purine-requiring mutants of bacteria that are blocked before the formation of the first complete nucleotide, inosine monophosphate (IMP), normally can satisfy their growth requirement with any of the four purine bases: adenine, hypoxanthine, guanine, and xanthine. This non-discriminatory behavior is made possible through a variety of interconversion events (22). A genetic deficiency in the utilization of guanine and xanthine as a purine source (designated *gxu*) first was revealed by the anomalous growth behavior of certain *purE* mutants. In our collection of about 100 *purE* mutants, those carrying the *proAB47* deletion differed from the others in that they were unable to grow with guanine or xanthine as the proffered purine. Their growth response to adenine or hypoxanthine was unaltered. The

proline requirement could not, per se, be responsible for the altered phenotype since no change occurred when point mutations such as *proA46* and *proC51* were present in the *purE* mutants. Figure 1 compares the dose responses to the four purine bases in the *purE66 proAB47* mutant with that of a normal *purE* mutant (*purE11 pro*$^+$). Growth in adenine and hypoxanthine were comparable, but the mutant carrying the *proAB47* deletion did not grow with xanthine, and though some growth with guanine was detectable it was less than 10% that of the normal response. Guanosine and deoxyguanosine, which are usually able to replace guanine as a growth factor for *purE* mutants, were also unable to support the growth of those mutants carrying the *proAB* deletion.

Genetic relationship of gxu and pro. The association of the *gxu* property with the *proAB* deletion suggested that the deletion might extend sufficiently beyond the *pro* region to cut out one or more genes necessary for the utilization of guanine and xanthine. That this is indeed the case was proven in several ways. First, utilization of both guanine and xanthine was completely restored in all *pro*$^+$ recombinants obtained from strain *purE66 proAB47* by either transduction with wild-type phage or conjugation with Hfr strains. In one transduction experiment, 100% (90/90) of all *pro*$^+$ *purE* recombinants were now able to grow on guanine or xanthine. In a conjugation experiment using Hfr SC19 (*pro*$^+$ *purE*$^+$) which transfers the *pro* region early and *purE*$^+$ late, 300 *pro*$^+$ *purE* recombinants were examined, and all were able to use guanine or xanthine for growth.

The second type of evidence comes from restoration of the deleted region in merodiploid strains carrying the F *prolac* episome. When the episome carrying *pro*$^+$ was introduced into strain *purE66 proAB47*, the merodiploid obtained had a normal response to guanine and xanthine. Thus, the F *prolac* episome also carried *gxu*$^+$, and this is dominant to *gxu*. Table 1 shows the growth response of the various *pro*$^+$ derivatives of strain *purE66 proAB47*. It can be seen that the *pro*$^+$ transductant and the F'*pro*$^+$ *lac*$^+$ merodiploid grew well on all four purines. Also shown is a merodiploid in which prototrophy with respect to the purine requirement was restored by introducing only the *purE*$^+$ gene (*purE66 proAB47*/F *purE*$^+$ *lac*$^+$). In this strain, the purine requirement is abolished, and growth is not affected by guanine or xanthine, indicating that the *gxu* deficiency is not due to any abnormal inhibitory effects.

Azaguanine resistance and deletion mapping. The inability of the *gxu* mutant to utilize guanine suggested that it also might be unable to utilize the guanine analogue, 8-azaguanine, and hence show resistance to the inhibitory action of the analogue. This was indeed the case in that resistance to azaguanine was found to be an additional phenotypic expression of the *proAB47* deletion. This phenotypic marker was particularly useful in moni-

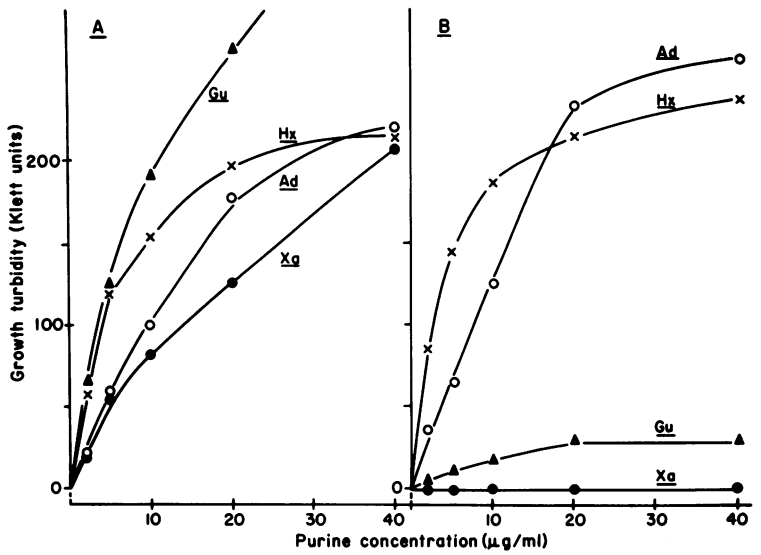

FIG. 1. *Dose response growth curves to adenine (Ad), hypoxanthine (Hx), guanine (Gu), and xanthine (Xa). A, purE11 (pro*$^+$*); B, purE66proAB47. Growth turbidity was measured after 24 hr of incubation.*

TABLE 1. *Growth response of genetically modified derivatives of strain purE66 proAB47*

Genetic alteration of *purE66 proAB47*	Growth response[a] to purines				
	Adenine	Hypoxanthine	Guanine	Xanthine	None
None	206	272	13	0	0
pro$^+$ (transductant)	220	285	271	206	0
F'*pro*$^+$*lac*$^+$	264	294	298	254	0
F'*lac*$^+$*purE*$^+$	280	288	296	284	280

[a] Growth is recorded as turbidity in Klett units after 18 hr in minimal glucose medium containing 0.1% casein hydrolysate and 20 μg of the purines per ml.

toring the deficiency in derivatives that did not require purines for growth and in screening a series of *pro* deletion mutants. In the original wild-type parent strain (strain LT-2), 50% inhibition of growth is obtained with azaguanine at about 2 μg/ml. Growth of strain *proAB47* (*purE*$^+$) is unaffected at concentrations of 100 μg/ml. Table 2 shows this and also shows that sensitivity to azaguanine inhibition was restored in both the *pro*$^+$ recombinant and in the F' merodiploid carrying the episomal *pro* region.

Table 2 also shows that other *pro* deletion mutants were either sensitive or resistant to azaguanine. With the assumption that azaguanine resistance is a second phenotypic expression of *gxu*, a tentative mapping of the *gxu* gene with respect to the *pro* deletions can then be made (Fig. 2). Thus, the deletions, *proAB47*, *proAB53*, and *proAB126*, extend sufficiently to the left to include *gxu*. On the other hand, *proAB21* and *proB25* presumably do not extend as far. The *proA107* deletion, which like *proAB47* includes the attachment site for phage P22 (*attP22*), is sensitive to azaguanine, thus indicating that *gxu* is not in the region between *proA* and *proC*.

This analysis was extended to include a series of *proAB* deletion mutants which was isolated by J. Kemper as *supQ* mutants (18). J. Kemper kindly supplied 48 independently isolated *proAB supQ* deletion mutants, and 20 of these were found to have the *gxu* phenotype on the basis of azaguanine resistance and defective uptake of guanine (see below).

GMP reductase. The first possibility that was considered to explain the mutant phenotype was a genetic loss in the direct interconversions at the nucleotide level. A defect in the conversion of GMP to adenosine monophosphate, through IMP, would not allow purine

TABLE 2. *Growth of various pro mutants and derivatives in presence of 8-azaguanine*

Strain	Percent inhibition of growth[a] in azaguanine	
	20 μg/ml	100 μg/ml
LT-2 (wild type)	100	100
proAB47[b]	0	0
proAB47[c]	0	0
proAB47/F 13	0	8
proAB47/F *pro*	100	100
proAB21	100	100
proB25	100	100
proA107	95	100
proC110	96	100
proAB53	0	9
proAB126	0	0

[a] Growth was measured in minimal glucose media containing 0.1% casein hydrolysate after 18 hr growth and expressed as percent inhibition with respect to control without azaguanine.

[b] Original *proAB47* strain.

[c] *purE*$^+$ transductant of *purE66 proAB47*.

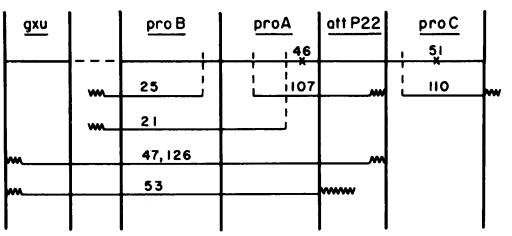

FIG. 2. *Partial genetic map of the proline (pro) region of Salmonella typhimurium showing representative deletion mutants and relation to gxu. Mapping data of pro deletions is based on a report by Itakawa and Demerec (13). attP22 is attachment site for phage P22.*

auxotrophs to use guanine or xanthine as alternate growth factors. This conversion is mediated by GMP reductase, and it is known that *guaC* mutants which lack this enzyme show the described *gxu* phenotype when present in purine auxotrophs (22, 25). Strain *purE66 proAB47* was compared with the wild type for GMP reductase activity, and the results shown in Table 3 do not reveal any deficiency in this reaction. In fact, the mutant showed a higher constitutive-like activity than the wild type which is known to require a guanine derivative for induction of the enzyme (2). The merodiploid F' strain carrying the *pro*$^+$ *gxu*$^+$ alleles was restored to the inducible wild-type pattern. Thus, the *gxu* deficiency is not due to a *guaC* mutation. Furthermore, azaguanine re-

sistance would not be expected in *guaC* mutants, and although *guaC* has been mapped near the *pro* region, it is closer to *thr* than it is to *pro* and it would be unlikely to be carried on the F *prolac* episome (10).

Incorporation of radioactive purines. The next possibility considered was a defect in the uptake mechanisms for guanine and xanthine. Figure 3 shows the difference between the wild-type LT-2 strain and strain *proAB47* in their ability to incorporate guanine-8-^{14}C. The *proAB47* strain carrying *purE*$^+$ was used to alleviate the purine requirement for growth. The marked deficiency of strain *proAB47* in the uptake of guanine-8-^{14}C is striking. Its differential rate of guanine uptake was less than 10% that of the wild-type activity. Table 4 shows further comparison between strains LT-2 and *proAB47* in the incorporation of adenine, hypoxanthine, guanine, and xanthine. The differential rates for adenine and hypoxanthine were comparable in the two strains, but those of guanine and xanthine were markedly decreased. Again, introduction of the F *pro* episome in the merodiploid F′ strain restores the ability to incorporate guanine.

TABLE 3. *Guanosine monophosphate (GMP) reductase activities of purE66 proAB47 and its pro*$^+$ *merodiploid derivative*[a]

Addition to media	GMP reductase (nmoles per min per mg of protein)		
	purE66 proAB47	*purE66 proAB47/F pro*$^+$	LT-2
None	4.86	1.59	0.69
Guanosine	5.25	3.10	3.49

[a] Cells were grown in minimal glucose media with and without guanosine (200 μg/ml) and with hypoxanthine (10 μg/ml), and proline (40 μg/ml) added where needed for growth.

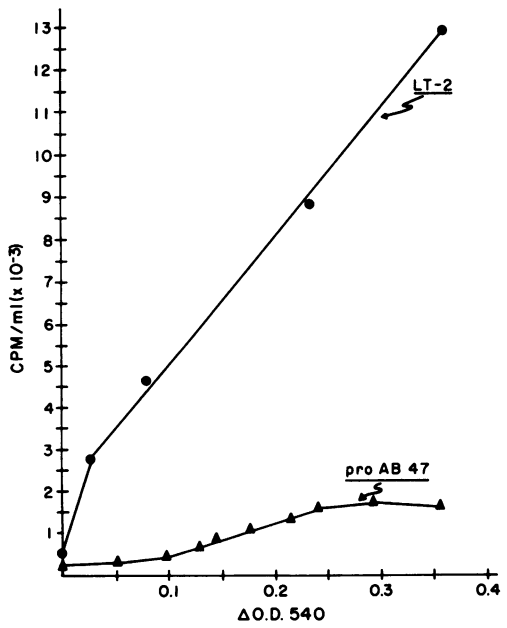

FIG. 3. *Differential rate of uptake of guanine-8-*^{14}C *by wild-type LT-2 and proAB47 mutant.*

Purine phosphoribosyltransferase activities. The efficiency of uptake of purine bases is known to be intimately associated with the purine phosphoribosyltransferase activities (3, 11, 15). Because of the often reported inseparability of hypoxanthine and guanine transferase, it was at first thought unlikely that the *gxu* lesion could be due to a defect in this enzyme. Nevertheless, phosphoribosyltransfer activities were examined, and the surprising results shown in Table 5 were obtained. There is no question that the *gxu* lesion is due to an impairment in the phosphoribosyl transfer activity for guanine and xanthine without affecting that for hypoxanthine. The table also compares activities in strains with various allelic combinations of the *gxu* locus. Thus, the strain with the *gxu*$^+$/*gxu*$^+$ combination (*proB25* F′ *pro*$^+$) shows a threefold increase in guanine and xanthine activities, indicating gene dosage effect. The hypoxanthine activity is also somewhat increased in this strain (1.7 times), suggesting that some hypoxanthine activity may be carried by the *gxu* product. This, however, is not found in the *gxu*/*gxu*$^+$ combinations which show wild-type levels of all activities.

TABLE 4. *Rates of uptake of purines by various strains*

Strain	Uptake[a] (nmoles per ml per Δ optical density)			
	Adenine	Hypoxanthine	Guanine	Xanthine
LT-2	106.0	94.5	80.0	46.3
proAB47	89.0	75.6	5.1	3.6
proAB47/F *pro*$^+$		130.7	84.9	

[a] Rate of uptake was calculated from slope of plots in the major linear range as shown in Fig. 3. Uptake in terms of counts per minute was converted to nanomoles on the basis of the following specific activities (counts per min per μmole) ÷ adenine-8-^{14}C (15.12), hypoxanthine-8-^{14}C (80.2), guanine-8-^{14}C (99.7), xanthine-8-^{14}C (109.4).

TABLE 5. *Phosphoribosyltransfer activities of various pro mutants and derivatives*[a]

Strain	Phosphoribosyltransfer activity (nmoles per min per mg of protein)		
	IMP	GMP	XMP
LT-2	28.0	18.2	24.1
proB25	28.9	15.7	17.8
proB25/F *pro*	49.6	45.7	54.7
proAB47	21.2	0.18	0.36
proAB47/F *pro*$^+$	23.0	18.6	23.7
proAB21	31.6	16.3	23.2
proAB107	28.4	19.7	16.2
proC110	28.2	17.4	16.1
proAB53	31.9	0.26	0.30
proAB126	34.9	0.28	0.32

[a] Abbreviations: IMP, inosine monophosphate; GMP, guanosine monophosphate; XMP, xanthosine monophosphate.

DISCUSSION

The deletion nature of the mutations described above obviates the possibility of a mutational modification in substrate specificities, shatters the concept of a single hypoxanthine-guanine enzyme in enteric bacteria, and clearly indicates the existence of at least two separate genes controlling the phosphoribosyl transfer of hypoxanthine, guanine, and xanthine. One of these, the product of the *gxu* gene, has unique specificity for guanine and xanthine. In a concurrent study, Chou and Martin (8) have described another mutation in *Salmonella* (designated *hpt*) that primarily affects hypoxanthine activity.

A number of earlier attempts to separate the hypoxanthine-guanine complex in bacterial extracts were unsuccessful (1, 7, 14). Krenitsky et al. (19) have recently reinvestigated the problem, and their results, based on chromatographic resolution and heat-inactivation patterns, indicated the existence of at least two different enzymes in *E. coli* preparations. One of these was strongly active with hypoxanthine as substrate and only weakly active with guanine. This enzyme is compatible with the *hpt* product described by Chou and Martin (8). The other enzyme worked primarily with either guanine or xanthine as substrate and had only slight activity for hypoxanthine. This enzyme is compatible with our findings as the candidate for the *gxu* product. That this enzyme possesses minor activity towards hypoxanthine is also indicated by the gene dosage effects in this paper and by the findings of Chou and Martin (8). The absence of significant guanine phosphoribosyltransferase activity in the extracts of our *gxu* mutants suggests that the hypoxanthine enzyme is unable to function with guanine as substrate. However, the weak, but detectable, growth response to guanine indicates that some guanine transferase activity may be present and that our inability to detect significant levels in the mutant extracts may be due to suboptimal conditions for the assay of this alternate substrate.

It is known that in order for 8-azaguanine to be inhibitory it must first be converted to its ribonucleotide via phosphoribosyl transfer, and, hence, the mutational loss of this activity leads to resistance to inhibition by the analogue (4, 5, 6, 14, 26). All of the deletion mutants that were resistant to inhibition by 8-azaguanine were also deficient in the guanine-xanthine phosphoribosyltransfer activity and were normal for hypoxanthine. Furthermore, sensitivity was readily restored by introduction of the normal *gxu* gene in the F′ merodiploids. Thus, it is obvious that the guanine-xanthine enzyme, rather than the hypoxanthine one, is required for inhibition by 8-azaguanine. Since selection for resistance to 8-azaguanine has not previously revealed the *gxu* phenotype, it is apparent that other mutational events may also give rise to this resistance (1, 14, 28).

The inability of the *gxu* mutant to use either guanosine or deoxyguanosine as a purine source indicates that phosphoribosyltransfer activity is required for the utilization of the guanine nucleosides. This would suggest that direct conversion of the nucleoside to its nucleotide does not occur readily and that the nucleosides are utilized primarily via the aglycone form obtained by the action of nucleoside phosphorylase. However, Hoffmeyer and Neuhard (12) have found that guanine-requiring *Salmonella* mutants that lack purine nucleoside phosphorylase can use guanosine as a growth factor, thus suggesting the existence of a guanosine kinase in *Salmonella*. One possibility to explain the inability of our mutant to use guanosine is that the gene controlling guanosine kinase may be linked closely to *gxu* and, hence, simultaneously deleted. Another possibility is that there is a marked difference in the K_m values for guanosine between the phosphorylase and the kinase, so that in our mutant guanosine is converted rapidly to irretrievable guanine and unavailable for conversion to the nucleotide by the kinase.

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