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Purine Salvage Pathways of *Bacillus subtilis* and Effect of Guanine on Growth of GMP Reductase Mutants

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We have isolated numerous mutants containing mutations in the salvage pathways of purine synthesis. The mutations cause deficiencies in adenine phosphoribosyltransferase (*adeF*), in hypoxanthine-guanine phosphoribosyltransferase (*guaF*), in adenine deaminase (*adeC*), in inosine-guanosine phosphorylase, (*guaP*), and in GMP reductase (*guaC*). The physiological properties of mutants containing one or more of these mutations and corresponding enzyme measurements have been used to derive a metabolic chart of the purine salvage pathway of *Bacillus subtilis*.

Purines and their nucleosides and nucleotides have been found to control numerous biochemical and developmental reactions. In particular, massive sporulation of *Bacillus subtilis* is induced by partial deprivation of the de novo synthesis of purine and specifically of guanine (Gua) nucleotides. The deprivation can be effected by limiting the Gua supply to *gua* auxotrophs or by inhibiting the GMP branch of the purine pathway (5, 6). Gua nucleotides are the only compounds whose concentration has always decreased whenever massive sporulation was initiated by any nutritional deprivation, including the stringent response to partial amino acid deprivation (5, 12, 13, 18). To understand the mechanisms controlling the concentration and interconversion of nucleotides, we have isolated *B. subtilis* mutants altered in enzymes involved in the salvage and degradation pathways of purines and examined their properties. Combining these and earlier studies, we present here a comprehensive scheme of the metabolic and inhibitory interrelationships between different purine intermediates in *B. subtilis*. In addition to the scientific interest, mutants altered in the purine pathway are of industrial importance, e.g., because some of them accumulate IMP or GMP, which are the most effective flavor enhancers (4).

MATERIALS AND METHODS

Organisms and media. The strains of *B. subtilis* used here are listed in Table 1. Synthetic medium contained 10 mM $(NH_4)_2SO_4$, 5 mM potassium phosphate (pH 7.0), 100 mM 3-(*N*-morpholino)propanesulfonic acid (pH adjusted to 7.0 by KOH), 2 mM $MgCl_2$, 0.7 mM $CaCl_2$, 50 μM $MnCl_2$, 5 μM $FeCl_3$, 2 μM thiamine-HCl, 1 μM $ZnCl_2$, 20 mM L-glutamic acid (pH adjusted to 7 by NaOH), 20 μg of L-methionine per ml, 50 μg of L-tryptophan per ml, and 55 mM D-glucose. Nutrients required by auxotrophic mutants were added at final concentrations of 50 μg/ml for amino acids and at the stated amounts for purines and purine nucleosides. We will use the following abbreviations: Ade, adenine; Ado, adenosine; Gua, guanine; Guo, guanosine; Hyp, hypoxanthine; Ino, inosine.

Growth conditions. Cells were precultured at 37°C on a tryptose blood agar base (Difco Laboratories, Detroit, Mich.) plate for 8 to 15 h and inoculated into synthetic medium, usually containing 0.5 mM Hyp (Hyp medium), at an optical density at 600 nm (OD_{600}) of 0.01. The cultures, occupying 1/10 or less of the flasks, were shaken in a reciprocating shaker at 120 strokes per min at 37°C. Growth was followed by measuring the OD_{600} in a Gilford spectrophotometer.

DNA was prepared and cells were transformed as described before (3). Resistance to 2-fluoro-Ado was determined on plates containing synthetic medium supplemented with 0.5 mM Hyp and 2 mM fluoro-Ado. Resistance to 8-thio-Guo was determined on plates containing synthetic medium and 2 mM thio-Guo.

Assay of various enzymes. Ade, Ado, and AMP deaminase activities were measured according to Coffee (2) and Nygaard (17). Cells were cultured in 100 ml of synthetic medium supplemented with 500 μM Ade, harvested at OD_{600} = 1.0, and washed three times with 50 ml of 10 mM Tris-acetate buffer (pH 7.6). The cells were suspended in Tris-acetate buffer to give an OD_{600} of 50 (about 25 mg of protein per ml) and kept frozen at −20°C. Later, the suspension was adjusted to 37°C and lysed with lysozyme (100-μg/ml final concentration) for 30 min, and the lysate was dialyzed against 300 ml of Tris-acetate buffer containing 20% ethylene glycol, 2 mM $CaCl_2$, and 50 μM EDTA for 2 h at 0°C. The dialysate was used without centrifugation for the enzyme assay. The reaction mixture (100 μl) contained

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TABLE 1. Characteristics and origin of *B. subtilis* strains used

Strain	Genotype[a]	Origin[b]
60001	*trpC2*	Spizizen 168
60015	*metC7 trpC2*	E. Nester SB26
61232	*purC1 trpC2*	B. Reilly BR5
61469	*lys-3 metB10 trpC2*	F. Young BR151
61639	*metC7 purH1 trpC2*	60015 + EMS
61684	*guaC3*[c] *metC7 purH1 trpC2*	61639 + EMS
61699	*adeC1 adeC1*[c] *metC7 purH1 trpC2*	61639 + cobalt irradiation of spores
61889	*guaC3*[c] *guaP1*[c] *metC7 purH1 trpC2*	61684 spontaneous
61911	*adeF2*[c] *purC1 trpC2*	61232 spontaneous
61914	*guaF1*[c] *lys-3 metB10 trpC2*	61469 spontaneous
62118	*guaC3*[c] *guaF1*[c] *metC7 trpC2*	Tf of 61684 by 61914
62121	*guaC3*[c] *metC7 trpC2*	Tf of 61684 by 61914
62178	*guaC3*[c] *metC7 trpC2*	Tf of 61684 by 60001
62221	*guaC3*[c] *metC7 trpC2*	Reversion of 61684
62235	*adeF2*[c] *guaC3*[c] *metC7 purH1 trpC2*	Tf of 61684 by 61911
62245	*guaP1*[c] *metC7 purH1 trpC2*	Reversion of 61889
62248	*adeC1*[c] *adeF2*[c] *metC7 purH1 trpC2*	Tf of 61699 by 61911

[a] Standard *B. subtilis* gene symbols have been used. *pur* indicates a block in the common purine pathway: such a mutant can use Hyp as a purine source. *ade* mutations are concerned with the specific synthesis and interconversion of Ade compounds, whereas *gua* mutations are similarly concerned with Gua and Hyp compounds. *adeC* causes a lack of Ade deaminase, an enzyme which deaminates Ade, Ado, and AMP. *adeF* causes resistance to 2 mM fluoro-Ade due to the lack of Ade PRTase activity. *guaC* produces a deficiency in the GMP reductase activity which is normally induced by addition of Gua or Guo. *guaF* causes resistance to 2 mM 8-thio-Guo and lacks Hyp-Gua PRTase activity. *guaP* causes a deficiency in Ino-Guo phosphorylase activity.

[b] EMS indicates mutagenesis by ethyl methane sulfonate. Tf means transformation by DNA obtained from the second strain and added to the first strain.

[c] Unmapped mutations.

22 mM Tris-acetate buffer (pH 7.6), 1.25 mM [8-^{14}C]Ade or [8-^{14}C]Ado (5 μCi/μmole), or 1 mM [*U*-^{14}C]AMP (4.6 μCi/μmol). The reaction at 37°C was initiated by the addition of the lysate (final concentration, 0.67 mg of protein per ml) and terminated by mixing 3 μl of 4 M formic acid with 10 μl of withdrawn reaction mixture. The reaction product was separated from the substrate on water-washed polyethyleneimine thin-layer plates (Brinkmann Instruments Inc., Westbury, N.Y.) developed with 0.2 M KH_2PO_4 (adjusted to pH 3.4 by H_3PO_4).

Ado, Guo, and Ino phosphorylase were assayed according to Hoffee et al. (9). Cells were cultured in 100 ml of Hyp medium. When the OD_{600} was 0.4, 0.1 mM Guo was added to the cultures used for the Guo phosphorylase and Ino phosphorylase assays and 0.1 mM Ado was added to the culture used for the Ado phosphorylase assay. The cells were harvested at OD_{600} = 1.0, washed twice with 10 ml of 0.05 M Tris-chloride (pH 7.5), and suspended at OD_{600} = 50 in 0.05 M Tris-chloride. The cells were lysed by lysozyme (100 μg/ml) at 37°C for 30 min, and the lysate was used as crude enzyme source without centrifugation. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 1 mM $MgCl_2$, and 2 mM [2-^{3}H]Ado (12.5 μCi/μmol), [*U*-^{14}C]Guo (0.25 μCi/μmol), or [*U*-^{14}C]Ino (0.25 μCi/μmol). The reaction was initiated at 37°C by the addition of the cell lysate (final concentration, 0.5 to 0.8 μg of protein per ml), and it was terminated by mixing 3 μl of 3 M formic acid with 10 μl of the withdrawn sample. The products of the enzyme reaction were separated from the substrate on polyethyleneimine thin-layer plates developed with 0.2 M KH_2PO_4 (adjusted to pH 3.4 by HCl).

Ade phosphoribosyltransferase (Ade PRTase) and Hyp-Gua phosphoribosyltransferase (Hyp-Gua PRTase) were assayed according to Hochstadt (8). Cells were cultured in Hyp medium containing a 0.5 mM concentration of any required amino acid. When the OD_{600} was 0.5, 500 μM Ado or Guo was added to the cultures used for Ade PRTase or Hyp-Gua PRTase assays, respectively. The cells were harvested at OD_{600} = 1.0, washed twice with 30 ml of 0.01 M Tris-hydrochloride (pH 7.9), and suspended in the same buffer at an OD_{600} = 50, and 0.5-ml aliquots were distributed into test tubes and kept at −20°C. Cells were lysed by lysozyme and the lysate was directly used as crude enzyme source. The reaction mixture contained 100 mM Tris-hydrochloride (pH 7.9), 2 mM $MgCl_2$, 1 mM EDTA, 2 mM [8-^{14}C]Hyp (10 μCi/μmol), [^{14}C]Gua (10 μCi/μmol), [8-^{14}C]Ade (5 μCi/μmol), and cell lysate (final concentration, 0.5 to 0.8 μg of protein per ml). The reaction was initiated at 37°C by the addition of 1 mM phosphoribosyl pyrophosphate and terminated by rapid mixing of 3 μl of 3 M formic acid with 10 μl of the withdrawn sample. Hyp and IMP were separated on polyethyleneimine plates developed by potassium phosphate buffer (1 M, pH 6.5). Gua and GMP or Ade and AMP were separated on polyethyleneimine plates developed by 0.2 M KH_2PO_4 (pH 3.4).

To assay GMP reductase activity of strains 61639 and 61684 (*guaC*), cells were cultured in 100 ml of Hyp medium. When the OD_{600} was 0.2, Guo was added (0.2 mM) to induce the reductase. Cells were harvested at OD_{600} = 1.0, washed once with 50 ml of 50 mM Tris-hydrochloride (pH 7.5), and suspended in the same buffer at OD_{600} = 50. The cells were lysed by lysozyme (200-μg/ml final concentration) for 30 min at 37°C, and the lysate was centrifuged at 0°C for 30 min at 15,000 × *g*. The supernatant was heated for 15 min at 54°C to reduce the activity of NADPH oxidase and was centrifuged again at 0°C and 15,000 × *g* for 30 min. The supernatant was used as the enzyme source. The enzyme activity was measured by the oxidation of NADPH according to Mager and Magasanik (14). The reaction mixture contained 80 mM Tris-chloride (pH 7.5), 2.5 mM cysteine, and 0.4 mM NADPH. The reaction was initiated by the addition of cell lysate at a final concentration of 0.34 to 0.49 mg of protein per ml, and the rate of decrease of the absorbance at 340 nm

was recorded. The concentration change of NADPH was determined with a molar extinction coefficient of 6.22×10^6. The protein concentration of crude cell lysates was measured according to Kalb and Bernlohr (11).

Chemicals. 2'-Fluoro-Ado was kindly given to us by J. A. R. Mead of the National Cancer Institute (NSC no. 30605). Methicillin as well as 8-thio-Guo and other nucleotides were purchased from Sigma Chemical Co., St. Louis, Mo. 3-(*N*-Morpholino)propanesulfonic acid was purchased from Leon Industries, St. Charles, Mo.

RESULTS

Isolation and characterization of mutants altered in the Ade salvage pathway. Spontaneous fluoro-Ado-resistant mutants were isolated from a *purC* strain (61232) on plates of Hyp medium containing 2 mM fluoro-Ado. Five independent isolates were investigated further. They all had normal Ado phosphorylase activity and two had normal Ade PRTase activity (their deficiency is unknown). The other three isolates had a low specific activity of Ade PRTase; one of them is strain 61911 (*adeF purC*) (Table 2). All five isolates grew as well as the parental *purC* strain (61232) in synthetic medium containing 0.3 to 0.5 mM Ade, Ado, Hyp, Ino, Gua, or Guo (for a summary showing strain 61911 and other mutants, see Table 3). The results show that Ade PRTase is necessary to convert fluoro-Ado to the active inhibitor (fluoro-AMP or a derived nucleotide) but is not necessary for the use of Ade or Ado as a general purine precursor.

Strains able to use Hyp or Ino but not Ade or Ado as a purine source were isolated after exposure of spores of the *purH* strain (61639) to gamma radiation by a ^{60}Co source. One of these strains (61699 = *adeC purH*) had reduced deaminase activity for Ade, Ado, and AMP, but it had normal activities of Ade PRTase and Ado phosphorylase (Table 2). It could not grow when Ade or Ado was used as sole purine source (Table 3). Both the parent and the mutant were inhibited (in Hyp medium) by 2 mM fluoro-Ado, which indicates that they could both actively transport Ado and convert it (via Ade) into AMP (not shown). The results indicate that the mutation in the *adeC* locus prevents the deamination of Ade, Ado, and AMP into the corresponding Hyp derivative whose production would enable growth. We have called the enzyme an Ade deaminase because in both the parent and the mutant the specific deamination activity with (1.25 mM) Ade was much higher than that with Ado or AMP (Table 2).

Isolation and characterization of mutants deficient in the Gua salvage pathway. Cells of the *purH* mutant (61639) were mutagenized by 280 mM ethyl methane sulfonate (to 0.1% survival). Mutants unable to provide all necessary purines from Gua or Guo were enriched by growth in synthetic medium containing 140 U of methicillin per ml and 100 μM Gua as purine source (methicillin was used because it is resistant to penicillin). One of the mutants (61684 = *guaC purH*), unable to grow with Gua or Guo as sole purine source, had one-fifth of the normal GMP reductase activity when it was grown in Hyp medium containing 200 μM Guo; the activities of Guo and Ino phosphorylase and of Hyp-Gua PRTase were normal (Table 4). When Gua or Guo was added to the Hyp medium, growth of the *guaC purH* mutant (61684) was inhibited (Table 5; Fig. 1a and b). It required 1 to 3 h until maximal inhibition was observed, apparently because some metabolite had to accumulate. No inhibition was observed when Ade or Ado was added. The parent (61639) was not inhibited by any of these additions (Table 5).

Inhibition of the mutant by Guo was used to isolate mutants deficient in Guo phosphorylase activity. The *guaC purH* mutant (61684) was plated on synthetic medium containing 200 μM Hyp and 500 μM Guo; colonies growing distinctly faster than the majority were isolated. The growth of these isolates (e.g., 61889 = *guaC GuaP purH*) in the presence of Hyp was still inhibited by Gua but no longer by Guo (Table 5; Fig. 1c and d). This strain had only one-third of the Guo phosphorylase and one-half of the Ino

TABLE 2. Activities of enzymes in the Ade salvage pathways[a]

Enzyme	Enzyme activity (nmol mg of protein^{-1} min^{-1})			
	61232 (*purC*)	61911 (*purC adeF*)	61639 (*purH*)	61699 (*purH adeC*)
Ade deaminase[b]	4.42	3.89	3.51	0.75
Ado deaminase[b]	0.41	0.59	0.45	<0.042
AMP deaminase[b]	0.53	0.42	0.48	0.14
Ade PRTase	19.2	2.8	22.9	20.2
Ado phosporylase	17.9	17.1	18.2	18.2

[a] For each enzyme, the cells were grown to $OD_{600} = 1$ in the presence of 0.5 mM substrate. Extracts were prepared and enzymes were assayed as described in the text.

[b] The deaminase reactions were linear for at least 45 min.

TABLE 3. Doubling times of different *ade* mutants[a]

Purine added	Doubling time (h)			
	61232 (*purC*)	61911 (*adeF purC*)	61699 (*adeC purH*)	62248 (*adeC adeF purH*)
None	>9	>6	>6	>9
Ade	1.0	1.0	>6	>5
Ado	1.0	0.87	>6	>4
Gua	1.0	1.2	1.6	1.5
Guo	1.0	1.1	1.4	1.5
Hyp	1.0	0.87	0.82	0.83
Ino	1.0	0.85	0.85	0.85

[a] The cells were grown in Hyp medium to OD_{600} = 0.1, collected on a membrane filter, and suspended in fresh synthetic medium without purine. Aliquots, 10 ml, were added to 125-ml Erlenmeyer flasks containing purines at 200 μM final concentration. For each flask, the OD_{600} was measured every 30 to 90 min, depending on the growth rate, in at least eight samples.

phosphorylase activities of the parent (61684) and the other strains investigated (Table 4). To show that the Guo phosphorylase mutation affected growth on Guo also in the absence of the GMP reductase mutation, we selected a rapidly growing *guaC*$^+$ revertant on synthetic medium containing 200 μM Gua. The growth of one such revertant (strain 62245 = *guaP purH*) is shown in Table 5. Whereas this strain grew with Gua at nearly the same rate as the original parent (61639), it grew with Guo at half the rate at which strain 61639 grew with Guo. With Ino (but not with Hyp), strain 62245 also grew more slowly than did strain 61639 (Table 5). These results show that Guo had to be converted to Gua to be efficiently used as a general purine precursor or to inhibit growth of the *guaC* mutant in the presence of Hyp.

Growth inhibition of GMP reductase mutants by Gua. To understand why Gua and Guo inhibited the growth of the *guaC purH* strain (61684), we compared its properties with those of the *guaC guaP purH* strain (61889). For both strains, the inhibition of growth caused by addition of 0.3 mM Gua to Hyp medium was counteracted by increased concentrations of Hyp but not by up to 1 mM Ino (Fig. 1). Whereas Guo inhibited growth of the *guaC purH* mutant (61684), it did not inhibit that of the *guaC guaP purH* mutant (61889). This shows that Guo competed with Hyp only if it was first converted by Guo phosphorylase to Gua. In contrast, growth on Ino was counteracted by Guo in both strains (Fig. 1), which indicates that Guo competed directly with Ino, either for transport or for Ino phosphorylase. Addition of Ade (0.2 mM) completely removed the inhibition by Gua or Guo (Fig. 1), which indicates that the inhibition resulted from a shortage of purines and not from the inhibition of some other metabolic reactions by a Gua derivative (such as the accumulated GMP; see below). This conclusion was supported by the fact that the growth in Hyp medium of *purH*$^+$ *guaC* strains, derived from 61684 by transformation (62178) or spontaneous reversion (62221), was not inhibited by 1 mM Guo or 0.3 mM Gua (not shown).

To determine the effect of Guo on the intracellular levels of Gua nucleotides, strains 61639 (*purH*) and 61684 (*guaC purH*) were grown in Hyp medium containing $^{32}P_i$ (59 μCi/ml) from OD_{600} = 0.1 to 1.0. The cells were collected by filtration, suspended in fresh medium (still containing the $^{32}P_i$) and distributed into flasks containing nothing, Guo (0.1 mM), or Guo plus Ade (0.1 mM). The concentration of intracellular nucleotides was measured 2 h later. The *guaC purH* (61684) cells accumulated 11 times more GMP if they were kept in the presence of Guo (0.1 mM) than without it (Table 6). However, the concentration of ATP and GTP had in the same time decreased by 80 and 30%, respectively. Addition of Ade (0.1 mM) prevented the accumulation of GMP and greatly reduced the decrease of ATP and GTP. Addition of Guo to a culture of the parent (*purH*) strain (61639) caused only a 30% increase of GMP, which was not prevented by Ade addition, and only a 34% decrease of ATP and a 15% decrease of GTP.

TABLE 4. Activities of enzymes in the Gua salvage pathways[a]

Enzyme	Enzyme activity (nmol mg of protein^{-1} min^{-1})				
	61639 (*purH*)	61684 (*purH guaC*)	61889 (*purH guaC guaP*)	61469 (*pur*$^+$)	61914 (*guaF*)
GMP reductase	5.1	1.1	ND[b]	ND	ND
Guo phosphorylase	43	47	14	46	46
Ino phosphorylase	ND	29	15	ND	ND
Hyp-Gua PRTase	19	18	ND	22	4.4

[a] For each enzyme, the cells were grown to OD_{600} = 1 in the presence of 0.5 mM relevant nucleoside (Guo for GMP reductase and Guo phosphorylase, Ino for Ino phosphorylase, and Hyp for Hyp-Gua PRTase). Extracts were prepared and enzymes were assayed as described in the text.

[b] ND, Not determined.

TABLE 5. Doubling times of different *gua* mutants[a]

Purine added	Concn (μM)	Doubling time (h)			
		61639 (*purH*)	61684 (*guaC purH*)	61889 (*guaC guaP purH*)	62245 (*guaP purH*)
None		>10	>10	>10	>10
Ade	300	0.78	1.0	0.83	0.78
Ado	300	0.97	0.98	0.80	0.87
Gua	300	1.9	14	>10	2.0
Guo	300	1.3	>10	>10	2.6
Hyp	150–300	0.78	0.80	0.75	0.63
Ino	300	0.85	0.92	1.3	1.7
Hyp + Gua	150–300 300	0.75	10	3.7	ND[b]
Ino + Gua	300 300	0.75	5.2	8.0	ND
Hyp + Guo	150–300 200–300	0.75	53	0.77	ND
Ino + Guo	300	0.75	7.5	9.0	ND

[a] The cells were grown in Hyp medium to OD_{600} = 0.1, collected on a membrane filter, and suspended in fresh synthetic medium (without purine). Aliquots, 10 ml, were added to 125-ml Erlenmeyer flasks containing purines at the stated final concentration. For each flask, the OD_{600} was measured every 30 to 90 min, depending on the growth rate, in eight or more samples.

[b] ND, Not determined.

Addition of Ade again prevented the decrease of ATP and GTP. These results indicated that Guo inhibited growth by inhibiting the conversion of Hyp to IMP and thus AMP and that the resulting growth inhibition caused an accumulation of GMP but not GTP.

In contrast to the above results obtained by culturing the *guaC purH* strain (61684) in synthetic medium containing 0.3 mM Ino, addition of Gua (0.3 mM) caused only weak growth inhibition if the medium also contained an amino acid mixture (not shown). Removing individual amino acids from this mixture and also adding individual amino acids to synthetic medium containing 0.3 mM Ino, we found that histidine (50 μg/ml) was the only amino acid able to prevent growth inhibition by Gua (0.3 mM) (Fig. 2a). Histidine also prevented growth inhibition by Guo in Hyp medium (Fig. 2b). When we measured the incorporation of [^{14}C]Ino and [^{3}H]Hyp into trichloroacetic acid-precipitable material (Fig. 2c and d), we observed a significant increase after histidine addition. But as histidine did not restore the purine incorporation to normal levels, it apparently functioned, at least partially, by reducing the need to produce new Ade nucleotides (note that histidine is the only amino acid whose synthesis consumes ATP, whereas ATP is merely converted to ADP during the synthesis of other amino acids). In fact, addition of Ade (0.2 mM) to a culture containing Hyp and Guo restored growth (Fig. 2b) and eliminated the uptake of Hyp into trichloroacetic acid-precipitable material (Fig. 2d).

Figure 2a shows that Gua plus histidine addition to a culture (61684) containing Ino produced a growth lag and enabled growth at the original rate only after 2 h. Apparently, cells had to increase the activity of some enzyme before they could grow. To examine the possibility that after their adaptation they might no longer need histidine for growth, we grew strain 61684 to OD_{600} = 1.0 with Ino alone or with Ino, Gua, and histidine. We collected the cells on a membrane filter and washed and suspended them in fresh synthetic medium containing various combinations of Ino, Gua, and histidine. Growth of the cells precultured with Ino alone was still inhibited by the addition of Gua, and it was restored by the addition of histidine. However, the cells precultured with Ino, Gua, and histidine grew with Ino plus Gua even in the absence of histidine (dashed line, Fig. 2a). Thus histidine was only needed until the cells had adapted to the presence of Gua, which caused a deficiency of adenine nucleotides by competing with the conversion of Ino to IMP.

Ino might be converted to IMP either by the combination of Ino phosphorylase and Hyp-Gua PRTase or by Ino kinase. We did not observe any Ino kinase activity in cell-free extracts. To ascertain that such an enzyme was not involved, we used a Hyp-Gua PRTase mutation (*GuaF*), which we had isolated as resistant to 8-thio-Guo (strain 61914). We transformed this mutation into strain 61684 (*guaC purH*), using a high enough DNA concentration to enable congression. However, thio-Guo, with which we wanted

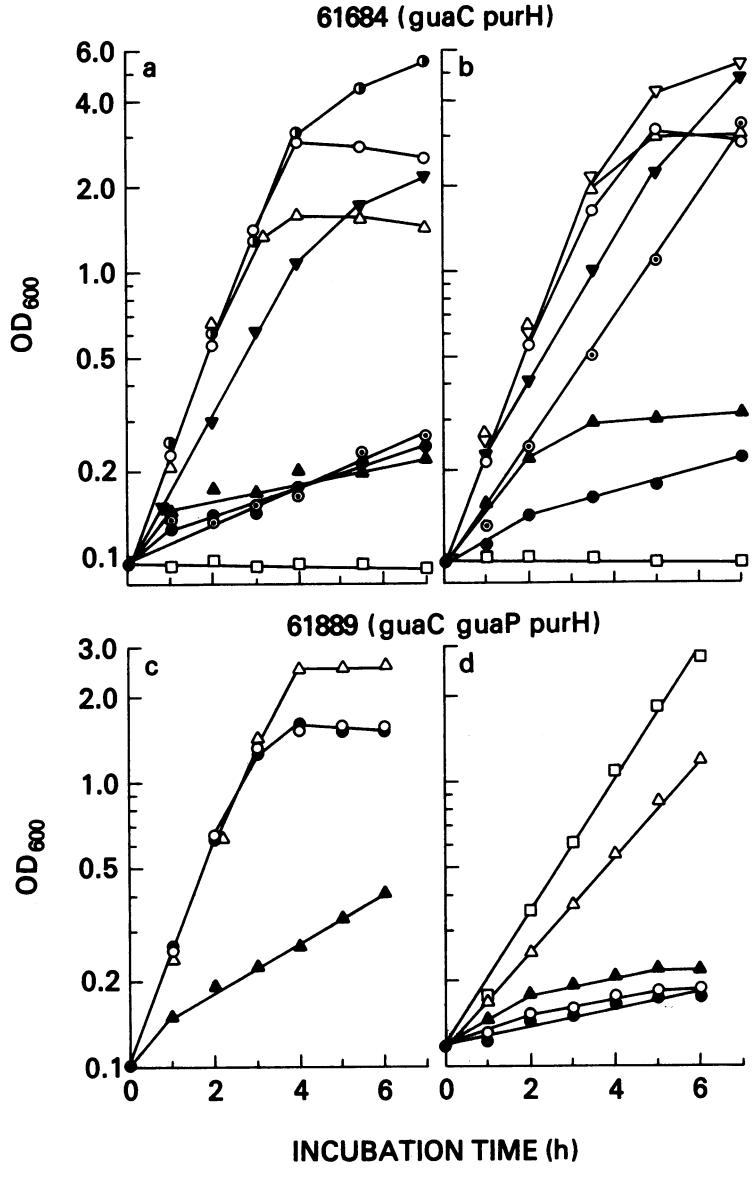

FIG. 1. Growth inhibition of GMP reductase mutants by Gua or Guo in the presence of other purines. Strains 61684 (*guaC purH*) and 61889 (*guaC guaP purH*) were cultured in synthetic medium containing 0.3 mM Hyp. When the OD_{600} was 0.1, the cells were collected on membrane filters and suspended in the same volume of synthetic medium without purine. Aliquots, 10 ml, were added to flasks containing various combinations of purines and purine nucleosides at the stated final concentration, and growth was followed by the increase in OD_{600}. (a) Strain 61684 with 0.3 mM Ino (○), 0.15 mM Hyp (△), and without purine (□). The effect of 0.3 mM Gua was examined in the presence of 0.15 mM Hyp (▲), 0.2 mM Hyp (▼), 0.3 mM Ino (●), 1 mM Ino (⊙), and 0.3 mM Ino plus 0.2 mM Ade (◑). (b) Strain 61684 with 0.3 mM Ino (○), 0.3 mM Hyp (△), or no purine (□). The effect of 0.2 mM Guo was examined in the presence of 1 mM Hyp (▼), 0.3 mM Hyp (▲), 0.7 mM Ino (⊙), 0.3 mM Ino (●), and 0.2 mM Ade plus 0.3 mM Hyp (▽). (c) Strain 61889 with 0.15 mM Hyp alone (○), plus 0.3 mM Guo (●) or Gua (▲); 0.3 mM Hyp plus 0.3 mM Gua (△). (d) Strain 61889 with 0.3 mM Ino alone (□) plus 0.3 mM Gua (●) or Guo (▲); 1 mM Ino plus 0.3 mM Gua (○) or Guo (△).

to identify Hyp-Gua PRTase-deficient mutants, inhibits growth (even at 2 mM) only in the absence of normal purines and purine nucleosides. Therefore, we first selected *pur*$^+$ transformants and then tested them for sensitivity to 2 mM thio-Guo. A sensitive strain (62121) and a

TABLE 6. Accumulation of GMP and decrease of other nucleotides in the *guaC purH* strain (61684) grown in the presence of Guo[a]

Strain	Addition	Intracellular nucleotide (pmol OD_{600}^{-1})		
		GMP	GTP	ATP
61684 (*purH guaC*)	None	109	371	1,483
	0.1 mM Guo	1,164	259	305
	0.1 mM Guo 0.1 mM Ade	100	353	1,042
61639 (*purH*)	None	126	332	1,395
	0.1 mM Guo	164	283	919
	0.1 mM Guo 0.1 mM Ade	169	369	1,439

[a] Cells were cultured from OD_{600} in Hyp medium containing 2 mM potassium phosphate and 59 μCi of $^{32}P_i$ per ml. When the OD_{600} was 1, the cells were collected on a membrane filter, washed, and suspended in the same volume of synthetic medium (without purine). The suspension was divided into three portions: one received 0.3 mM Hyp, the second received 0.3 mM Hyp plus 0.1 mM Guo, and the third received 0.3 mM Hyp, 0.1 μM Guo, and 0.1 mM Ade. The intracellular concentrations of ATP, GTP, and GMP were measured 2 h after the shift according to the method of Lopez et al. (13).

resistant strain (62118) were isolated and grown in synthetic medium containing amethopterin (100 μM) and an excess of those methylated compounds (except purines) whose synthesis is inhibited by amethopterin (Table 7, footnote *a*). This caused a partial deficiency of purines as was demonstrated by normal growth when Ade (0.3 mM) was added (Table 7). Addition of 0.3 mM Ino also enabled normal growth of the *guaC* strain (62121) but not of the Hyp-Gua PRTase-deficient *guaF GuaC* strain (62118) (Table 7). (The same was true with Hyp [not shown].) Strain 62118 also grew badly with the combination of Ino, Gua, and histidine, in contrast to the Hyp-Gua PRTase$^+$ strain (62121). These observations show that Hyp-Gua PRTase was required for the conversion of Ino into IMP (via Hyp).

To determine whether Gua affected the activity of the phosphorylase converting Ino (plus P_i) to Hyp (plus ribose-1-P), we used a crude cell lysate of strain 61684 (*guaC purH*) grown in Hyp medium. Gua (0.3 mM) did not inhibit the phosphorylase reaction at Ino concentrations of 0.1 to 1.0 mM. A concentration of 1 mM GMP inhibited the reaction by <6% (not shown).

The above observations have demonstrated that Gua inhibited the growth on Ino or Hyp by inhibiting Hyp-Gua PRTase. This was demonstrated by measuring the effect of Gua and GMP on Hyp-Gua PRTase in a crude cell lysate, using Hyp as a substrate. Figure 3 shows that the apparent K_m for Hyp was 26 μM and the reaction was competitively inhibited by Gua. GMP inhibited the reaction much less than Gua did; e.g., for 80 μM inosine, 200 μM GMP caused 16% inhibition.

Because Hyp-Gua PRTase was the only enzyme significantly inhibited by Gua, it should also be the enzyme whose activity increased during the lag period during which cells adapted to growth in the presence of Gua and histidine. A factor of 2 increase [from 17.9 to 35.9 nmol min^{-1} mg of $protein^{-1}$ for cells grown as in Fig. 2a) was actually observed.

It remains necessary to explain why growth inhibition of the *guaC purH* mutant (61684) by Gua could be counteracted by high concentrations of Hyp but not Ino. The effect of Hyp can be explained by the competition between Gua and Hyp for Hyp-Gua PRTase (Fig. 3); a competition between Hyp and Gua transport is also conceivable (but uptake and conversion to the nucleotide could not be experimentally separated). Because Gua did not inhibit Ino posphorylase, the inability of Ino to compete with Gua implies that Ino must be transported or converted to Hyp so slowly that the intracellular concentration of Hyp just sufficed to produce IMP (by Hyp-Gua PRTase) at the rate needed for normal growth but was not high enough to overcome the inhibition of Hyp-Gua PRTase by the added Gua. To demonstrate this, we measured the intracellular Hyp concentration for cells grown at various concentrations of Ino or Ado. To prevent conversion of Ado to AMP, we first removed the Ade PRTase activity by transforming strain 61684 with DNA of the *adeF* mutant (61911). If the resulting strain 62235 (*adeF guaC purH*) was grown in synthetic medium containing different concentrations of [^{14}C]Ado, the cells contained 12 to 19 times more intracellular Hyp than if the cells were grown in synthetic medium containing the same concentrations of [^{14}C]Ino (Table 8). Even with 1 mM extracellular Ino, the intracellular Hyp concentration was five times lower than with 0.3 mM extracellular Ado. Thus, Gua inhibited growth on Ino because the conversion of Ino to Hyp was so slow that Gua could effectively compete with the intracellular Hyp for Hyp-Gua PRTase.

DISCUSSION

The biochemical pathway of purine interconversions in *B. subtilis* and the presently known mutations affecting some of the enzymes are summarized in Fig. 4. We have found no activities for kinases which might convert Ado, Guo, or Ino directly into the respective nucleotides, and our mutant data show that such enzymes do

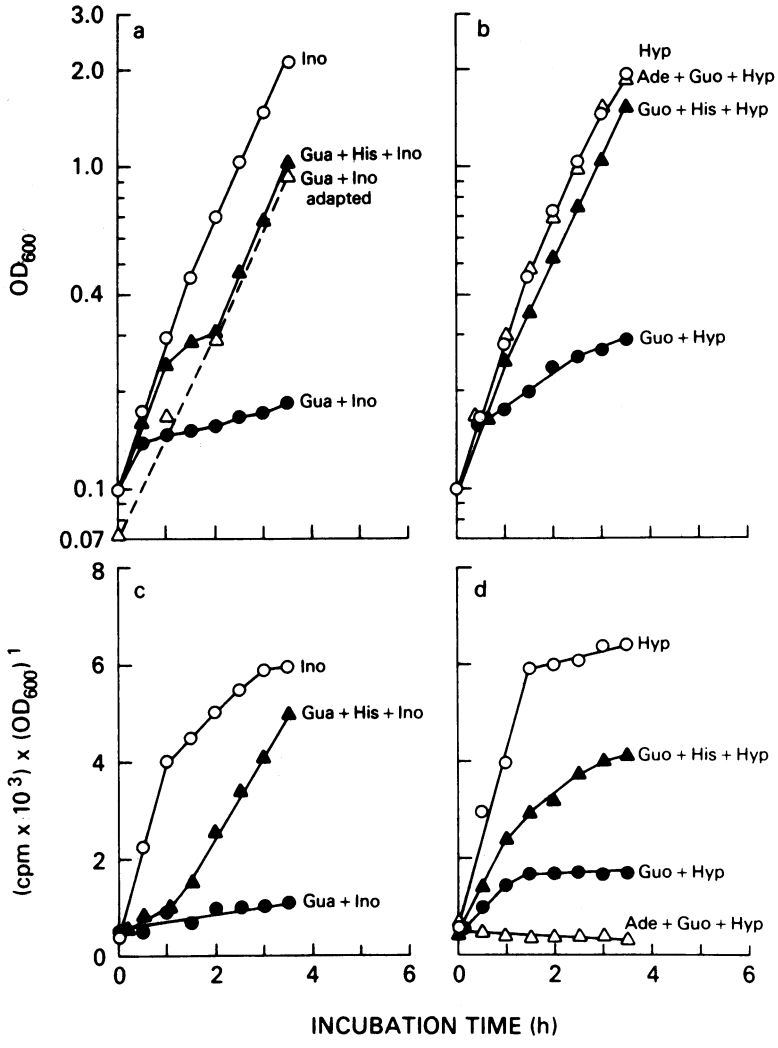

FIG. 2. Effect of histidine on growth inhibition caused by Gua or Guo. Cells of strain 61684 (*guaC purH*), grown in synthetic medium containing 0.3 mM Hyp to OD_{600} = 0.1, were collected by filtration and suspended in synthetic medium at the same OD_{600}. Aliquots, 10 ml, were added to flasks containing the following compounds at the stated final concentrations: (a and c) 0.3 mM [8-^{14}C]Ino (0.4 μCi/μmol) (○), 0.3 mM [8-^{14}C]Ino plus 0.3 mM Gua (●), and 0.3 mM [8-^{14}C]Ino plus 0.3 mM Gua plus 50 mM L-histidine (▲); (b and d) 0.3 mM [*U*-^{3}H]Hyp (0.4 μCi/μmol) (○), 0.3 mM [*U*-^{3}H]Hyp plus 0.3 mM Guo (●), 0.3 mM [*U*-^{3}H]Hyp plus 0.3 mM Guo plus 50 mM histidine (▲), and 0.3 mM [*U*-^{3}H]Hyp plus 0.3 mM Guo plus 0.2 mM Ade (△). (a, b) OD_{600}. (c, d) Incorporation of radioactivity into trichloroacetic acid-precipitable material: 0.5 ml of culture was mixed with 10% cold trichloroacetic acid. After 1 h on ice, the precipitate was collected on a membrane filter (0.45-μm pore size; 25-mm diameter) and washed with 10 ml of ice-cold 5% trichloroacetic acid, and the radioactivity was measured. To measure the effect of adaptation to the presence of Gua, cells were transferred to synthetic medium containing 0.3 mM Ino, 0.3 mM Gua, and 0.25 mM histidine, grown to OD_{600} = 1.0 (for 260 min), collected on a membrane filter, washed, and suspended in fresh synthetic medium at OD_{600} = 0.075. Aliquots, 10 ml, were added to flasks with synthetic medium containing 0.3 mM each Ino and Gua (in a).

not exist in cells grown in synthetic medium containing various purine nucleosides. The three purine nucleosides are converted to the corresponding nucleotides in two steps, the first being the ribose removal by a nucleoside phosphorylase (using P_i) and the second the conversion of the base to the nucleotide by a PRTase, using phosphoribosyl pyrophosphate. *B. subtilis* has two nucleoside phosphorylases: one reacts specifically with Ado whereas the other, in which we obtained a mutation (*guaP*), reacts with Guo and Ino. If the (leaky) *guaP* mutation was pres-

TABLE 7. Effects of Gua and histidine on growth of the *guaC guaF* strain (62118) and the *guaC* strain (62121) in the presence of amethopterin[a]

Compound added					Doubling time (h) for strain:	
ade (300 μM)	Amethopterin (100 μM)	Gua (300 μM)	His (250 μM)	Ino (300μM)	62118 (guaC guaF)	62121 (guaC)
−	−	−	−	−	0.75	0.75
−	+	−	−	−	1.8	1.8
+	+	−	−	−	0.75	0.75
−	+	−	−	+	1.6	0.75
−	+	+	−	+	1.7	1.7
−	+	+	+	+	1.7	0.75

[a] Strains were cultured in synthetic medium to OD_{600} = 0.1, collected on a membrane filter, washed, and suspended at the same OD_{600} in synthetic medium containing 80 μg of glycine, 2 μg of pyridoxine, 2 μg of riboflavin, and 1 μg of thiamine per ml and 300 μM thymine. Aliquots (10 ml) of the suspension were added to 125-ml flasks containing the stated compounds.

ent in a general purine (*purH*) auxotroph, it greatly reduced the rate at which the cells grew on Ino or Guo as sole purine source, whereas the growth on Ado as well as on Ade, Gua, or Hyp was not affected. Our inhibition studies have shown that Guo efficiently competes with Ino for the Ino-Guo phosphorylase, which has a higher affinity for Guo than for Ino. The finding of two phosphorylases agrees with earlier reports on the physical separation of an Ado from an Ino-Guo enzyme (10, 19). Because these enzymes were found in extracts free of cell wall and membranes and because *B. subtilis* does not have an outer membrane layer, which in gram-negative bacteria defines a periplasmic envelope, the two phosphorylases are probably locat-

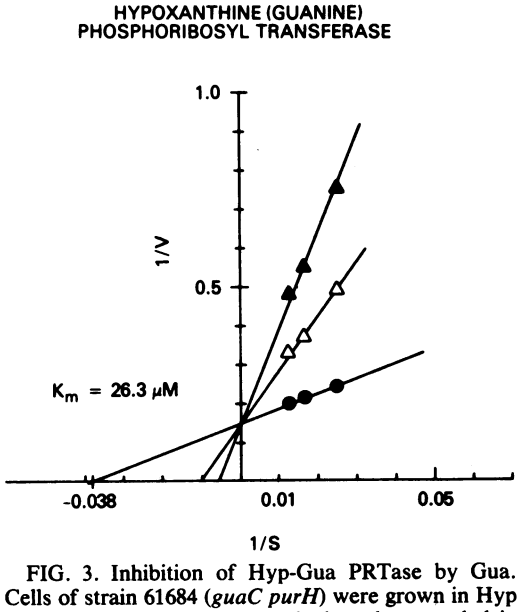

FIG. 3. Inhibition of Hyp-Gua PRTase by Gua. Cells of strain 61684 (*guaC purH*) were grown in Hyp medium to OD_{600} = 0.1, washed, and suspended in synthetic medium plus 0.3 mM Hyp. When the OD_{600} was 1, the cells were harvested by centrifugation, washed with 50 mM Tris-chloride (pH 7.5), suspended in Tris-chloride at an OD_{600} of 50, and lysed by lysozyme (100-μg/ml final concentration) for 30 min at 37°C. A 0.675-mg portion of protein of the crude lysate was used per ml of Hyp-Gua PRTase assay mixture which contained [^{14}C]Hyp (see text). The Hyp substrate concentrations were 40, 60, and 80 μM (●), and the effect of Gua (100 μM, △; and 200 μM, ▲) on the reaction was measured. The velocity, V, of IMP production is expressed in nanomoles per minute. In the presence of 80 μM Hyp, 200 μM Gua inhibited 57.8% and 200 μM GMP inhibited 16.3% (not shown).

TABLE 8. Intracellular Hyp concentration of strain 62235 (*adeF guaC purH*) cultured in the presence of various concentrations of Ino and Ado[a]

Purine source	Concn of purines added in the culture (mM)	Intracellular Hyp concn (nmol OD_{600}^{-1})
[^{14}C]Ino	0.3	0.75
	0.6	0.90
	1.0	1.77
[^{14}C]Ado	0.3	9.21
	0.6	29.20
	1.0	33.60

[a] Cells were grown in synthetic medium containing 0.3 mM Hyp to OD_{600} = 0.2, collected on a membrane filter, washed once with 10 ml of purine-free synthetic medium, and then suspended in this medium at the same OD_{600}. Aliquots, 1.5 ml, received 0.3, 0.6, and 1.0 mM [8-^{14}C]Ino (10 μCi/ml) or [8-^{14}C]Ado (400 μCi/ml). After 110 min of shaking at 37°C, the cells of 0.5 ml of each culture were collected on a membrane filter which was immediately placed upside down onto 50 μl of ice-cold 1 M formic acid. After 30 min of extraction, 10 μl of extract was applied to the corner of a polyethyleneimine thin-layer plate. To separate Hyp from the other compounds, the plate was eluted with 0.2 M KH_2PO_4 (pH 3.4) in the first dimension, washed with acetone, and, after drying, eluted with distilled water in the second dimension.

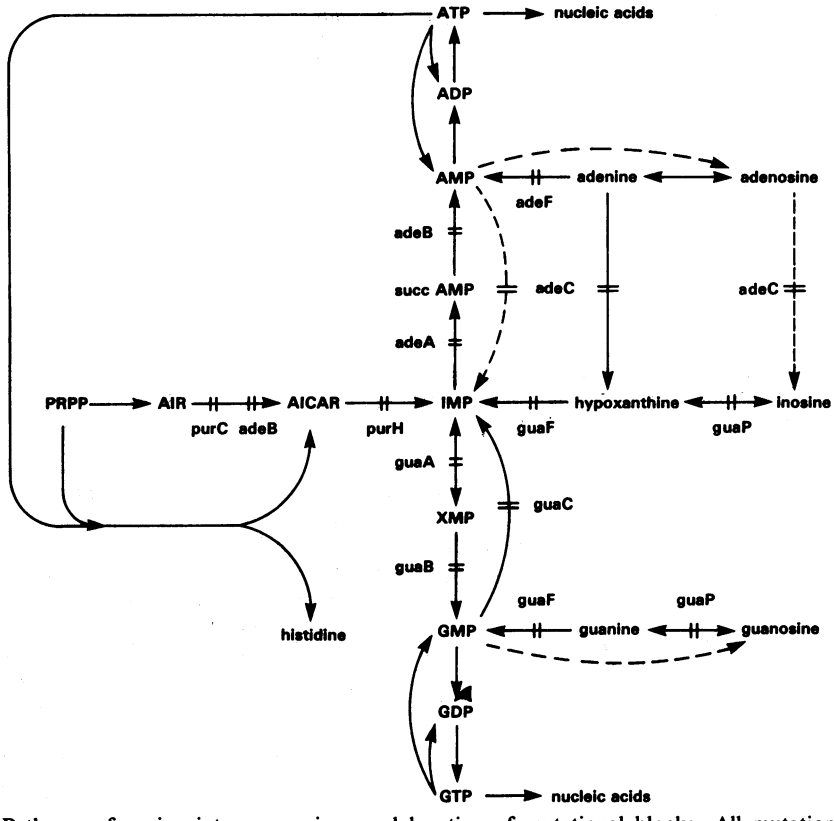

FIG. 4. Pathway of purine interconversions and location of mutational blocks. All mutations causing a specific alteration of metabolism of Ade and its derivatives are named *ade*. Similarly, mutations causing a specific alteration of the metabolism of Gua and its derivatives are named *gua*. Mutations in the general purine pathway which cause a deficiency that can be overcome by addition of Hyp are called *pur*.

ed in the cytoplasm of *B. subtilis*. However, it is possible that they are frequently associated with the membrane, for if a Gua auxotroph (*guaA*) is supplied with Gua, this compound is rapidly converted into Guo which appears in the medium (6).

B. subtilis has two PRTases, one for Ade, for which we obtained a mutation (*adeF*), and another for Hyp and Gua, for which we also obtained a mutation (*guaF*). The PRTase activities of *B. subtilis* were described by Berlin and Stadtman (1), but the authors did not ascertain whether the activities for Hyp and Gua resulted from one or two enzymes. Our competition and mutant results demonstrate that *B. subtilis* has, like mammalian tissue and yeast (8, 9), one enzyme for both activities. This should be contrasted to *Salmonella typhimurium* which has two separate activities for Hyp and Gua (7, 9). An Ade PRTase-deficient purine auxotroph (*adeF purC*) could still use Ade or Ado as sole purine source because *B. subtilis* has an Ade deaminase that acts on Ade, Ado, or AMP. The same enzyme deaminates all three compounds because an *adeC* mutation caused the absence of all three deaminating reactions and prevented growth on Ade or Ado in an *adeC purH* mutant (Table 3). A similar mutation was described by Nishikawa et al. (16).

Most interesting were the observations with a *guaC* mutation which caused the absence of GMP reductase, i.e., prevented the conversion of GMP to IMP. A *guaC purH* mutant did not grow on Gua or Guo as sole purine source because no enzyme remained which could convert these compounds into IMP; the mutant could grow on all other purines (Table 5). Addition of Gua or Guo to a culture of the mutant growing on Hyp or Ino inhibited growth because these compounds competed for Ino phosphorylase and Hyp-Gua PRTase, preventing the synthesis of IMP and thus of Ade nucleotides (see the 80% decrease of ATP, Table 6). The inhibition of growth caused an accumulation of intracellular GMP, apparently because this compound was now faster produced than consumed.

In contrast, the concentration of GTP decreased, presumably because ATP, which is required for the conversion of GMP to GDP and GTP, decreased. *pur*$^+$ transformants or revertants of this strain (still having the *guaC* marker) grew without any purine in the medium; Gua or Guo addition did not inhibit its growth and caused only a slight (35%) decrease of ATP. This contrasts with the observation of Momose et al. (15), who found that their *pur*$^+$ transformant of a *guaC pur* mutant grew without any purine only after a 2-h lag, which was avoided by the addition of Ino, and it did not grow in the presence of Guo for at least 6 h. The reason for this difference may be that in our experiments the cells were actively growing and thus producing all nucleotides; when Gua or Guo was added, they continued to grow while they immediately adapted to the increase of GMP by shutting off the de novo synthesis of GMP from IMP, thus avoiding excessive feedback inhibition of all de novo purine synthesis which would have prevented AMP and thus ATP synthesis. In the experiments of Momose et al. (15), resting cells may have been challenged to grow in the presence of Guo. Unfortunately, we were unable to obtain those strains to compare their properties with ours. Addition of Ade or Ado enabled growth of both the *guaC* and the *guaC purH* mutants in the presence of Gua or Guo. This addition also prevented all other phenomena caused by Gua or Guo addition; this shows that the inhibition resulted from a deficiency of Ade nucleotides.

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