

Comparative Action of Glyphosate as a Trigger of Energy Drain in Eubacteria

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Escherichia coli, *Bacillus subtilis*, and *Pseudomonas aeruginosa*, each possessing a 5-enolpyruvylshikimate 3-phosphate synthase that is sensitive to inhibition by glyphosate [*N*-(phosphonomethyl)glycine], provide a good cross-section of organisms exemplifying the biochemical diversity of the aromatic pathway targeted by this potent antimicrobial compound. The pattern of growth inhibition, the alteration in levels of aromatic-pathway enzymes, and the accumulation of early-pathway metabolites after the addition of glyphosate were distinctive for each organism. Substantial intracellular shikimate-3-phosphate accumulated in response to glyphosate treatment in all three organisms. Both *E. coli* and *P. aeruginosa*, but not *B. subtilis*, accumulated near-millimolar levels of shikimate-3-phosphate in the culture medium. Intracellular backup of common-pathway precursors of shikimate-3-phosphate was substantial in *B. subtilis*, moderate in *P. aeruginosa*, and not detectable in *E. coli*. The full complement of aromatic amino acids prevented growth inhibition and metabolite accumulation in *E. coli* and *P. aeruginosa* where amino acid end products directly control early-pathway enzyme activity. In contrast, the initial prevention of growth inhibition in the presence of aromatic amino acids in *B. subtilis* was succeeded by progressively greater growth inhibition that correlated with rapid metabolite accumulation. In *B. subtilis* glyphosate can decrease prephenate concentrations sufficiently to uncouple the sequentially acting loops of feedback inhibition that ordinarily link end product excess to feedback inhibition of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase by prephenate. The consequential unrestrained entry of energy-rich substrates into the aromatic pathway, even in the presence of aromatic amino acid end products, is an energy drain that potentially accounts for the inability of end products to fully reverse glyphosate inhibition in *B. subtilis*. Even in *E. coli*, after glyphosate inhibition and metabolite accumulation were allowed to become fully established, a transient period where end products were capable of only partial reversal of growth inhibition occurred. The distinctive metabolism produced by dissimilation of different carbon sources also produced profound effects upon glyphosate sensitivity.

Many antimetabolites that act against biosynthetic pathways mimic end products, causing inappropriate regulatory effects upon enzymes having early-pathway positions. In contrast, *N*-(phosphonomethyl)glycine (PMG), an herbicide known as glyphosate, is an exceedingly effective inhibitor of a mid-pathway enzyme of aromatic amino acid biosynthesis, 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase. EPSP synthase has been shown to be a sensitive target of PMG action in prokaryotes, lower eucaryotes, and higher plants (see reference 34 for reference citations). Although PMG is now under intensive study in higher plants by virtue of its potent herbicidal properties, antimicrobial studies are few. The presence of PMG during growth can produce massive accumulation of shikimate-3-phosphate behind the blocked EPSP synthase (Fig. 1), an effect caused by decreased levels of end product molecules that normally feedback inhibit or repress the initial enzyme of the pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase.

Three molecular events triggered by PMG could account in part for growth inhibition: (i) starvation for aromatic amino acids, (ii) an energy drain imposed by utilization of one phosphoenolpyruvate (PEP) molecule and one ATP molecule for every molecule of shikimate-3-phosphate accu-

mulated, and (iii) the possible toxicity of accumulated common-pathway intermediates, particularly phosphorylated compounds such as DAHP or shikimate-3-phosphate. In this study side-by-side comparisons have been made of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* to evaluate the energy drain effects of PMG in organisms exhibiting diversity of aromatic-pathway construction and regulation.

In an organism such as *E. coli*, the administration of aromatic amino acids would be expected to reverse the action of PMG completely, regardless of which effects listed above contribute to growth inhibition. This follows because the three aromatic amino acids act directly as feedback inhibitors and repression signals for the three regulatory isozymes of DAHP synthase in *E. coli* (7) (Fig. 1). Although *P. aeruginosa* differs substantially from *E. coli* in the enzymic construction and overall regulation of the aromatic amino acid pathway (1, 20), complete reversal of PMG inhibition of growth by aromatic amino acids is also expected in *P. aeruginosa* because the two DAHP synthase isozymes present (Fig. 1) are directly controlled by L-tyrosine and L-tryptophan (39).

Expectations are markedly different for *B. subtilis*, an organism in which end products control DAHP synthase by an indirect mechanism, employing a sequentially acting network of allosteric enzymes (23, 30). DAHP synthase of *B. subtilis* is feedback inhibited by chorismate and prephenate (Fig. 1), metabolites whose intracellular levels ordinarily

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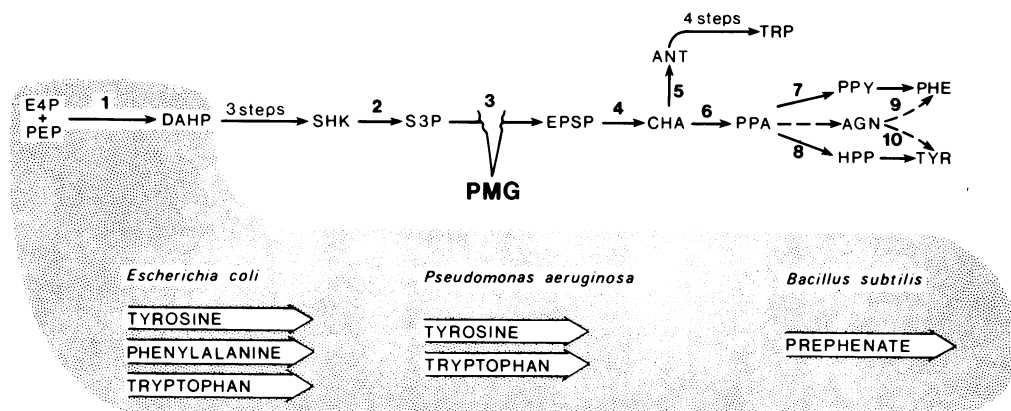


FIG. 1. Allosteric control of DAHP synthase and PMG inhibition of EPSP synthase in *E. coli*, *B. subtilis*, and *P. aeruginosa*. The wedge-shaped symbol indicates EPSP synthase as the target of PMG action, and the shaded area portrays the comparative allostery of DAHP synthase in the three microbes. *E. coli* possesses three isozymes of DAHP synthase, each differentially controlled by one of the aromatic amino acids (6). *P. aeruginosa* possesses two isozymes of DAHP synthase, a major species that is feedback inhibited by L-tyrosine and a minor species that is feedback inhibited by L-tryptophan or by chorismate (39). *B. subtilis* possesses a single DAHP synthase enzyme, subject to feedback inhibition by prephenate (23). Enzymes are numbered as follows: 1, DAHP synthase; 2, shikimate kinase; 3, EPSP synthase; 4, chorismate synthase; 5, anthranilate synthase; 6, chorismate mutase; 7, prephenate dehydratase; 8, chorismate dehydrogenase; 9, arogenate dehydratase; 10, arogenate dehydrogenase. Abbreviations: E4P, D-erythrose-4-phosphate; SHK, shikimate; S3P, shikimate-3-phosphate; CHA, chorismate; ANT, anthranilate; TRP, L-tryptophan; PPA, prephenate; PPY, phenylpyruvate; PHE, L-phenylalanine; AGN, L-arogenate; HPP, 4-hydroxyphenylpyruvate; TYR, L-tyrosine.

reflect overall end product levels. In this study we pursue the question whether PMG (through prevention of chorismate and prephenate formation in *B. subtilis*) has the potential to uncouple the normal control of DAHP synthase by aromatic amino acid end products.

MATERIALS AND METHODS

Microorganisms. All bacterial strains used were wild-type prototrophs. *E. coli* K-12 EMG2 (3, 13) was obtained from B. Bachmann of the *E. coli* Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn. *P. aeruginosa* PAO1 (19) and *B. subtilis* 168 strain NP1 (27) were from the culture collection of our laboratory.

Growth of bacteria and culture conditions. *E. coli* was grown in M9 minimal medium (29) containing 0.2% (wt/vol) glucose. *B. subtilis* was grown on the minimal medium of Spizizen (36), containing glucose (0.2%, wt/vol) as the carbon source. In addition, both media were supplemented with $ZnCl_2$, $MnCl_2 \cdot 4H_2O$, and $CoCl_2 \cdot 6H_2O$ at final concentrations of 1, 2, and 7 $\mu g/ml$, respectively. *P. aeruginosa* was grown in a minimal salts medium containing (per liter) 7 g of K_2HPO_4 , 3 g of KH_2PO_4 , 0.1 g of $MgSO_4 \cdot 7H_2O$, 1 g of $(NH_4)_2SO_4$, and 0.5% (wt/vol) fructose as the carbon source. Agar (Difco Laboratories, Detroit, Mich.) was added to give a final concentration of 1.5% (wt/vol) for solid media.

Culture turbidities for growth studies were determined with a Klett-Summerson colorimeter (Klett Manufacturing Co., Inc., New York, N.Y.) equipped with a no. 54 green filter for *P. aeruginosa* or a no. 66 red filter for *E. coli* and *B. subtilis*. Exponentially growing cultures were used to inoculate either 20 ml (for *E. coli* and *B. subtilis*) or 10 ml (for *P. aeruginosa*) of fresh medium containing supplements as indicated. These cultures were grown in 125-ml sidearm flasks with vigorous shaking at 37°C.

Extracts were prepared from 1,000-ml cultures contained in 2,800-ml Fernbach flasks which were shaken at 300 rpm at 37°C. Cultures in the late-exponential phase of growth were

harvested by centrifugation, washed twice with cold 50 mM potassium phosphate buffer (pH 7.0), and stored as whole cell pellets at $-20^\circ C$.

Preparation of crude extracts. Extracts for DAHP synthase determinations were prepared in 50 mM potassium phosphate buffer (pH 7.0) containing either 1 mM $MgCl_2$ and 100 mM KCl for *B. subtilis* or 1 mM dithiothreitol for *E. coli*. All EPSP synthase activities were determined from extracts prepared in 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.5 at 37°C). The pH of the HEPES buffer was adjusted with tetramethylammonium hydroxide to avoid the introduction of cations. The extract from *E. coli* to be used for anthranilate synthase determinations was prepared in 10 mM Tris hydrochloride buffer (pH 7.8 at 25°C) containing 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. Extracts from *B. subtilis* to be used for anthranilate synthase assays were prepared in the buffer described by Kane et al. (25), except that 20 mM L-glutamine was added to the extract buffer. The extracts for determining prephenate dehydrogenase activities were prepared in 100 mM Tris hydrochloride buffer (pH 8.5 at 25°C) containing 50 mM KCl plus 0.5 mM dithiothreitol.

Cell pellets, suspended in 3 to 5 ml of the required buffer, were disrupted by either ultrasound or lysozyme. For *E. coli* and *P. aeruginosa*, cells were disrupted at 4°C with three 20- and 30-s bursts, respectively, of ultrasound with an intensity of 100 W from a Lab-Line Ultratip Labsonic System (Lab-Line Instruments, Inc., Melrose Park, Ill.). Sonication largely inactivated EPSP synthase, anthranilate synthase, and prephenate dehydrogenase in *B. subtilis* crude extracts, and only extracts of *B. subtilis* to be used for DAHP synthase assays could be prepared by sonication. With *B. subtilis* two 20-s bursts at 100 W were sufficient to achieve a degree of disruption comparable to that obtained with *E. coli* and *P. aeruginosa*. *B. subtilis* extracts to be used for EPSP synthase, anthranilate synthase, and prephenate dehydrogenase assays were prepared by gentle lysis with lysozyme (100 $\mu g/ml$) at 37°C for 15 min. DNase at a final concentration of 25 $\mu g/ml$ was added to degrade DNA. The disrupted

cell suspensions were ultracentrifuged at $145,000 \times g$ for 60 min at 4°C to remove cell debris. With the exception of the extracts used for assay of EPSP synthase from *B. subtilis* and *P. aeruginosa*, the resulting supernatants were then desalted by passage over a Sephadex G-25 column (PD-10; Pharmacia Fine Chemicals, Uppsala, Sweden). The *B. subtilis* and *P. aeruginosa* extracts used for EPSP synthase assays were extensively dialyzed against 25 mM HEPES buffer (adjusted to pH 7.5 as described above) to ensure removal of potential activating cations.

Analytical procedures. Protein concentrations were estimated by the method of Bradford (5) as described in Bio-Rad technical bulletin 1051 (Bio-Rad Laboratories, Richmond, Calif.). DAHP synthase was assayed by the method of Srinivasan and Sprinson (37) as modified by Jensen and Nester (24). Reaction mixtures for the *B. subtilis* enzyme were prepared as described by Jensen and Nester (24), but were modified for *E. coli* to contain 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol, 1 mM PEP, 1 mM D-erythrose-4-phosphate, and 1 mM MgSO_4 . Anthranilate synthase from *B. subtilis* was assayed by the method of Kane and Jensen (26), whereas anthranilate synthase from *E. coli* was assayed as described by Calhoun et al. (11). EPSP synthase was assayed by following the disappearance of PEP (17) as modified by Rubin et al. (34), except that standard reaction mixtures contained 3 mM PEP, 3 mM shikimate-3-phosphate, 25 mM HEPES buffer (pH 7.5 at 37°C), and the necessary activating cation as designated below. Prephenate dehydrogenase was assayed as described by Champney and Jensen (12) for *B. subtilis*, whereas the method of Byng et al. (9) was used for *E. coli*.

Determination of metabolite levels. For quantitation of intracellular metabolites, whole cell pellets were washed twice with 50 mM potassium phosphate buffer (pH 7.0), resuspended in the buffer, and disrupted by sonication (four 30-s bursts at 100 W). Cell debris was removed from the extract by ultracentrifugation at $145,000 \times g$ for 60 min at 4°C . The protein concentration of each extract was then determined, and accumulated DAHP was quantitated by the method of Jensen and Nester (23). To determine intracellular levels of shikimate and shikimate-3-phosphate, samples of each extract were heated in a 100°C water bath for 2 min, centrifuged at $12,000 \times g$ for 10 min, and passed through a $0.2\text{-}\mu\text{m}$ polycarbonate membrane (Nuclepore Corp., Pleasanton, Calif.) to remove most of the protein, nucleic acid, and particulates. Shikimate and shikimate-3-phosphate levels were determined in these preparations by using high-performance liquid chromatography. Samples were injected into a $20\text{-}\mu\text{l}$ loop and eluted from a Baker Amino column (4.6 by 250 mm, $5\text{-}\mu\text{m}$ particle size) (J.T. Baker Chemical Co., Phillipsburg, N.J.) with a mobile phase consisting of acetonitrile- H_2O -phosphoric acid (95:4:1) at a flow rate of 1.0 ml/min. Shikimate and shikimate-3-phosphate were detected at 215 nm and quantitated using a standard curve generated from the recorded peak heights obtained with authentic shikimate and shikimate-3-phosphate.

Extracellular shikimate and shikimate-3-phosphate levels were determined directly from the cell-free media after harvesting. A sample of each medium was passed through a $0.4\text{-}\mu\text{m}$ polycarbonate membrane (Nuclepore) before processing as described above for measuring intracellular accumulation.

Biochemicals and chemicals. Unless indicated otherwise, all biochemicals and commercially prepared enzymes were obtained from Sigma Chemical Co., St. Louis, Mo. Analytical-grade PMG (99.9% pure) was a gift from Monsanto Co.,

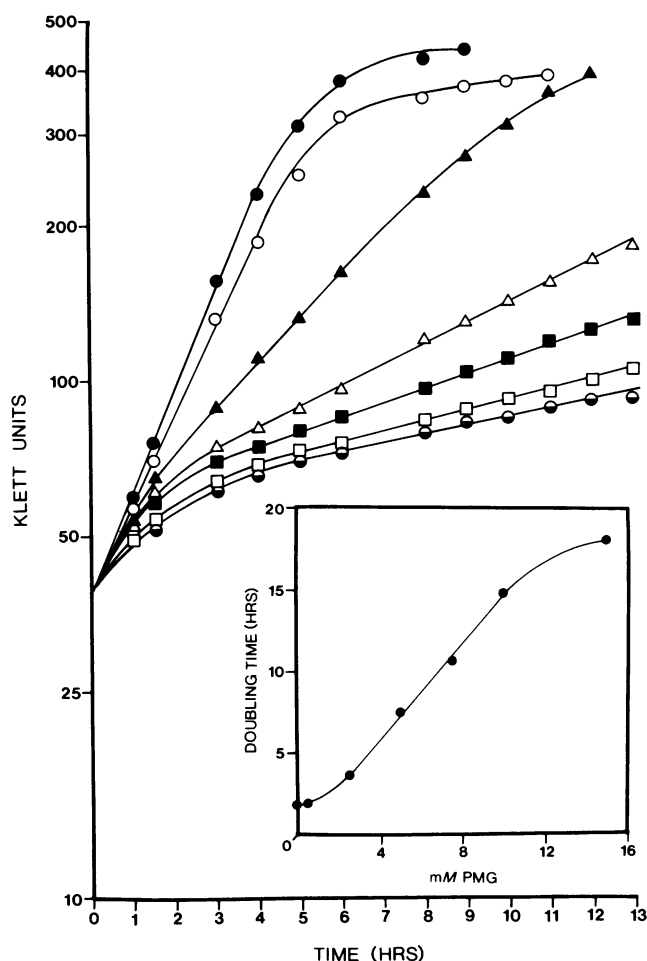


FIG. 2. PMG inhibition of growth of *E. coli*. Growth conditions were outlined in Materials and Methods. PMG concentrations: ●, none; ○, 0.5 mM; ▲, 2.5 mM; △, 5.0 mM; ■, 7.5 mM; □, 10.0 mM; ●, 15.0 mM. The inset shows the doubling times plotted as a function of PMG concentration.

St. Louis, Mo. Shikimate-3-phosphate was prepared as previously described (28), except that it was converted to a tetramethylammonium salt instead of the conventional sodium or potassium salt. Barium prephenate was prepared from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* (14) and was converted to the potassium salt with a twofold excess of K_2SO_4 before use. Chorismate was prepared from culture supernatants of *Klebsiella pneumoniae* by the method of Gibson (16). Acetonitrile and phosphoric acid for high-performance liquid chromatography and the standard reagent-grade chemicals were obtained from Fisher Scientific Co., Springfield, N.J.

RESULTS

Inhibition of growth by PMG. PMG was capable of exerting effective antimetabolite action against all three microorganisms under study. Figure 2 illustrates the family of growth curves generated in *E. coli* in the presence of PMG concentrations ranging between 0.5 and 15 mM. Care was taken to ensure that experiments were started with exponentially dividing cell populations. Reports of initial growth lags after the addition of PMG (2, 32) probably resulted from the

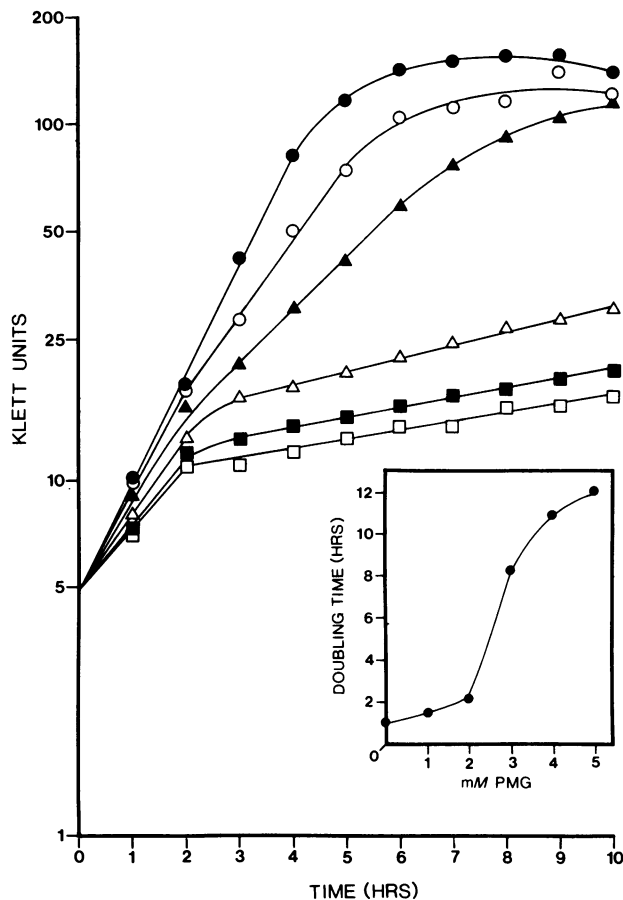


FIG. 3. PMG inhibition of growth of *B. subtilis*. Culture conditions were as described in Materials and Methods. PMG concentrations: ●, none; ○, 1.0 mM; ▲, 2.0 mM; △, 3.0 mM; ■, 4.0 mM; □, 5.0 mM. The inset shows a replot of doubling time versus PMG concentration.

use of growth inocula that were either approaching or already in stationary-phase physiology. Subcultured stationary-phase cells are well known to lag longer than normal upon subculture in the presence of a variety of antimetabolites. An initial period of progressively increasing inhibition was succeeded by transition to a constant rate of inhibited growth. The duration of the initial phase of progressively increasing inhibition varied with increasing PMG dose, e.g., lasting about 2 h at a PMG concentration yielding an ultimate growth inhibition of 50% and lasting about 6 h at a PMG concentration yielding an ultimate growth inhibition of 90%. Once the stable rate of inhibited growth characteristic of any given concentration of PMG was achieved, it endured for at least 24 h. The inset of Fig. 2 shows a replot in which the doubling time eventually achieved is plotted against PMG concentration. About 2 mM PMG was required to inhibit the growth rate of *E. coli* by 50%.

The family of growth curves generated in *B. subtilis* in the presence of PMG is shown in Fig. 3. An initial phase of progressively increasing inhibition similar to that seen in *E. coli* was observed, although the time required for transition to a stable rate of inhibited growth appeared to be shorter, e.g., no longer than 4 h at the highest PMG concentration used. Although the concentration of PMG required (about 1.8 mM) to produce 50% inhibition was similar to that seen

in *E. coli*, the responsivity of *B. subtilis* to PMG is compressed within a smaller concentration range than in *E. coli*. This is shown by the sigmoid inhibition curve plotted in the inset of Fig. 3. Thus, at 1, 3, and 5 mM concentrations of PMG the growth inhibitions of *E. coli* and *B. subtilis* were 30 and 25%, 65 and 88%, and 79 and 92%, respectively. The rate of growth ultimately obtained in *B. subtilis* as a function of PMG concentration did not endure with the stability observed with *E. coli*. After 16 h of growth in the presence of 2.5 mM PMG, a transition to the wild-type growth rate was observed in *B. subtilis*. At higher PMG concentrations the transitions were less abrupt and became apparent at progressively later times. It is not known whether this phenomenon reflects the selective growth of a fractional population of resistant mutants or whether it reflects a physiological adaptation. The uniformity of the response seems to favor the latter alternative.

P. aeruginosa is about 50-fold less sensitive to PMG on glucose-based medium than are either of the other two organisms, but it exhibits much greater sensitivity to PMG on fructose-based medium (A. Berry and R. Jensen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K169, p. 221). On fructose-based medium the family of growth-inhibition curves generated for *P. aeruginosa* was similar to those obtained with *E. coli* and *B. subtilis* on glucose-based medium (Fig. 4). Relative to the doubling time on fructose-based medium, the duration of the period of progressively

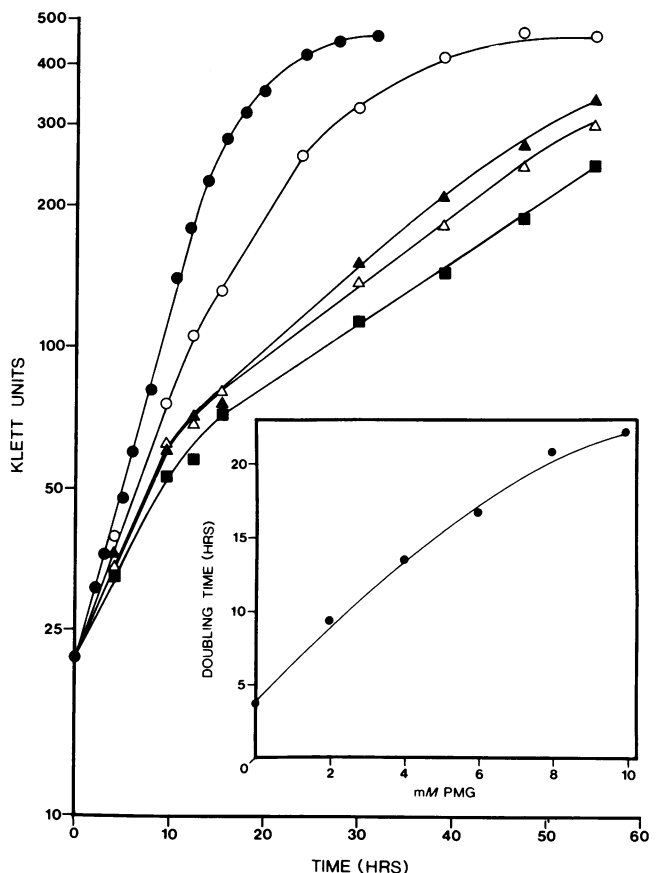


FIG. 4. PMG inhibition of growth of *P. aeruginosa* grown on fructose as the sole carbon source as described in Materials and Methods. PMG concentrations: ●, none; ○, 2.0 mM; ▲, 6.0 mM; △, 8.0 mM; ■, 10.0 mM. The inset shows a replot of doubling time versus PMG concentration.

increasing inhibition was roughly similar to the other organisms. The ultimate rates of growth attained at any given concentration of PMG were quite stable as in *E. coli*, not exhibiting the apparent adaptation in *B. subtilis*. The inset of Fig. 4 shows that on fructose-based medium only 1.4 mM PMG was required for 50% inhibition of growth of *P. aeruginosa*.

Comparative sensitivities of microbial EPSP synthases to PMG. Each EPSP synthase enzyme examined was sensitive to inhibition by PMG, confirming the expectation that the molecular target of PMG action is EPSP synthase. Under standard in vitro assay conditions (substrate saturation), the *E. coli* enzyme was the most sensitive to inhibition by PMG based on the criterion of concentration required to produce 50% inhibition (75 μ M for *E. coli*, 174 μ M for *B. subtilis*, and 1.1 mM for *P. aeruginosa*). The K_m of EPSP synthase for PEP and the K_i for PMG inhibition with respect to PEP would be expected to be the most salient kinetic parameters with respect to in vivo sensitivity of EPSP synthase to PMG. These values are as follows: *E. coli* (15), K_m of 1.5 μ M, K_i of 1 μ M; *B. subtilis* (manuscript submitted), K_m of 12.7 μ M, K_i of 22 μ M; *P. aeruginosa* (this study), K_m of 213 μ M, K_i of 200 μ M. The EPSP synthases from *B. subtilis* (R. S. Fischer, J. L. Rubin, and R. A. Jensen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K174, p. 222) and *P. aeruginosa* (data not shown) require monovalent cations for activity. The *B. subtilis* and *P. aeruginosa* enzymes were assayed under conditions of maximal activation by ammonium (100 mM NH_4Cl) or potassium (200 mM KCl), respectively.

Metabolite accumulation during growth of PMG-treated bacteria. The data presented in Table 1 illustrate properties of metabolite accumulation in response to PMG treatment that proved to be distinctive for each organism. Intracellular accumulation of shikimate-3-phosphate was marked in each organism. However, in *P. aeruginosa* and *B. subtilis* metabolites along the entire common pathway probably back up, judging from the presence of both shikimate and DAHP within the cells. No intracellular shikimate or DAHP was detected in PMG-treated *E. coli* cultures. In both *E. coli* and *P. aeruginosa*, high levels of shikimate-3-phosphate were exported into the medium. The data indicate that *P. aeruginosa* cultures grown on fructose-based medium in the presence of PMG might be a good source for isolation of shikimate-3-phosphate that is contaminated with relatively little shikimate. In contrast, *B. subtilis* appears unable to export the phosphorylated intermediate into the culture

TABLE 1. Intracellular and extracellular accumulation of aromatic-pathway metabolites in PMG-treated microbes^a

Species	Intracellular accumulation (nmol/mg of protein) of:			Extracellular accumulation (μ M concn in supernatant) of:	
	DAHP	Shikimate	Shikimate-3-phosphate	Shikimate	Shikimate-3-phosphate
<i>E. coli</i>	0	0	126	35	305
<i>P. aeruginosa</i>	1.3	2.9	32.6	10	480
<i>B. subtilis</i>	17.0	7.9	97.2	14	0

^a The PMG concentrations used for *E. coli* (2 mM), *P. aeruginosa* (3 mM), and *B. subtilis* (2 mM) inhibited growth rates 56, 82, and 50%, respectively. PMG was added to exponentially growing cells diluted to an initial turbidity of about 0.04 at 600 nm. Analyses of metabolite levels were carried out at growth yields corresponding to absorbances of 0.8, 1.0, and 0.7 at 600 nm for *E. coli*, *P. aeruginosa*, and *B. subtilis*, respectively. Values of 0 indicate levels of DAHP (<0.7 nmol), shikimate (<0.1 nmol), and shikimate-3-phosphate (<0.2 nmol) that are below the sensitivity of the assay.

TABLE 2. Response of aromatic-pathway enzyme levels to PMG treatment^a

Species	PMG	Sp act (nmol/min per mg)			
		DAHP synthase	EPSP synthase	Prephenate dehydrogenase	Anthranilate synthase
<i>E. coli</i>	-	62.7	71.6	31.0	0.32
	+	156.8	74.5	274.0	1.14
<i>B. subtilis</i>	-	28.9	7.9	20.0	0.12
	+	101.4	18.4	29.0	0.04

^a Crude extracts were prepared as described in Materials and Methods from cultures of *E. coli* and *B. subtilis* grown in the presence of 2 mM PMG and harvested at growth yields corresponding to turbidities of 0.8 and 0.7 at 600 nm, respectively.

medium. Modest extracellular levels of shikimate were detected in all three organisms.

Effect of PMG upon aromatic-pathway enzyme levels. Except for the enzymes of the tryptophan pathway (11), aromatic-pathway enzymes (and indeed biosynthetic-pathway enzymes in general) of *P. aeruginosa* appear not to exhibit repression-derepression behavior in response to end product levels. Since aromatic amino acid biosynthesis is subject to repression control in both *B. subtilis* and *E. coli*, a number of enzymes were assayed after growth in the presence and absence of PMG (Table 2). In *E. coli* the derepression of DAHP synthase undoubtedly reflects the selective elevation of DAHP synthase-Tyr, an isozyme that is largely repressed in minimal medium (22). EPSP synthase of *E. coli* has been reported to be constitutive (38). Prephenate dehydrogenase activity in *E. coli* was increased almost ninefold after growth in PMG, whereas anthranilate synthase exhibited only modest derepression compared with its potential level during maximal derepression.

DAHP synthase, EPSP synthase, and prephenate dehydrogenase activities of *B. subtilis* (grown in the presence of PMG) were all found to be at levels that were close to maximal derepression (31). Interestingly, anthranilate synthase was repressed more than 60% in the presence of 2 mM PMG. Since chorismate mutase inevitably elevates with DAHP synthase in strain 168 of *B. subtilis* by virtue of both being shared catalytic activities of a bifunctional protein (7), the results indicate that when chorismate levels are lowered by PMG action against EPSP synthase, anthranilate synthase can still maintain a competitive advantage over chorismate mutase—even with an eight- to ninefold decrease in the ratio of anthranilate synthase to chorismate mutase. This indicates the relatively better ability of *B. subtilis* anthranilate synthase to compete with chorismate mutase at low levels of chorismate, compared with *E. coli*.

Reversal of PMG inhibition by aromatic amino acids. The data shown in Fig. 5 are consistent with the evidence cited above for superior ability of the tryptophan pathway to scavenge limited intracellular chorismate in *B. subtilis* compared to *E. coli*. The inhibition of growth of *E. coli* established in the presence of PMG for 2 h was almost completely reversed by the combination of all three aromatic amino acids (Fig. 5A). The addition of L-phenylalanine plus L-tyrosine, however, led to only partial reversal of growth inhibition (40% inhibition remaining), indicating that tryptophan synthesis must be rate limiting to growth.

In contrast, the combination of L-phenylalanine and L-tyrosine was as effective as all three aromatic amino acids in reversing PMG-inhibited growth of *B. subtilis* (Fig. 5B).

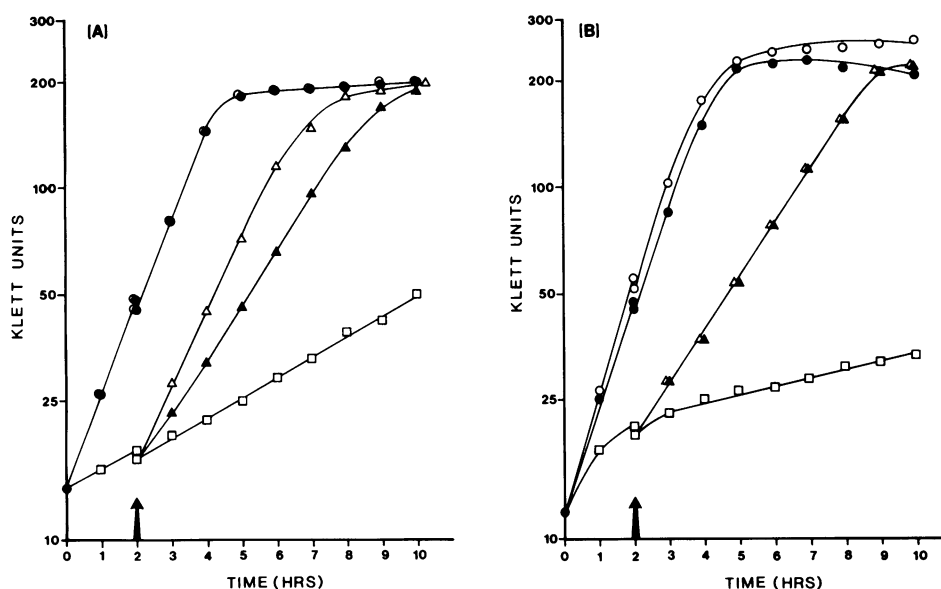


FIG. 5. Reversal of PMG inhibition of growth of *E. coli* (A) and *B. subtilis* (B) by aromatic amino acids; PMG concentrations were 10 and 5 mM, respectively. L-Tryptophan (Trp), L-tyrosine (Tyr), and L-phenylalanine (Phe) were added to yield final concentrations of 0.25 mM. The various end product combinations were added to cultures after growth for 2 h (arrow) in the presence of PMG. Symbols: ●, untreated control; ○, Trp + Tyr + Phe; ▲, PMG + Tyr + Phe; △, PMG + Trp + Tyr + Phe; □, PMG-treated control.

Presumably, still higher concentrations of PMG causing more than the 92% inhibition of growth observed in this experiment would be required to demonstrate tryptophan limitation in the presence of L-phenylalanine and L-tyrosine.

Table 3 shows a comparison of the abilities of all possible combinations of aromatic amino acids to reverse growth inhibition by PMG in *B. subtilis* and *E. coli*. A striking difference between *E. coli* and *B. subtilis* was that in *B. subtilis*, established PMG inhibition of growth (92%) was not completely reversed by aromatic amino acids. A residual inhibition of about 45% persisted under these conditions (Fig. 5B and Table 3). The possibility was considered that in *B. subtilis* the normal regulation of DAHP synthase might become uncoupled from proper responsiveness to end product levels in the presence of PMG. In *B. subtilis* end products regulate DAHP synthase indirectly via their effect upon intracellular levels of prephenate (23, 30). In contrast, end product supply in *E. coli* directly regulates the DAHP synthase isozymes. Thus, the inhibition of growth that persists in the presence of all three aromatic amino acids (Fig. 5B) reflects the consequences of massive drain of energy (and possibly of reducing power) within the shikimate pathway of *B. subtilis*.

The latter idea predicts that accumulation of common-pathway intermediates would be observed in *B. subtilis* during growth on sufficiently high concentrations of PMG, even if aromatic amino acids were supplied simultaneously. In *E. coli* and *P. aeruginosa*, on the other hand, effective early-pathway control by end products ought to prevent any accumulation of common-pathway intermediates in the presence of even high concentrations of PMG. These expectations were fully realized (Table 4).

DISCUSSION

Energy drain. Inhibition of EPSP synthase in its mid-pathway position has two effects that seem to apply universally. First, starvation for aromatic-pathway end products is

initiated. Compensatory regulatory adjustments occur, resulting in elevation of repressible enzymes and relief of allosteric enzymes from feedback inhibition. This produces the second effect: unrestrained entry of PEP and erythrose-4-phosphate into the aromatic pathway. The largely futile utilization of significant amounts of PEP in the DAHP synthase reaction must actually promote increased sensitivity of EPSP synthase to PMG in vivo. The metabolic impact of wasteful loss of PEP and ATP is likely to vary somewhat for every organism. If energy drain is sufficiently drastic, other pathways could actually become more limiting to growth than aromatic amino acid limitation. This might happen at PMG concentrations just adequate to slow EPSP formation enough to promote unregulated activity of DAHP synthase. Such indirect effects upon other pathways should still be reversed by aromatic amino acids through restoration of early-pathway regulation. Even in *B. subtilis* early-pathway regulation can be restored by aromatic amino acids

TABLE 3. Reversal by aromatic amino acid combinations of growth inhibition previously established in the presence of PMG^a

Amino acid additions ^b	% Inhibition of growth rate of:	
	<i>B. subtilis</i>	<i>E. coli</i>
None (control)	92	78
Trp	92	74
Tyr	88	75
Phe	78	69
Trp + Tyr	87	67
Trp + Phe	77	63
Tyr + Phe	45	40
Trp + Tyr + Phe	45	15

^a PMG concentrations of 5 and 10 mM were introduced to exponential-phase cultures of *B. subtilis* and *E. coli*, respectively, at t_0 . Doubling times for the control cultures were 12.6, and 5.2 h, respectively.

^b Aromatic amino acids were added singly or in combination as indicated to give final concentrations of 0.25 mM each. Additions were made after 2 h of growth in the presence of PMG.

under conditions of minimal inhibition of EPSP synthase. This is because intracellular prephenate (feedback inhibitor) levels are dictated by the balance between slowed formation of prephenate in the presence of PMG and slowed entry of prephenate into tyrosine and phenylalanine branches due to feedback inhibition of prephenate dehydratase and prephenate dehydrogenase. However, if prephenate formation is severely restricted (i.e., at high PMG concentrations), early-pathway regulation cannot be restored, even in the presence of L-tyrosine and L-phenylalanine, and energy drain persists (Table 4).

Dramatic differences in sensitivity to growth inhibition by PMG were observed in correlation with nutritional variables. Thus, *E. coli* and *B. subtilis* were both similarly sensitive to PMG on glucose-based medium, whereas *P. aeruginosa* was about 50-fold less sensitive on this medium. However, *P. aeruginosa* exhibited roughly the same sensitivities seen with *E. coli* and *B. subtilis* when fructose was substituted for glucose as the carbon source. That alteration of sensitivity to growth inhibition by PMG through manipulation of carbon and energy source is not an isolated phenomenon in *P. aeruginosa* is apparent by results showing that the PMG sensitivity of *B. subtilis* (and *P. aeruginosa*) can be markedly decreased by supplying L-glutamate and, to a lesser extent, L-glutamine, L-proline, and L-arginine (R. Fischer, unpublished data). Although the initial molecular event underlying PMG action is simple (i.e., inhibition of EPSP synthase), the resulting energy burden occasioned by massive investment of PEP and ATP in shikimate-3-phosphate accumulation must initiate other metabolic vulnerabilities. The dramatic ability of L-glutamate and L-glutamine to increase PMG resistance in *B. subtilis* and *P. aeruginosa* fits with a possible consequence of energy drain upon nitrogen assimilation. Each transformation of ammonia and 2-ketoglutarate in the glutamate synthase-glutamine synthetase system of L-glutamate formation requires one ATP in the glutamine synthetase reaction.

Incomplete inhibition of EPSP synthase by PMG could indirectly create other points of vulnerability within the aromatic pathway. For example, if L-glutamate or L-glutamine became limiting as supposed above, then glutamate-requiring transamination reactions leading to L-phenylalanine and to L-tyrosine (or the glutamine-requiring anthranilate synthase reaction leading to L-tryptophan) might become limiting. These reactions could be especially vulnerable under conditions where the levels of keto-acid substrates (i.e., phenylpyruvate and 4-hydroxyphenylpyruvate) have already become depressed in the presence of PMG. Another possibility is that phosphoribosylpyrophosphate shortage in response to energy drain could limit the second reaction in the tryptophan pathway. The enzyme catalyzing this step (anthranilate-phosphoribosylpyrophosphate phosphoribosyltransferase) probably already encounters decreased cosubstrate (anthranilate) levels in the presence of PMG. Other biochemical pathways that could become stressed by energy drain include those for the biosynthesis of L-histidine, aspartate-derived amino acids, purines, and pyrimidines.

Metabolite reversal of growth inhibition by PMG. *E. coli*, *P. aeruginosa*, and any microbe having a regulatory pattern where aromatic amino acid end products directly control DAHP synthase have the following characteristics. Inhibition of growth by PMG can be prevented indefinitely in the presence of aromatic amino acids since they prevent any possibility of energy drain. However, if PMG inhibition is first established in the absence of end products, energy drain

TABLE 4. Ability of aromatic amino acids to prevent PMG-promoted accumulation of aromatic-pathway metabolites^a

Species	Intracellular accumulation (nmol/mg of protein) of:			Extracellular accumulation (μM concn in supernatant) of:	
	DAHP	Shikimate	Shikimate-3-phosphate	Shikimate	Shikimate-3-phosphate
<i>E. coli</i>	0	0	0	0	0
<i>P. aeruginosa</i>	0	0	0	0	0
<i>B. subtilis</i>	3.4	7.1	54.3	10.9	0

^a The PMG concentrations used for *E. coli* (15 mM), *P. aeruginosa* (6 mM), and *B. subtilis* (7.5 mM) inhibited growth rates by 0, 6, and 14%, respectively, in the presence of 0.25 mM concentrations of all three aromatic amino acids (only L-tyrosine plus L-phenylalanine for *B. subtilis*). Values of 0 are as described in footnote a of Table 1.

results in only a partial ability of aromatic amino acids to reverse growth inhibition. Such residual inhibition not reversed by aromatic amino acids is transient, however, and full reversal becomes established after restoration of early pathway control and relief of the energy burden.

In *B. subtilis* and other microbes (8) having a pattern of indirect control over DAHP synthase (sequential feedback inhibition), aromatic amino acids can only prevent growth inhibition by PMG for a short initial period that is succeeded by progressively greater inhibition. At sufficiently high PMG concentrations, aromatic amino acid supplementation can only partially reverse PMG inhibition of growth, and residual inhibition endures indefinitely.

It is quite possible that supplementation with nonaromatic metabolites that spare the need for energy-rich compounds will prove to reverse residual inhibition of growth in the presence of aromatic amino acids. If so, all systems may prove to be fully reversible, given the appropriate nutritional regimen.

***B. subtilis* as a model for plant systems.** In higher plants a chloroplast-localized DAHP synthase exists that is feedback inhibited by L-arogenate, thus constituting another variation of sequential feedback inhibition (35; R. J. Ganson, T. A. d'Amato, and R. A. Jensen, *Plant Physiol.*, in press). The inhibition of plant cell culture systems by PMG is characteristically reversed only partially by aromatic amino acids, and it has been hypothesized that energy drain within the chloroplast compartment plays a significant role in the herbicidal mechanism of PMG action (21, 33). The analogous pattern of early pathway regulation in *B. subtilis* makes it ideal as a model for examination of the impact of energy drain effects upon metabolic pathways of higher plants.

The variety of prevention and reversal results obtained with yeast (4), algae (18), plants (18), photosynthetic eucaryotes (10), and bacteria (2, 18, 32) suggests that the patterns of reversal of PMG inhibition observed in individual organisms may indeed reflect the diversity of aromatic amino acid biosynthesis in nature (8).

PMG as a physiological probe. PMG can be used as a tool to manipulate wild-type cultures for derepression. For example, tryptophan-pathway enzymes can be derepressed in *E. coli* by growth of PMG-treated cultures in the presence of L-phenylalanine plus L-tyrosine. In *P. aeruginosa* growth in the presence of 100 mM PMG in glucose-based medium results in limitation of the tryptophan pathway. In contrast, the tryptophan-pathway enzymes in *B. subtilis* actually become repressed during partial inhibition of growth in the presence of PMG. The latter result indicates that under conditions of precursor limitation, anthranilate synthase of

B. subtilis is able to outcompete chorismate mutase for the available substrate molecules. *E. coli*, by comparison, must undergo a modest derepression of anthranilate synthase to maintain adequate levels of L-tryptophan.

If the energy-drain proposal is correct, then the order of nutritional requirements for small molecules imposed by PMG treatment may provide a useful approach to gaining insight into relationships between connecting networks of biochemical pathways (25, 27).

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