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 gxl). 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 gxl (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 photoelectric meter 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 car-
ried out at 37 C on a roller drum. Measurement of
growth inhibition by 8-azaguanine required a small
initial inoculum of about 10^8 bacteria per ml for re-
producible 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 typhimu-
rium, 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 was obtained from A. Newton, and the epis-
ome was transferred to strain SL751 (proA46 purC7
purE66 flaA45 rha461 flaA56 strA) by selecting for
Pro^+ and streptomycin resistance. This then served
as the standard F^+ donor for other pro recipients.
The F1 episome carrying the lac-purE region was
transferred directly from Escherichia coli strain
W3747 (obtained from A. Garen) to strain purE66
purAB47 by selecting for Pur^-.

Chemicals. The tetrasodium salt of 5-phosphor-
ibosyl-1-pyrophosphate was purchased from Sigma
Chemical Co. (St. Louis, Mo.), and 8-azaguanine (2-
amino, 6-oxo, 8-azapurine) was from Calbiochem
(Paris, Los Angeles, California). Hypoxanthine-8-14C
(3.07 mCi/mole) and guanine-8-14C (54 mCi/mole)
were obtained from New England Nuclear (Boston,
Mass.); xanthine-2-14C (48 mCi/mole) was from
Schwarz-Mann (Orangeburg, N.Y.), and adenine-8-
14C (17.9 mCi/mole) was from Calbiochem. The
scintillation fluid used in the Packard Tri-Carb scin-
tillation counter consisted of the Packard fluor, di-
methyl POPOP (1, 4-bis-2-[5-phenylazoxyl] ben-
ze; 100 mg) and (2,5-diphenyloxazole; 2 g) dis-
solved 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 centri-
fugation, washed three times (30 ml per wash) with
0.03 m sodium phosphate buffer (PH 7.4), and sus-
pended in 1.0 ml of fresh buffer containing 2-mer-
captoethanol (2 mm). The cell suspension was rup-
tured 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 × 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 oxidore-
ductase; EC 1.6.9.5) was assayed by the spectropho-
tometric method previously described (2) using con-
tinuous recording at 340 nm.

Purine phosphoribosyltransferase activities were
assayed in a mixture volume of 0.5 ml containing
0.05 μmole (1 μCi/mole) of 14C-labeled purine, 0.5
μmole of tetrasodium 5-phosphoribosyl-1-pyrophos-
phate, 0.1 m tris(hydroxymethyl)aminomethane-hy-
drochloride buffer (pH 8.0), 0.01 m magnesium sul-
fate, and 0.1 to 0.3 mg of protein of the cell-free ex-
tract. 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 μl-
ters 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-isomyl alcohol (1:1) until the solvent
front reached approximately 1.5 inches (3.81 cm)
from the top of the sheet. Nucleotides were identi-
fied by ultraviolet absorption, cut from the sheet,
immersed in 10 ml of scintillation fluid, and
counted. At the same time, 25 μl of unpurified
mixture was also counted, and the ratio of radioactivity of the isolated nucleotide and un-
chromatographed 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 over-
night culture was diluted 1/20 in fresh minimal-gluc-
sose 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 14C-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. Fil-
ters 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 com-
plete nucleotide, inosine monophosphate (IMP),
normally can satisfy their growth re-
quirement with any of the four purine bases:
adename, hypoxanthine, guanine, and xanthine.

This non-discriminatory behavior is made pos-
sible 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.](http://jb.asm.org/Downloaded fromhttp://jb.asm.org/ on October 26, 2017 by guest)
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 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 presented 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-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percent inhibition of growth* in azaguanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 μg/ml</td>
</tr>
<tr>
<td>LT-2 (wild type)</td>
<td>100</td>
</tr>
<tr>
<td>proAB47</td>
<td>0</td>
</tr>
<tr>
<td>proAB53</td>
<td>0</td>
</tr>
<tr>
<td>proAB47/F 13</td>
<td>0</td>
</tr>
<tr>
<td>proAB47/F pro</td>
<td>100</td>
</tr>
<tr>
<td>proAB21</td>
<td>100</td>
</tr>
<tr>
<td>proB25</td>
<td>100</td>
</tr>
<tr>
<td>proA107</td>
<td>95</td>
</tr>
<tr>
<td>proC110</td>
<td>96</td>
</tr>
<tr>
<td>proAB53</td>
<td>0</td>
</tr>
<tr>
<td>proAB126</td>
<td>0</td>
</tr>
</tbody>
</table>

* 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.

Original proAB47 strain.

purE+ transductant of purE66 proAB47.

**Table 2. Growth of various pro mutants and derivatives in presence of 8-azaguanine**
stance 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-8-14C. 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-8-14C 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.

**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 inescapability of hypoxanthine and guanine transferase, it was at first thought unlikely that the *gxx* 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 *gxx* 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 *gxx* locus. Thus, the strain with the *gxx*+/*gxx* 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 *gxx* product. This, however, is not found in the *gxx*/*gxx* combinations which show wild-type levels of all activities.

**Table 3. Guanosine monophosphate (GMP) reductase activities of purE66 proAB47 and its pro* merodiploid derivative**

<table>
<thead>
<tr>
<th>Addition to media</th>
<th>GMP reductase (nmoles per min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>purE66</td>
</tr>
<tr>
<td>None</td>
<td>4.86</td>
</tr>
<tr>
<td>Guanosine</td>
<td>5.25</td>
</tr>
</tbody>
</table>

* 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.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Adenine</th>
<th>Hypoxanthine</th>
<th>Guanine</th>
<th>Xanthine</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT-2</td>
<td>106.0</td>
<td>94.5</td>
<td>80.0</td>
<td>46.3</td>
</tr>
<tr>
<td><em>proAB47</em></td>
<td>88.0</td>
<td>75.6</td>
<td>5.1</td>
<td>3.6</td>
</tr>
<tr>
<td><em>proAB47</em> F pro*</td>
<td>130.7</td>
<td>84.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 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 nmoles on the basis of the following specific activities (counts per min per μmole) × adenine-8-14C (15.12), hypoxanthine-8-14C (80.2), guanine-8-14C (99.7), xanthine-8-14C (109.4).
Table 5. Phosphoribosyltransfer activities of various pro mutants and derivatives

<table>
<thead>
<tr>
<th>Strain</th>
<th>IMP (nmoles per min per mg of protein)</th>
<th>GMP (nmoles per min per mg of protein)</th>
<th>XMP (nmoles per min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT-2</td>
<td>28.0</td>
<td>18.2</td>
<td>24.1</td>
</tr>
<tr>
<td>proB25</td>
<td>28.9</td>
<td>15.7</td>
<td>17.8</td>
</tr>
<tr>
<td>proB25/F pro</td>
<td>49.6</td>
<td>45.7</td>
<td>54.7</td>
</tr>
<tr>
<td>proAB47</td>
<td>21.2</td>
<td>0.18</td>
<td>0.36</td>
</tr>
<tr>
<td>proAB47/F pro</td>
<td>23.0</td>
<td>18.6</td>
<td>23.7</td>
</tr>
<tr>
<td>proAB21</td>
<td>31.6</td>
<td>16.3</td>
<td>23.2</td>
</tr>
<tr>
<td>proAB107</td>
<td>28.4</td>
<td>19.7</td>
<td>16.2</td>
</tr>
<tr>
<td>proC110</td>
<td>28.2</td>
<td>17.4</td>
<td>16.1</td>
</tr>
<tr>
<td>proAB53</td>
<td>31.9</td>
<td>0.26</td>
<td>0.30</td>
</tr>
<tr>
<td>proAB126</td>
<td>34.9</td>
<td>0.28</td>
<td>0.32</td>
</tr>
</tbody>
</table>

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 *Km* 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.
ACKNOWLEDGMENTS

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LITERATURE CITED