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Initiation of Spore Germination in *Bacillus subtilis*: Relationship to Inhibition of L-Alanine Metabolism

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The inhibitory effects of anthranilic acid esters (methyl anthranilate and *N*-methyl anthranilate) on the L-alanine-induced initiation of spore germination was examined in *Bacillus subtilis* 168. Methyl anthranilate irreversibly inhibited alanine initiation by a competitive mechanism. In its presence, the inhibition could be reversed only by the combined addition of D-glucose, D-fructose, and K^+. Both L-alanine dehydrogenase and L-glutamate-pyruvate transaminase, enzymes which catalyze the first reaction in L-alanine metabolism, were competitively inhibited by methyl anthranilate. The K_i values for germination initiation (0.053 mM) and of L-glutamate-pyruvate transaminase (0.068 mM) were similar, whereas that for L-alanine dehydrogenase (0.4 mM) was six to seven times higher. Since a mutant lacking L-alanine dehydrogenase activity germinated normally in L-alanine alone, it is speculated that the major pathway of L-alanine metabolism during initiation may be via transmination reaction.

It has been reported previously that methyl anthranilate (MA) inhibits the initiation of spore germination of aerobic bacilli (15). MA inhibited the initiation of germination in nutrient broth or by L-alanine under conditions in which it did not affect ougrowth, cell division, or sporulation. This communication shows that the analogues of MA also inhibit germination, and the enzymes inhibited by these drugs are characterized.

MATERIALS AND METHODS

Bacteria. All strains were derived from the transformable *Bacillus subtilis* Marburg strain 168 of Spizizen. Standard strain 60015 (which requires indole and methionine) from this laboratory was used in most of the experiments. An L-alanine dehydrogenase-minus mutant (60229) was also used in some studies (3).

Sporulation, harvesting, heat activation, and initiation procedures. The procedures for sporulation, harvesting, heat activation, and initiation were described previously (22). The initiating agents were used at the following millimolar concentrations: KCl, 400; L-alanine, 5; L-asparagine, 2.5; D-glucose, 5; D-fructose, 5; and tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.4), 100. In some experiments nutrient broth (Difco) was also used.

The rate of germination initiation was measured by the decrease of absorbance at 625 nm (A_{625}). The data are presented as percent reduction in A with the initial reading taken as 100%. In some cases the value A/A° ($A^\circ = A_{625}$ at zero time) was also plotted. The percent inhibition of germination was computed as follows:

$$\% \text{ Inhibition of germination} = \left(1 - \frac{A_c^\circ \cdot \Delta A_i}{A_i^\circ \cdot \Delta A_c}\right) \times 100$$

where A_i° and $A_c^\circ = A^\circ$ values for samples with and without (control) inhibitor, respectively, and ΔA_i and $\Delta A_c = (A^\circ - A)$ values for samples with and without inhibitor. Unless indicated otherwise, the germination was measured at 37 C, and all germination mixtures received enough concentrated spore suspension to get $A^\circ = 0.3$-0.5. The calculation of K_m for germination was made by using maximal rate of decrease in A_{625}.

Preparation of spore extract. Spore extracts for enzyme assays were prepared as described earlier (14).

Enzyme assays. L-Leucine and L-alanine dehydrogenases were assayed as described earlier (14).

L-Glutamate-pyruvate transaminase was assayed by measuring the formation of [^{14}C]glutamate from [^{14}C]α-ketoglutarate. The reaction mixture (100 μliters) contained (micromoles): Tris-hydrochloride buffer (pH 7.4), 10.0; L-alanine, 5.0; [^{14}C]α-ketoglutarate (3.2 μCi/μmol), 0.15; pyridoxal phosphate, 10^{-4}; and crude extract (100 to 200 μg of protein). The reaction was run at 37 C in a glass tube (10 by 75 mm) and stopped by immersing the tube in boiling water for 2 min. The tube was cooled and then spun at 3,000 $\times$ g for 10 min at room temperature. A 5- to 10-μliter volume of supernatant was spotted on an MN 300 cellulose plate, dried, and chromatographed in the following solvent system: isopropanol-88% formic

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acid-water-8-quinolinol (40:2:10:0.026, vol/vol/vol/wt). After the plates were dried, the spot corresponding to glutamic acid was cut, and the radioactivity was counted in 10 ml of scintillation fluid (4 pints of toluene [ca. 1.9 liters], 2 pints of Triton-X 100 [ca. 0.55 liter] and 150 ml of Liquifluor).

All other enzymes were assayed as described by Bergmeyer (1).

Enzyme specific activity is expressed as nanomoles of substrate transformed, or product formed, per minute per milligram of protein.

Protein was measured by the method of Lowry et al. (9) using bovine serum albumin, fraction V (Armour Pharmaceutical Co., Kankakee, Ill.), as standard.

RESULTS

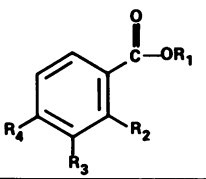
Inhibition of initiation in nutrient broth by analogues of anthranilic acid. Previous work (15) had indicated that the addition of MA to a suspension of *B. subtilis*, *B. cereus*, *B. megaterium*, or *B. coagulans* spores in G medium (11) inhibits the initiation of germination. In the present work, experiments were undertaken to delineate conditions and characteristics of this inhibition. To determine the relationship between the structure and inhibitory potential of different analogues of anthranilic acid, heat-shocked spores and inhibitor were incubated in nutrient broth at 37 C for 30 min, and the A_{625} was measured. The percent inhibition of germination was calculated. Table 1 shows the effect of different anthranilic acid analogues on the initiation of germination. The compounds were tested at concentrations of 1 or 2.5 mM, except *N*-phenylanthranilic acid which was tested at 10.0 mM. Of 10 compounds examined, only MA, ethyl anthranilate, and *N*-methyl MA inhibited the initiation by 90% or more at a concentration of 1.0 mM or less. On the basis of these results, MA was selected for study of its mode of action.

Variation of inhibition with MA concentration. The degree of inhibition of germination initiation in nutrient broth containing 100 μg of chloramphenicol per ml increased linearly with increasing concentrations of MA (Fig. 1). The temperature of incubation and pH of nutrient broth did not show any significant effect on the degree of inhibition.

Time course of MA inhibition. The inhibition of initiation of spores by MA (1 mM) is shown in Fig. 2. If MA was added any time after the initiation of germination, it took about 10 to 12 min before further reduction of the A_{625} ceased. An explanation for such a lag may be that it takes about 10 to 12 min for MA to penetrate the spores and inhibit the reaction(s) leading to initiation.

Nature of inhibition of alanine-induced germination and its reversal by glucose, fructose, and K^+. The germination of 60015 spores can be initiated by L-alanine alone or by a combination of D-fructose, D-glucose, L-asparagine, and K^+ (21). Previous experiments indicated that the metabolism of these compounds in spores produces at least three common inter-

TABLE 1. EFFECT OF BENZOIC ACID ESTERS AND THEIR ANALOGUES ON THE INITIATION OF GERMINATION OF *BACILLUS SUBTILIS* SPORES



Inhibitor	R_1	R_2	R_3	R_4	Concn (mM)	Inhibition of Germination[a] (%)
Ethylbenzoate	$-C_2H_5$	-H	-H	-H	1.0	23.2
Ethylanthranilate	$-C_2H_5$	$-NH_2$	-H	-H	1.0	90.0
Ethyl-m-aminobenzoate	$-C_2H_5$	-H	$-NH_2$	-H	1.0	<1
					2.5	3.5
Ethyl-p-aminobenzoate	$-C_2H_5$	-H	-H	$-NH_2$	1.0	30.3
Methylbenzoate	$-CH_3$	-H	-H	-H	1.0	21.0
Methylanthranilate	$-CH_3$	$-NH_2$	-H	-H	0.5	85.4
					1.0	98.0
Methyl-p-aminobenzoate	$-CH_3$	-H	-H	$-NH_2$	1.0	33.3
N-methyl methyl anthranilate	$-CH_3$	$-NHCH_3$	-H	-H	1.0	95.0
Anthranilic acid	-H	$-NH_2$	-H	-H	1.0	1.0
					2.0	7.4
N-phenylanthranilic acid	-H	-NH-⬡	-H	-H	10.0	<1

[a] The percent inhibition of germination was calculated after 30 min of incubation at 37 C in nutrient broth.

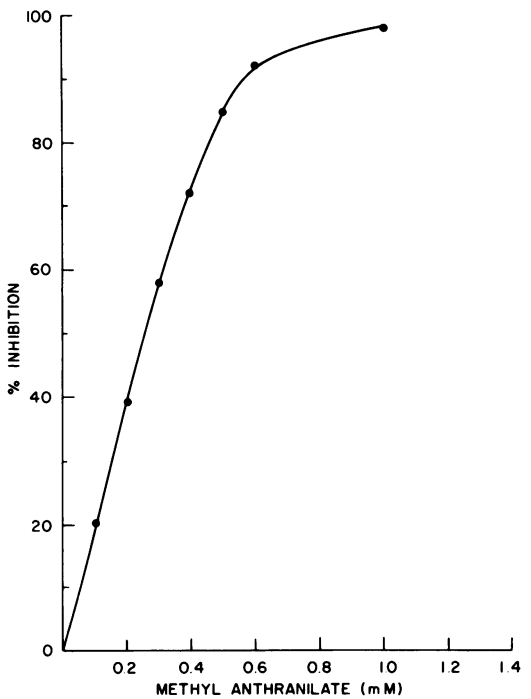


FIG. 1. *Effect of methyl anthranilate concentration on the initiation of germination in 1% nutrient broth plus 100 μg of chloramphenicol per ml. Incubation, 60 min at 37 C. The percent inhibition of germination was calculated on the basis of final yield of spores that germinate in 60 min.*

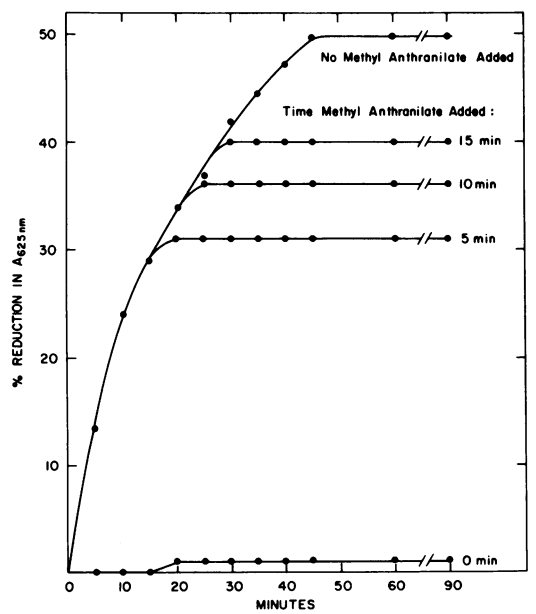


FIG. 2. *Bacillus subtilis spore germination in 1% nutrient broth plus 100 μg of chloramphenicol per ml to which methyl anthranilate (1 mM) was added during initiation. Incubation at 37 C.*

mediates needed for the initiation (14; see Fig. 7 in reference 21). To determine which of the above reactions are inhibited by MA, initiation of 60015 spores by different compounds was measured in Tris-hydrochloride buffer in the presence and the absence of 5.0 mM MA (Fig. 3). The L-alanine initiation was completely inhibited by 5.0 mM MA, but further addition of K^+, D-fructose, and D-glucose allowed initiation. However, L-alanine plus K^+ and either D-glucose or D-fructose did not allow initiation in the presence of MA, and further addition of L-asparagine also had no effect (Fig. 3).

The initiation by a combination of D-glucose, D-fructose, K^+, and L-asparagine was not inhibited by MA. Incubation in MA irreversibly destroyed the ability of spores to be initiated by L-alanine alone. When spores of 60015 were incubated at 37 C for 30 to 180 min with 10 mM MA in 100 mM Tris-hydrochloride (pH 7.4), washed twice with cold 95% ethanol followed by distilled water, and suspended in 5 mM L-alanine and 100 mM Tris-hydrochloride (pH 7.4), initiation of germination did not take place. However, the initiation by the above combination was unaffected in the presence of, or after pretreatment with MA. The spores of 60015 incubated in the absence of MA and then processed as described above did not loose their property of L-alanine initiation.

The Lineweaver-Burk plot (Fig. 4) indicated competitive inhibition of L-alanine initiation of germination (K_m for L-alanine = 2.94×10^{-3} M and K_i for MA = 5.3×10^{-5} M).

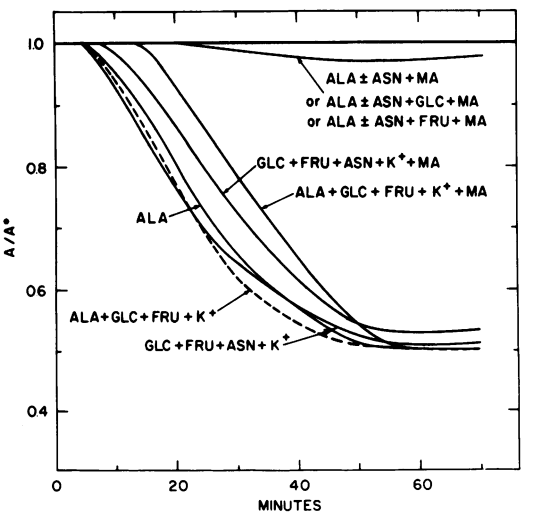


FIG. 3. *Effect of methyl anthranilate (MA; 5 mM) on initiation of germination by L-alanine (ALA) or D-glucose (GLC) plus D-fructose (FRU) plus L-asparagine (ASN) plus K^+ or their various combinations.*

MA inhibition of enzymes converting L-alanine to pyruvate. The conversion of L-alanine to pyruvate could be catalyzed by one of two enzymes: L-alanine dehydrogenase and L-glutamate-pyruvate transaminase (14). Figure 5 shows the inhibition of both of these enzymes from 60015 spores by 0.5 mM MA. The substrate (L-alanine) saturation curve for both enzymes revealed normal Michaelis-Menten kinetics, as indicated by the double-reciprocal plots (Fig. 6 and 7). The Michaelis constants (K_m) with respect to L-alanine were calculated to be 1.53×10^{-3} M for L-alanine dehydrogenase and 0.5×10^{-3} M for L-glutamate pyruvate. The kinetics of inhibition at different MA concentrations indicated that MA was acting as a competitive inhibitor of the enzymes (K_i for L-alanine dehydrogenase = 4.2×10^{-4} M and K_i for L-glutamate dehydrogenase = 6.8×10^{-5} M). Thus, MA appears to have a greater affinity for L-glutamate dehydrogenase than for L-alanine dehydrogenase.

Hermier et al. (7) have shown the presence of two separate dehydrogenases for L-alanine (L-al-

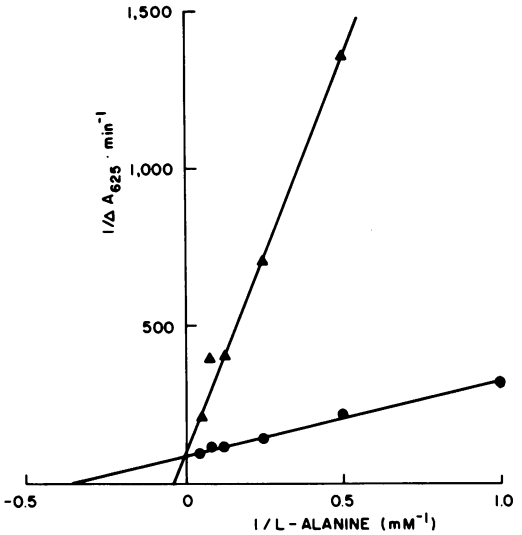


FIG. 4. *Kinetics of inhibition of L-alanine initiation of germination by methyl anthranilate (0.5 mM). Symbols: ●, no addition; and ▲, methyl anthranilate. The K_m for germination was calculated using maximal rate of decrease in A_{625}.*

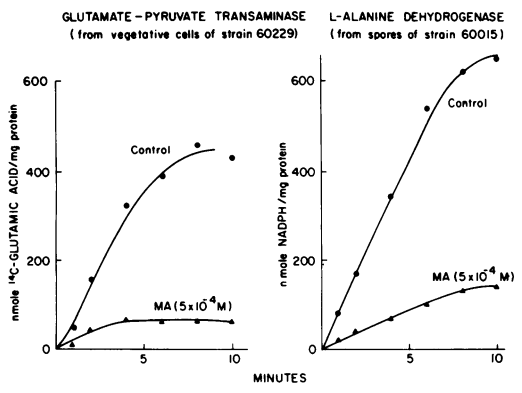


FIG. 5. *Time course of inhibition of L-glutamate-pyruvate transaminase (in strain 60229) and L-alanine dehydrogenase (in strain 60015) by methyl anthranilate (0.5 mM).*

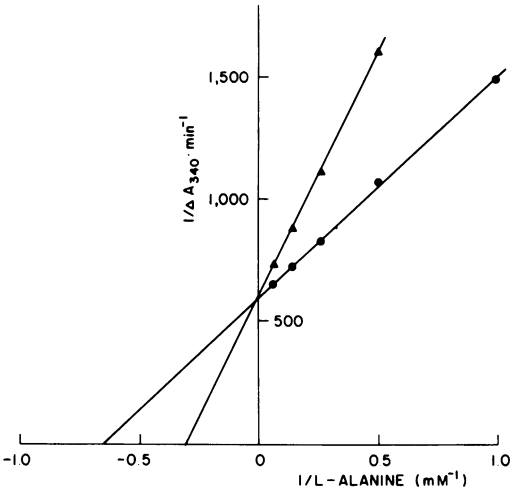


FIG. 6. *Kinetics of inhibition of L-alanine dehydrogenase from spores of Bacillus subtilis (60015) by methyl anthranilate (0.5 mM). Symbols: ●, no addition; and ▲, methyl anthranilate.*

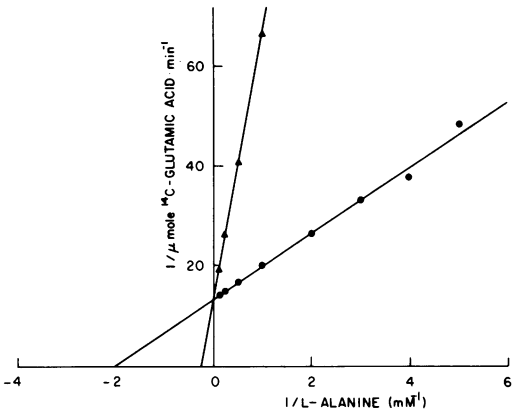


FIG. 7. *Kinetics of inhibition of L-glutamate-pyruvate transaminase from vegetative cells of Bacillus subtilis (60229) by methyl anthranilate. Symbols: ●, no addition; and ▲, methyl anthranilate. The L-glutamate-pyruvate assay reaction mixture was the same as described in Materials and Methods except that [^{14}C]α-ketoglutarate concentration was set at a saturating level (15 mM).*

anine, L-α-aminobutyrate, and L-serine) and L-leucine (L-α-aminobutyrate, L-valine, L-norvaline, L-leucine, L-isoleucine, and L-norleucine) in *B. subtilis* (with a substrate specificity shown in parentheses). Since initiation of germination of 60015 spores in 1% nutrient broth (Difco) (containing 0.3 to 1.0 mM L-alanine, L-serine, L-leucine, L-valine, and L-isoleucine; see reference 18) was inhibited by MA and its analogues (Table 1), several L-amino acid dehydrogenases were examined for their inhibition by MA (Table 2). MA appeared to inhibit specifically the L-amino acid dehydrogenases and L-glutamate pyruvate (and possibly some other enzymes not measured here). It showed no significant effect on the activities of glucose-6-phosphate dehydrogenase, glucose dehydrogenase, or malate dehydrogenase.

DISCUSSION

It is apparent from the data presented in Table 1 that one of the inhibitors of L-alanine-induced initiation of germination must satisfy at least two minimal structural requirements. It must be an arylcarboxylic ester and have an amino group preferably in the *ortho* position to the ester group (presence of an amino group in the *meta* position was totally ineffective, whereas in the *para* position it was slightly effective). The nature of the alkyl group in ester linkage or *N*-methylation of the amino group did not affect the potency of the inhibitor.

The germination of many bacillus spores is initiated by L-alanine, either alone or in combination with inosine (8, 19). In *B. subtilis* 168, L-alanine alone is sufficient to initiate germination. However, L-asparagine or L-glutamine (which by themselves can not initiate the germination of *B. subtilis* 168) can do so in a combination with D-glucose, D-fructose, and K^+ (21). In each case, concurrent with initiation, these compounds are metabolized to produce reduced nicotinamide adenine dinucleotide, fructose-6-phosphate, and some aminated compound (21). Reduced nicotinamide adenine dinucleotide can be produced by D-glucose dehydrogenase, L-alanine dehydrogenase, or any of the several other dehydrogenases present in spores (14). Pyruvate, formed from L-alanine by L-alanine dehydrogenase or L-glutamate-pyruvate transaminase, can be converted to fructose-6-phosphate through gluconeogenesis. Methyl anthranilate inhibits L-alanine dehydrogenase and L-glutamate-pyruvate transaminase and thus prevents L-alanine initiation of germination. However, MA does not inhibit the other reactions needed for initiation (14), i.e., the D-glucose dehydrogenase and amination reaction and the formation of fructose-6-phosphate from D-fructose, because the germination in L-alanine plus MA (or in L-asparagine MA) could be initiated by addition of D-glucose, D-fructose, and K^+.

The metabolism of L-alanine through L-glutamate-pyruvate transaminase reaction may be speculated to play a major role in L-alanine initiation of germination, for the following reasons. Although MA inhibited initiation by L-alanine as well as L-alanine dehydrogenase and L-glutamate-pyruvate transaminase activities, a mutant lacking L-alanine dehydrogenase activity germinated normally in L-alanine alone (2, 14). Furthermore, the K_i for germination and Δ-glutamate-pyruvate transaminase (53 and 68 μM respectively) are in the same range, whereas the K_i for L-alanine dehydrogenase is higher (420 μM; Fig. 4, 6, 7).

TABLE 2. *Specific activities of some dehydrogenases in Bacillus subtilus spores and their inhibition by methyl anthranilate*

Enzyme	EC no.	Sp act (nmol/min/mg of protein)		Inhibition (%)
		No inhibitor	Methyl anthranilate (0.5 mM)	
L-Alanine dehydrogenase[a] (NAD^+)	1.4.1.1	76.2	18.9	75.2
L-α-Aminobutyrate dehydrogenase (NAD^+)		75.4	15.1	80.0
L-Leucine dehydrogenase (NAD^+)		122.4	19.6	84.0
L-Valine dehydrogenase (NAD^+)		90.5	9.1	90.0
Glucose-6-phosphate dehydrogenase ($NADP^+$)	1.1.1.49	137.1	115.4	15.8
Malate dehydrogenase (NAD^+)	1.1.1.37	300.0	223.0	25.7
Glucose dehydrogenase (NAD^+)	1.1.1.47	123.0	107.0	13.0
L-Alanine-α-ketoglutarate transaminase[b]	2.6.1.2	70.0	10.0	85.7

[a] This enzyme was also inhibited by ethyl anthranilate and N-methyl methyl anthranilate. NAD^+, Nicotinamide adenine dinucleotide.

[b] Assayed in the crude extract of log-phase cells of strain 60229 grown in nutrient sporulation agar (3).

If preservatives are to prevent spoilage caused by bacterial spores, the spores must be prevented from germinating. A variety of metabolic inhibitors have been reported in recent years which can inhibit one or more steps in the process of initiation and outgrowth (5, 10, 13, 15–17, 20). However, most of these inhibitors are either very toxic or are needed at a relatively high concentration. In contrast, the analogues of MA are required at a very low concentration and are nontoxic (6, 12). *N*-methyl methyl anthranilate has already been approved as a flavoring agent in a wide range of foods particularly in flour and sugar confectionary (6).

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