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Inhibition of *Bacillus subtilis* Growth and Sporulation by Threonine

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A 1-mg/ml amount of threonine (8.4 mM) inhibited growth and sporulation of *Bacillus subtilis* 168. Inhibition of sporulation was efficiently reversed by valine and less efficiently by pyruvate, arginine, glutamine, and isoleucine. Inhibition of vegetative growth was reversed by aspartate and glutamate as well as by valine, arginine, or glutamine. Cells in minimal growth medium were inhibited only transiently by very high concentrations of threonine, whereas inhibition of sporulation was permanent. Addition of threonine prevented the normal increase in alkaline phosphatase and reduced the production of extracellular protease by about 50%, suggesting that threonine blocked the sporulation process relatively early. 2-Ketobutyrate was able to mimic the effect of threonine on sporulation. Sporulation in a strain selected for resistance to azaleucine was partially resistant. Seventy-five percent of the mutants selected for the ability to grow vegetatively in the presence of high threonine concentrations were found to be simultaneously isoleucine auxotrophs. In at least one of these mutants, the threonine resistance phenotype could not be dissociated from the isoleucine requirement by transformation. This mutation was closely linked to a known *ilvA* mutation (recombination index, 0.16). This strain also had reduced intracellular threonine deaminase activity. These results suggest that threonine inhibits *B. subtilis* by causing valine starvation.

Much information on the regulation of gene expression has been obtained from the study of metabolite inhibition of microbial growth. Probably the most familiar example is the inhibition of *Escherichia coli* K-12 by valine, but the effects of metabolites or metabolite analogs on a wide variety of organisms have been studied (15, 25, 30). Because inhibition generally results from an imbalance of normal regulatory mechanisms (25), it is a useful tool for physiologically and genetically dissecting control mechanisms.

This approach was applied to the study of the biochemical genetics of *Bacillus subtilis*. Metabolites that inhibited growth would be of use in studying metabolic regulation in this species. Furthermore, if a metabolite showed a differential inhibition of growth and sporulation, it might prove useful in studying regulatory factors controlling metabolic changes that occur during bacterial differentiation. In light of previous reports (5, 10) which showed that the nutritional requirements of various auxotrophic strains were different for sporulation than for vegetative growth, the approach seemed possible and promising.

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High concentrations (0.5%) of a variety of amino acids were tested for their inhibitory effect on sporulation. Threonine, valine, and isoleucine were the most inhibitory, while tryptophan, phenylalanine, arginine, and glycine were much less inhibitory. Threonine was chosen for further characterization because it caused a high level of inhibition, and previous work (5) showed that synthesis of threonine was essential for sporulation. The experiments described here show that threonine inhibited both vegetative growth and sporulation, but that there were differences between inhibition of vegetative growth and sporulation.

(A preliminary account of this work has appeared [D. H. Lamb and K. F. Bott, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, I54, p. 163]).

MATERIALS AND METHODS

Bacterial strains. Strains of *B. subtilis* used in this study were all derivatives of 168 and are listed in Table 1. 168T$^+$, a prototrophic transformant of 168, was used for physiological characterization of threonine inhibition to obviate the possibility that inhibition was due to prevention of tryptophan uptake. With the exception of the tryptophan requirement, the two strains are isogenic.

Media. For measurement of sporulation or related

TABLE 1. *Strains used in this study*

Strain	Relevant phenotype	Source (reference)
168T$^+$	Prototroph	Stock strain
168	Trp$^-$	Stock strain
Spc-9	Trp$^+$ Ilv$^+$ Spc^r	Stock strain
Mu8u5u1	Met$^-$ Leu$^-$ Ile$^-$ Str^r	N. Sueoka
CU 457	Trp$^-$ Azl^r	S. Zahler (35)
Thl-12	Trp$^-$ Thl^r	This study

events, the growth medium (GM) and resuspension (MRM) medium of Sterlini and Mandelstam (28) were used. Auxotrophic requirements were added at 50 to 100 μg/ml when needed. Cultures were grown to late exponential phase (an absorbance at 500 nm (A_{500}) of 0.7 to 0.8 as measured by a Bausch and Lomb Spectronic 20 colorimeter) in GM, collected by centrifugation, and resuspended in an equal volume of MRM.

Growth curves were obtained in the minimal medium of Spizizen (27). Cells were grown overnight on minimal plates and inoculated into liquid medium to an A_{500} of ca. 0.05. Samples of 3 ml were withdrawn hourly, and absorbancies were determined. For most experiments, liquid medium was supplemented with $FeCl_3 \cdot 6H_2O$ (6 mg/liter), $CaCl_2 \cdot 2H_2O$ (0.2 g/liter), and $MnSO_4$ (0.047 g/liter). In all cases, cultures occupied 10% of the flask volume and were grown at 37°C with vigorous agitation. Threonine was A grade (allo-free) from Calbiochem. Stock solutions of threonine were neutralized with KOH and sterilized by filtration through 0.45-μm Nalgene filters. All amino acids were of the L-form unless otherwise noted.

Determination of sporulation. The level of sporulation was determined at T_{20} to T_{24} by diluting cells in 2× Davis minimal salts (Difco) and spreading 0.1-ml portions of the appropriate dilutions (in duplicate) on Difco tryptose blood agar base medium (TBAB). T_0 represents the time at which cells were resuspended in MRM. T*n* equals *n* hours after resuspension. Heat resistance was determined by heating capped 0.5-ml samples at 80°C for 10 min. Percent sporulation is expressed as heat-resistant colony-forming units/total colony-forming units × 100.

Mutant selection. Strain 168 (*trpC2*) was grown in Difco antibiotic assay medium 3 (A3) until mid-logarithmic growth phase. Cells were washed once with 2× Davis minimal salts and plated on minimal plates containing 20 mg of threonine per ml and 4 mg of leucine per ml and supplemented with appropriate auxotrophic requirements. Colonies arising on this medium after 24 to 36 h at 37°C (designated Thl^r) were streaked on TBAB medium to give single colonies and one colony was transferred to selective medium. Clones which survived two such purification cycles were used for further study. Solutions of amino acids and salts were sterilized by filtration after neutralization with KOH and added to autoclaved agar solutions asceptically.

Transformation. Procedures used for DNA extraction and transformation have been described previously (36). To test transformants for Thl^r phenotype, recipient cells were transferred to minimal agar supplemented with the appropriate auxotrophic requirements. Colonies with the proper phenotype were transferred onto TBAB plates. After 20 to 24 h at 37°C, colonies were transferred onto threonine-leucine medium and scored after 24 to 48 h at 37°C. The cycle of growth on TBAB plates was required to give reliable discrimination between Thl^s and Thl^r phenotypes. Transformation experiments were carried out at several DNA concentrations to ensure that all genetic linkage data derived from transformation experiments employed limiting DNA concentrations.

Enzyme assays. Alkaline phosphatase was assayed by the method of Grant (13) as described previously (2). Total extracellular protease activity was assayed by using azocoll as substrate by the method of Millet (22). To assay threonine deaminase, L-threonine hydro-lyase (deaminating), EC 4.2.1.16, cells were grown overnight on minimal agar and inoculated at a low initial A_{500} into minimal medium. Cells were allowed to grow until late logarithmic phase. Cells were harvested by centrifugation and washed once in 2× Davis minimal salts and frozen until the next day. Cells were resuspended in 0.1 M tris(hydroxymethyl)aminomethane and 0.1 M EDTA (pH 8) with 20 μg of pyridoxal phosphate per ml and lysed by incubating with 1 mg of lysozyme per ml for 30 to 60 min at 37°C. Lysates were centrifuged at 20°C at 12,000 rpm for 10 min and assayed as described by Umbarger and Brown (31). Specific activity is expressed as micromoles of 2-ketoglutarate per hour per milligram of protein. Protein was measured by the method of Lowry et al. (21).

RESULTS

Inhibition of sporulation. In all experiments involving threonine inhibition of sporulation, threonine was added to 1 mg/ml (8.4 mM) because this concentration was shown to give maximal inhibition of sporulation (Fig. 1). Addition of this concentration of threonine at T_0 allowed less than 5% as much sporulation as in control cultures, but about 60% as many colony-forming units at T_{24} as that of the controls. After several days of incubation, inhibited cultures showed extensive lysis, indicating that cells under sporulation conditions were unable to overcome the inhibition imposed by threonine. Thre-

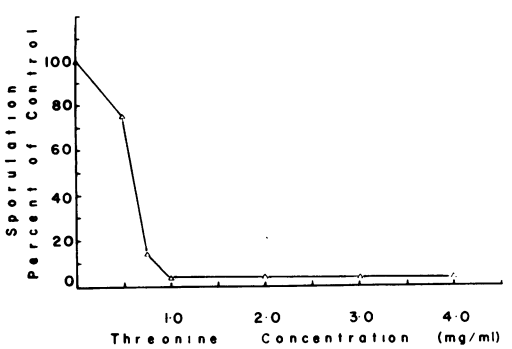

FIG. 1. *Effect of various threonine concentrations on B. subtilis 168T$^+$.*

onine did not induce germination of normally formed spores because addition of 5 mg of threonine per ml to spores caused no decline in heat-resistant colony-forming units (i.e., no germination) over a 5-h incubation period.

It was observed that addition of threonine at the end of logarithmic growth in a medium containing casein hydrolysate did not inhibit sporulation. The common amino acids were therefore tested for their ability to reverse inhibition when added to MRM with threonine, at T_0. When tested at 50 μg/ml, only valine and, to a lesser extent, isoleucine, could reverse inhibition (Table 2). Addition of lysine, methionine, LL-DD-*meso*-diaminopimelate (100 μg/ml), or a combination of methionine and diaminopimelate was unable to reverse inhibition as judged by the absence of phase-bright spores at T_{24}. Amino acids which reversed inhibition of vegetative growth (see below) were tested for their ability to reverse inhibition of sporulation when added at 500 μg/ml. Glutamine and arginine allowed some sporulation at the higher concentration but were not as effective as addition of 50 μg of valine per ml (as estimated by the percentage of phase-bright spores). Aspartate was ineffective at the higher concentration. Glutamine was present at 2 mg/ml as carbon source in MRM.

To characterize more fully the point at which threonine blocked the developmental sequence, two early sporulation-associated biochemical markers were assayed. Addition of threonine at T_0 reduced the production of total extracellular protease activity by about 50% (Fig. 2). Under these same conditions, the normal increase in alkaline phosphatase was not observed (Fig. 2). These data show that threonine blocks sporulation relatively early. Figure 3 shows that after $T_{2.5}$, the sporulation process was no longer sensitive to threonine inhibition. The biochemical basis of this resistance is uncertain.

TABLE 2. *Reversal of threonine inhibition of sporulation by various amino acids*[a]

Conditions[b]	S	V	(S/V) × 100	(S_e/S_c) × 100
Control (6)	6.8×10^8	7.9×10^8	86.1	100
Threonine (6)	4.3×10^6	4.4×10^6	1.0	0.6
Threonine +				
Valine (6)	3.7×10^8	4.1×10^8	90.2	54.4
Isoleucine (3)	5.7×10^7	2.7×10^8	21.1	8.4
Leucine (3)	5.1×10^6	2.4×10^8	2.1	0.8
Methionine (3)	4.7×10^6	1.6×10^8	2.9	0.7

[a] Values under S represent the number of heat-resistant colony-forming units (spores) per milliliter. V represents number of total colony-forming units per milliliter. (S/V) × 100 represents percent sporulation. (S_e/S_c) × 100 represents the number of spores in the experimental cultures expressed as a percentage of the number of spores in the control cultures.

[b] Strain 168T+ was grown in GM and transferred to MRM as described in the text. Threonine and amino acids to be tested were added to MRM at T_0. Threonine was added to 1 mg/ml, and the others were added to 50 μg/ml. The numbers in parentheses are the number of experimental values averaged to give the values shown.

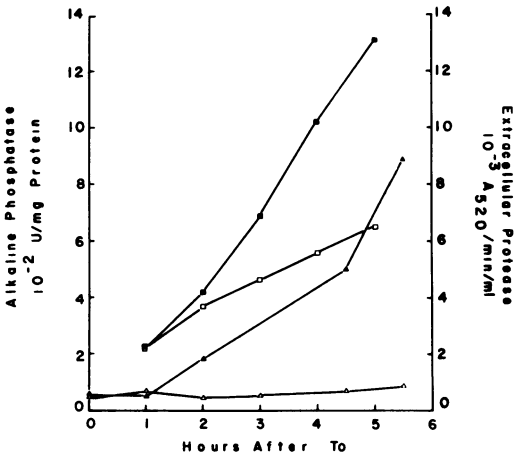

FIG. 2. *Production of alkaline phosphatase and extracellular protease by strain 168T+ in the presence and absence of threonine. Cells were grown in GM medium and resuspended in MRM as described in the text. Squares represent production of extracellular protease. Triangles represent production of alkaline phosphatase. Closed symbols represent control cultures. Open symbols represent cultures containing 1 mg of threonine per ml.*

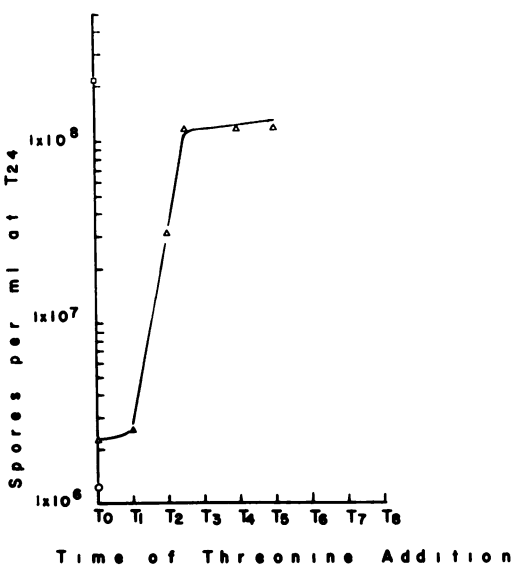

FIG. 3. *Level of sporulation of 168T+ at T_{24} as a function of time of threonine addition. □, Number of spores at T_{24} in control culture; ○, number of spores at T_0 in control culture.*

Inhibition of vegetative growth. The effect of threonine on vegetative growth was studied to determine whether there were any differences between inhibition of vegetative growth and sporulation. Increasing the threonine concentration of a minimal glucose-salts medium increased the lag phase but did not prevent eventual growth. The extent by which the lag phase was increased was dependent on the threonine concentration up to ca. 100 μg/ml (Fig. 4), but even on plates containing 20 mg of threonine per ml, cells showed good growth after incubation overnight at 37°C. The length of the lag phase of growth was estimated by the time taken for the first cell doubling to occur in liquid medium. Two hundred micrograms per milliliter was taken as an approximate minimal inhibitory concentration, and the other common amino acids were tested to determine whether any were able to reverse inhibition. Simultaneous addition of 50 μg of valine per ml reversed inhibition as did addition of 50 μg of glutamate, glutamine, aspartate, or arginine per ml (not shown). Addition of methionine, methionine plus diaminopimelate (100 μg/ml), lysine, or any of the other common amino acids was ineffective. To more directly compare inhibition of vegetative growth and sporulation, amino acids that reversed inhibition imposed by 200 μg of threonine per ml were tested for their ability to reverse inhibition imposed by 1 mg of threonine per ml (Fig. 5). All were found to be at least partially effective. Subsequently it was found that addition of 50 μg of aspartate, arginine, glutamate, or glutamine per ml to minimal medium showed a stimulation of growth in the absence of threonine (data not shown). Consequently, the apparent reversal of threonine inhibition by these amino acids probably represents the combined effect of growth stimulation by these four amino acids and growth inhibition by threonine. Addition of 50 μg of valine per ml in the absence of threonine did not stimulate growth.

Examination of the mechanism of inhibition of sporulation. The fact that valine, but not methionine, is able to efficiently reverse inhibition of sporulation suggests that threonine may be disrupting normal branched-chain amino acid biosynthesis. Consequently, experiments were done to examine this possibility. It was found that addition of 1.19 mg of 2-ketobutyrate (8.4 mM) per ml inhibited sporulation to a slightly greater degree than did threonine and that this inhibition was also reversed by valine (Table 3).

Because pyruvate serves as precursor for valine synthesis, pyruvate was tested for its ability to reverse inhibition of sporulation. Addition of

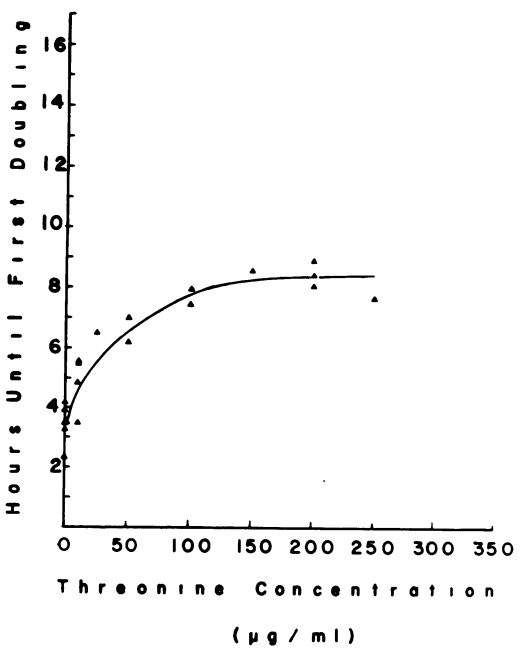

FIG. 4. *Effect of various threonine concentrations on the length of the lag phase of 168T⁺ in minimal medium.*

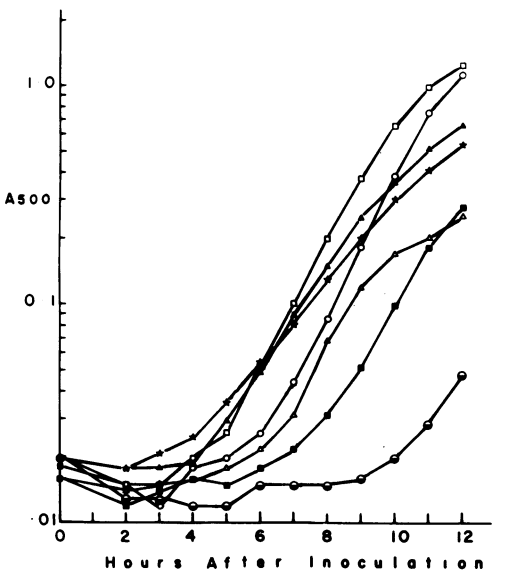

FIG. 5. *Ability of various amino acids to reverse threonine inhibition of vegetative growth of 168T⁺. Threonine was added to 1 mg/ml; other amino acids were added to 50 μg/ml. All amino acids were added before inoculation of cultures.* ○, *No additions;* ◒, *threonine alone;* △, *threonine plus arginine;* ★, *threonine plus aspartate;* ▲, *threonine plus glutamate;* ■, *threonine plus glutamine;* □, *threonine plus valine.*

TABLE 3. *Effect of various compounds on sporulation of 168T+ and CU 457*[a]

Strain/ conditions[b]	S	V	(S/V) × 100	(S_e/S_c) × 100
168T+	2.7×10^8	3.5×10^8	77.1	100
168T+ + 2-keto-butyrate	4.1×10^5	2.2×10^8	0.2	0.2
168T+ + 2-keto-butyrate + valine	1.4×10^8	1.7×10^8	82.4	51.9
168T+	6.1×10^8	5.8×10^8	105.2	100
168T+ + threonine	1.2×10^7	2.5×10^8	4.8	2.0
168T+ + threonine + pyruvate	1.4×10^8	2.9×10^8	48.3	23.0
CU 457	4.8×10^8	4.4×10^8	109.1	100
CU 457 + threonine	1.2×10^8	3.2×10^8	37.5	25.0

[a] See footnote *a* of Table 2. Values represent the average of three or four experiments.

[b] Compounds to be tested were added to MRM at T_0 at the following concentrations: 2-ketobutyrate (8.4 mM) 1.19 mg/ml; valine, 50 μg/ml; threonine, 1 mg/ml. Pyruvate was at T_0 and at hourly intervals through T_3 (see text).

300 μg of pyruvate per ml at T_0 and at hourly intervals through T_3 partially reversed threonine inhibition. Addition of 300 μg of pyruvate per ml at T_0 alone was ineffective. The requirement for such high levels of pyruvate might be at least partially explained by the fact that other cellular processes (e.g., the trichloroacetic acid cycle) would be competing with valine biosynthesis for pyruvate. Sporulation in a strain of *B. subtilis* selected for resistance to the leucine analog azaleucine (strain CU 457) and showing elevated levels of acetohydroxyacid synthetase as well as the leucine biosynthetic enzymes was partially resistant.

Genetic studies. The above observations are consistent with a working hypothesis of threonine inhibition in which exogenously added threonine is broken down to 2-ketobutyrate by threonine deaminase. This large amount of 2-ketobutyrate would then restrict the flow of carbon into valine. This hypothesis predicts that selecting for threonine-resistant variants in the presence of isoleucine should yield threonine deaminase-less mutants. High concentrations of threonine inhibit vegetative growth only transiently, making selection of vegetatively resistant strains inconvenient. However, it was found serendipitously that simultaneous addition of high concentrations of leucine potentiated threonine inhibition. No other amino acid showed this potentiation nor did leucine alone inhibit growth. Also, spotting one drop of a 10-mg/ml valine solution on plates containing threonine and leucine allowed a zone of growth. Variants were selected for the ability to grow in medium containing threonine and leucine (Thl^r) in the presence of growth factor quantities of isoleucine and tryptophan as described in Materials and Methods. Of 56 resistant colonies picked at random, 42 (75%) were found to be auxotrophic for isoleucine. One of these mutants, Thl-12, was chosen for further study. The isoleucine requirement of Thl-12 was inseparable from resistance to threonine-leucine by transformation. All *ile*+ transformants (146/146) lost the resistant phenotype. The isoleucine requirement was closely linked (Table 4) to a known *ilvA* mutation (recombination index, 0.16). This shows that the isoleucine requirement of Thl-12 is due to a mutation in, or at least near, the structural gene for threonine deaminase. Assay of cell extracts of 168 and Thl-12 (Table 5) showed that Thl-12 had reduced threonine deaminase activity. These results strongly suggest that threonine per se is not the compound responsible for growth inhibition and that a functional threonine deaminase is required for conversion of threonine to the inhibitory compound(s). This is supported by the results presented in Table 3 that show that 2-ketobutyrate can mimic threonine inhibition.

DISCUSSION

Many reports have appeared which show changes in the levels of certain enzymes during differentiation of various *Bacillus* species (1, 7, 9, 19, 34); however, the molecular factors controlling these changes and their role in sporulation are unknown. Some amino acid analogs can differentially affect growth and sporulation (20, 24). It seems, therefore, that the elucidation of the basis of the differential action of these ana-

TABLE 4. *Linkage of Ile⁻ phenotypes of Mu8u5ul and Thl-12 by transformation*

Recipient strain	Donor strain	Transformants/ml		*ile*+/ *leu*+	RI[a]
		ile+	*leu*+		
Mu8u5ul	Spc 9	1.0×10^3	1.43×10^3	0.70	0.16
Mu8u5ul	Thl-12	1.2×10^2	1.06×10^3	0.11	

[a] RI, Recombination index as defined by Ephrati-Elizur *et al.* (8).

TABLE 5. *Specific activities of threonine deaminase from 168 and Thl-12*[a]

Strain	Sp act
168	3.04
Thl-12	0.43
168 + Thl-12[b]	1.49

[a] Crude extracts were prepared and assayed for threonine deaminase activity as described in the text.

[b] Equal volumes of cells harvested from cultures at approximately equal optical densities were mixed before cell lysis.

logs and similarly acting compounds might give a clearer understanding of the factors which control changes in enzyme synthesis during sporulation. This report describes experiments designed to characterize threonine inhibition of vegetative growth and sporulation.

We have observed that inhibition of sporulation can be efficiently reversed by simultaneous addition of valine and somewhat less efficiently reversed by pyruvate, isoleucine, glutamate, or arginine. Inhibition of vegetative growth could be reversed by glutamate or aspartate, as well as by valine, glutamine, or arginine (pyruvate was not tested for its ability to reverse vegetative inhibition). Addition of aspartate or glutamate was not observed to reverse inhibition of sporulation. Glutamine and arginine appeared to be less effective in reversing sporulation inhibition than inhibition of vegetative growth. The apparent reversal of growth inhibition by glutamate, aspartate, glutamine, and arginine probably represents a nonspecific stimulation of growth rather than a specific reversal of threonine inhibition. We have also observed that cells in MRM were unable to overcome inhibition, but that no concentration of threonine tested was able to permanently inhibit vegetative growth. Nevertheless, we propose that threonine inhibits growing and sporulating cells by the same mechanism. This speculation is supported by the fact that valine could reverse inhibition of both vegetative growth and sporulation and that results from physiological experiments on sporulating cells correctly predicted the occurrence of resistant variants deficient in threonine deaminase when selection was applied against vegetatively growing cells. The differences noted above are thought to arise from metabolic differences between growing and sporulating cells. For example, vegetatively growing cells would be expected to have higher intracellular concentrations of pyruvate than do sporulating cells (9). Because pyruvate serves as a precursor of valine, this lowered intracellular pyruvate concentration might be expected to contribute to increased sensitivity to threonine. These differences are probably not due to sporulation-specific metabolism, but to changes brought about by the poor carbon source used to induce sporulation. It is known that differences in carbon source can affect antimetabolite sensitivity in a variety of microorganisms (3, 4, 16). This conclusion is supported by the fact that the extent of growth inhibition by threonine was less if threonine was added to cells growing exponentially than if threonine was added to liquid medium at the same time as an inoculum taken from a plate incubated overnight at 37°C and, presumably, in stationary phase (data not shown).

Threonine inhibition of vegetative growth was first observed by Teas (29), who reported that it could be reversed by glutamate, aspartate, or arginine. Vapnek and Greer (32) have reported that a mutation (*sprA*) which results in elevated levels of homoserine dehydrogenase (EC 1.1.1.3), homoserine kinase (EC 2.7.1.39), and threonine synthetase (EC 4.2.99.2) conferred sensitivity of vegetative growth to low concentrations of threonine (2 to 25 μg/ml) and that this inhibition could be reversed by valine. They also reported that methionine was able to reverse threonine inhibition and ascribed inhibition to feedback inhibition of homoserine dehydrogenase (26, 33). We were not able to confirm this finding because we have not observed that methionine reversed inhibition of either sporulation or vegetative growth. The reason for this discrepancy is unclear; however, the following observations argue against the idea that feedback inhibition of homoserine dehydrogenase is responsible for inhibition by threonine. (i) Valine, but not methionine, reverses inhibition; (ii) if threonine were inhibited by feedback inhibition of homoserine dehydrogenase, mutants with lowered amounts of threonine deaminase would be expected to be more, not less, sensitive to threonine as we have observed, due to a lessened ability to catabolize threonine to 2-ketobutyrate; (iii) homoserine dehydrogenase in *B. subtilis* 168, when assayed in the forward direction, is only weakly inhibited by addition of threonine (J. Yeggy and D. P. Stahly, personal communication); (iv) earlier work by Doering and Bott (5) showed that a methionine auxotroph resuspended in MRM in the absence of methionine still showed ca. 60% sporulation. Consequently, one would not expect that starvation of cells for methionine would result in as severe an inhibition (of sporulation) as that imposed by threonine. By contrast, withholding valine from a valine auxotroph resuspended in MRM almost completely prevented the appearance of phase-bright spores (our unpublished data).

The fact that valine efficiently reversed inhibition of growth and sporulation suggests that threonine may be disrupting normal branched-chain amino acid biosynthesis. A mutant selected for resistance to growth inhibition was found to have reduced levels of threonine deaminase. This strongly suggests that threonine, per se, is not the inhibitory compound but that threonine is broken down, via threonine deaminase, to a compound(s) that antagonizes valine synthesis. This conclusion is supported by the observation that 2-ketobutyrate can mimic threonine inhibition. 2-Ketobutyrate could antagonize valine synthesis by one of several possible mechanisms. The amination product of 2-keto-

butyrate, 2-aminobutyrate, has been shown to block valine synthesis in *E. coli* (11) by inhibiting acetohydroxy acid synthetase [acetolactate pyruvate-lyase (carboxylating), EC 4.1.3.18] and valyl-tRNA synthetase [L-valine: $tRNA^{Val}$ ligase (AMP-forming), EC 6.1.1.9]. 2-Ketobutyrate has also been shown to act as a valine antagonist in *B. anthracis* (12). It is possible that 2-ketobutyrate (and possibly 2-aminobutyrate) are acting in a similar manner in *B. subtilis*. Aminobutyric acid has been shown to inhibit growth of *B. subtilis* (29), but the mechanism of this inhibition is uncertain. Alternatively, 2-ketobutyrate might be expected to compete with pyruvate for the active site of acetohydroxyacid synthetase. A large intracellular concentration of 2-ketobutyrate could thus channel carbon into isoleucine synthesis and away from valine synthesis. Cells resuspended in MRM would be expected to have low levels of branched-chain amino acid biosynthetic enzymes and diminishing pyruvate levels and so be particularly susceptible to this type of substrate competition inhibition. The partial resistance of strain CU 457 and the ability of pyruvate to partially reverse threonine inhibition are also consistent with the second model. It should be noted that metabolite inhibition can result from complex, and unexpected, mechanisms (17). Consequently, results from experiments designed to explain the mechanisms of metabolite inhibitions must be interpreted with care.

The results presented suggest that threonine inhibits growth and sporulation by inhibition of valine synthesis. This inhibition is similar in some respects to the feedforward inhibition suggested as a possible mechanism of growth inhibition of *Pseudomonas putida* by branched-chain amino acids (4) in that a biochemical step subsequent to the added metabolite is inhibited. The mechanism by which glutamate, aspartate, arginine, and glutamine can reverse inhibition appears to be a general stimulation of growth rather than a specific reversal of threonine inhibition. The mechanism of this stimulation was not determined; however, it may be due to a sparing of pyruvate derived from glucose (with a concomitant rise in intracellular pyruvate levels). Hatfield and Umbarger (Bacteriol. Proc., p. 113, 1967) have reported that derepression of threonine deaminase by valine limitation in *B. subtilis* requires the presence of aspartate. Also, Holtzclaw and Chapman (14) have reported that some mutants of *B. subtilis* selected for resistance to norvaline appeared to excrete small amounts of glutamate as well as valine. These reports, taken together with those of Teas (29) and ourselves (this report) are also consistant with the possibility that a regulatory interlock (18) exists between aspartate, glutamate, and possibly arginine and glutamine, with branched-chain amino acid biosynthesis in *B. subtilis*.

The transient inhibition of growth, in contradistinction to the permanent inhibition of sporulation, suggests that the response of vegetatively growing cells to threonine is different than the response of cells under sporulation conditions. The partial resistance of strain CU 457 further suggests that this difference cannot be explained entirely in terms of the lowered intracellular pyruvate levels found in sporulating cells. Consequently, the regulation of valine synthesis may be altered during sporulation. In this regard it is interesting that the relative amounts of two valyl-tRNA species have been reported to change during sporulation of *B. subtilis* (6). Also, it has been reported that *B. subtilis* contains two active forms of valyl-tRNA synthetase (23) and that the activities of these two forms are altered during sporulation.

The results presented show that threonine inhibition has the potential to serve as a tool for the study of the biochemical genetics of growth in *B. subtilis*. Threonine inhibition may also be useful in exploring regulation of metabolic changes that occur during sporulation. Even though the differences noted are probably not unique to sporulation-specific metabolism but are due to poor nutrient sources that also induce sporulation, changes of this type cannot be dismissed a priori as unimportant for the sporulation process.

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