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Media Dependence of Commitment in *Bacillus subtilis*

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At some time during sporulation development, cells of *Bacillus subtilis* develop a commitment to continue sporulation even after addition of or dilution into a fresh nutrient. The extent of commitment was measured by the titer of spores produced at the time at which the original culture sporulated maximally. Since newly formed spores of *B. subtilis* soon germinate in the replenished medium, the measurement of their titer, especially of heat-resistant spores, gave low values. This problem was avoided by the germination-delaying effect of methyl anthranilate (1 mM) when added together with the fresh nutrients. In a given culture, the titer of committed cells was then independent of the method by which it was measured, i.e., by the phase-bright, octanol-resistant, or heat-resistant spore titer. The time of commitment depended on the type of nutrient added. Commitment occurred earliest for glucose, later for aspartate, glycerol, or malate, and latest for casein hydrolysate. The rates at which non-metabolizable amino acid analogues or the ^{14}C from an amino acid mixture were taken up by the cells increased toward the end of growth and later declined. This decline occurred slowly and was only weakly correlated with the commitment time of an analogous amino acid.

After a certain time of development, the sporulation process can no longer be interrupted by the addition of fresh nutrients (4, 8, 9). Cells are then committed with respect to the particular replenished medium to complete the sporulation process in spite of the presence of nutrients capable of supporting vegetative growth. In *Bacillus megaterium*, the time (and the morphological stage) at which cells become committed to continue the development of phase-bright and later heat-resistant spores depends on both the medium in which the cells have grown and the type of nutrient with which the medium is replenished (2) (P. H. Cooney and E. Freese, J. Gen. Microbiol., in press). The latest commitment, observed with respect to dilution into very rich media, coincides with the complete engulfment of the prespore by a double membrane (2, 6). In *Bacillus subtilis*, commitment occurs near the end of exponential growth with respect to glucose addition (5) and later (apparently about the time of complete prespore engulfment) with respect to dilution into fresh nutrient sporulation medium (7). With respect to casein hydrolysate addition, commitment to form phase-bright spores was observed during development, but when measured by the formation of heat-resistant spores, a less distinct commitment seemed to occur later, shortly before heat-resistant spores appeared (13).

We show here that the seeming differences of commitment to form phase-bright or heat-resistant spores are caused by the immediate germination of spores before or as soon as they become heat resistant. This germination can be delayed by methyl anthranilate, which has been shown in this laboratory to inhibit germination by alanine (12) and which, at the concentration used, does not inhibit growth or sporulation. Using this technique, commitment with respect to different carbon sources is examined. Since the timing of commitment is correlated with the decrease of transport of glucose in *B. subtilis* (5) and of sucrose and glucose in *B. megaterium* (2), the possible correlation between commitment and the transport of amino acids is also examined.

MATERIALS AND METHODS

Bacteria and media. *B. subtilis* strain 61501 was derived from the transformable *B. subtilis* 168 strain for its ability to grow rapidly on aspartate as sole carbon source; it has a constitutive aspartase (M. Diesterhaft and E. Freese, manuscript in preparation). The nutrient sporulation medium used here contained 8 g of nutrient broth (Difco) per liter, 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (Sigma) adjusted to pH 6.8 with KOH, 10 mM NaCl, 10 mM potassium acetate, 10 mM potassium phosphate, pH 6.8, 25 μg of L-tryptophan per ml, 1 mM $MgCl_2$, 0.7 mM $CaCl_2$, 50 μM

$MnCl_2$, and 1 μM $FeCl_3$. Sodium ions stimulated growth on aspartate (Diesterhaft and Freese, in preparation) and acetate improved sporulation of this mutant so that the sporulation frequency was routinely higher than 80%. All commitment studies presented here were carried out with strain 61501 and completely agree with results obtained with strain 60015 in slightly different media.

For uptake and oxygen consumption studies, *B. subtilis* strain 60015 (also derived from the 168 strain), which requires L-tryptophan and L-methionine for growth, was used. For these studies, the strain was grown in the above nutrient sporulation medium, but NaCl and potassium acetate were replaced with 20 mM sodium DL-α-glycerophosphate (Sigma), which allowed excellent sporulation and induced glycerophosphate dehydrogenase (nicotinamide adenine dinucleotide [NAD] independent).

Cultures were inoculated at an absorbancy at 600 nm (A_{600}) of 0.1 from an overnight plate (14 to 16 h) containing tryptose blood agar base (33 g/liter, Difco). The medium always occupied less than 20% of the volume of the culture flasks, which were shaken at 100 to 125 strokes/min in a reciprocating water bath shaker (New Brunswick Scientific) at 37°C. Growth was followed by measuring A_{600}, using a Gilford spectrophotometer (model 220). Samples were diluted into growth medium to keep actual readings below 0.7.

Commitment and sporulation. The pattern of sporulation in an unsupplemented (control) culture was determined by counting the appearance of phase-bright spores in a Petroff-Hausser chamber at time intervals between 5 and 10 h after the end of exponential growth (t_5 to t_{10}) (Fig. 1). A total of 50 squares was counted for each sample. To determine the effect of methyl anthranilate (Aldrich Chemical Co.) on the measurement of commitment, duplicate samples of a culture were diluted fivefold at t_2 into fresh growth medium + 0.5% casein hydrolysate (Nutritional Biochemicals, vitamin free) with and without 1 mM methyl anthranilate (Fig. 1). Sporulation in the diluted cultures was monitored in the same manner as for the control. The frequency of phase-bright spores in the diluted cultures was multiplied by 5 and then expressed as a percentage of the maximum number of phase-bright particles found in the undiluted culture.

The effect of methylanthranilate on the measurement of commitment was also determined after a 50-fold dilution into fresh growth medium + 0.5% casein hydrolysate. Since such a large dilution makes the use of the Petroff-Hausser chamber impractical (due to the low number of phase-bright particles in a large background of vegetative cells), sporulation was monitored by plate counts of octanol- and heat-resistant particles. Octanol-sensitive particles were killed by mixing 1 ml of culture with 5 μl of octanol and incubating for 5 min at 37°C. The octanol-resistant titer and the titer of spores resistant to heating (15 min at 75°C in dilution medium) were determined on tryptose blood agar base plates after 18 to 24 h of incubation at 37°C. The dilution medium contained 100 mM potassium phosphate, pH 6.8, and 1 mM $MgSO_4 \cdot 7\ H_2O$.

To time commitment with respect to addition of different nutrients, portions (1.8 ml) of the culture were added to prewarmed tubes (25 by 150 mm) containing 0.2 ml of 50 mg of aspartic acid, casein hydrolysate, glucose, glycerol, or malic acid per ml neutralized with KOH when necessary. Methyl anthranilate was always included to give a final concentration of 1 mM. After addition of the test compound and methyl anthranilate, the samples were shaken until $t_{8.5}$ of the unsupplemented culture; the samples and portions of the control culture were then frozen and stored at −20°C for no longer than 3 days. After thawing, the titer of phase-bright spores was determined.

Uptake studies. At different times during growth and sporulation, samples (2 or 4 ml) of the culture were removed, centrifuged for 1 min at room temperature in an Eppendorf microfuge, and resuspended in prewarmed (37°C) buffer composed of 50 mM TES and 30 mM potassium phosphate, pH 6.8, containing 1 mM $MgCl_2$ and 100 μg of chloramphenicol (Sigma) per ml. Portions (2 ml) of the suspension were incubated at 37°C in 25-ml flasks in a water bath shaker for 5 min. Sodium DL-α-glycerophosphate (20 mM, final concentration) was added as an energy source, and uptake was initiated by the addition of the ^{14}C-labeled compounds at the following final concentrations: L-[U-^{14}C]malic acid (Amersham/Searle Corp.), 0.2 mM, 200 nCi/ml, neutralized with KOH; α[3-^{14}C]aminoisobutyric acid (AIBA) (New England Nuclear), 0.1 or 0.2 mM, 200 nCi/ml; and threo-β-hydroxy-DL-[3,4-^{14}C]aspartic acid (THA), 0.2 mM, 70 nCi/ml. THA was prepared as described by Kornguth and Sallach (10). The product (7.5 mCi/mmol) was neutralized with KOH to give a solution containing 28.6 μCi/ml. The amino acid mixture (New England Nuclear) was added to give a final concentration of 200 nCi/ml and of 0.2 mM for each amino acid (with the exception of L-tyrosine, which was approximately 0.03 mM).

The uptake assays were carried out as described previously (2) by filtration on membrane filters (type GN-6, 0.45-μm pore size, Gelman Instrument Co., Ann Arbor, Mich.). The filters were washed three times with 5 ml of the TES-phosphate-$MgCl_2$ buffer and counted in a liquid scintillation spectrometer. Scintillation fluid contained, per liter: 758 ml of toluene; 200 ml of Triton X-100; and 42 ml of Spectrafluor (Amersham/Searle). The initial rate of uptake (nanomoles/A_{600} per minute) was determined from the linear portion of the uptake curves.

Oxygen consumption. The rate of oxygen consumption of strain 60015 was measured at 15-min intervals during growth and sporulation with a Clark oxygen electrode. At A_{600} values of more than 1.3, however, the rate of oxygen consumption became too rapid to measure accurately. Samples were therefore diluted either 1:4 or 1:10 into prewarmed (37°C) supernatants collected from a parallel culture at analogous times (and A_{600} values). Errors introduced by the addition of fresh nutrients in the diluent could be avoided by this procedure.

Oxygen consumption was also measured in starved cells in the presence of 20 mM DL-α-glycerophosphate or 10 mM reduced NAD (NADH). Cells

were starved by centrifugation and incubation two times for 10 min each time in the TES-PO_4-Mg buffer described above. The cells were resuspended to approximately the same A_{600} (0.5) for each determination. The endogenous rate of respiration (in the absence of energy source) was less than 5% of the prestarved level under these conditions. Glycerophosphate was also used as energy source for the uptake studies.

RESULTS

Effect of methyl anthranilate on the measurement of committed cells. Normal growth and sporulation of strain 61501 in a nutrient sporulation medium are shown in Fig. 1. Phase-bright spores were first detected microscopically near t_5, and they reached their maximal titer between t_8 and t_9. If the culture was diluted (fivefold) into fresh nutrient sporulation medium supplemented with casein hydrolysate, most noncommitted cells resumed growth as followed from the absence of a growth lag early in development and a short lag later. When the diluted culture again reached the end of exponential growth, it started another round of sporulation. The committed cells continued their sporulation process and produced phase-bright spores slightly earlier than cells in the undiluted control (Fig. 1). In the absence of a germination inhibitor, the titer of phase-bright spores declined again until the second round of sporulation took place. This germination of spores made it difficult to measure the titer of comitted cells accurately. To avoid this problem, methyl anthranilate (1 mM) was added to inhibit germination (12). In its presence, neither growth nor sporulation was impaired, but the decrease of the phase-bright spore titer was avoided (Fig. 1).

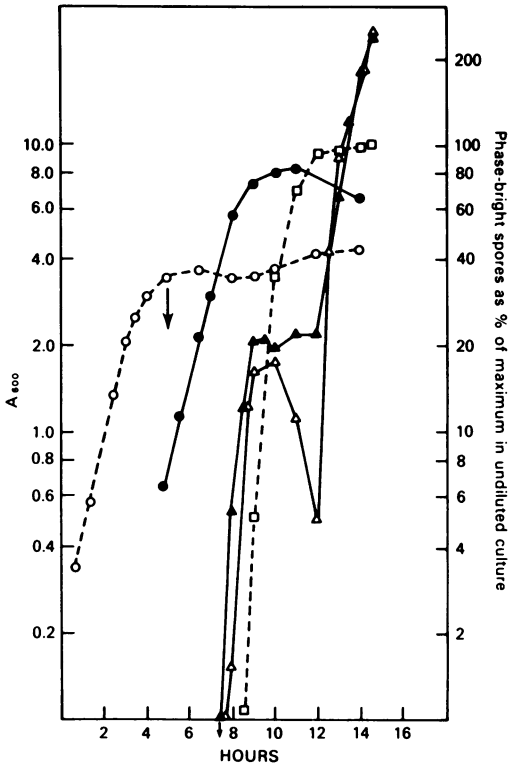

FIG. 1. *Sporulation of committed and noncommitted cells after dilution into fresh medium with and without methylanthranilate. Dashed lines illustrate growth, measured by* A_{600} (○), *and sporulation, monitored by the titer of phase-bright spores* (□), *of strain 61501 in nutrient sporulation medium. The end of exponential growth* (t_0) *occurred at 3 h. At 5 h* (t_2), *indicated by the arrow, cells were diluted fivefold into two flasks containing fresh nutrient sporulation medium supplemented with casein hydrolysate; one flask* (▲) *also contained methylanthranilate, whereas the other* (△) *did not. Both diluted cultures showed the same* A_{600} *increase* (●), *whereas their phase-bright spore titers (triangles, solid line) differed.*

Commitment can also be determined by measuring the increase of octanol- or heat-resistant spores. Since particles with these properties are counted on petri plates, much lower titers can be measured than when phase-bright particles are counted in a Petroff-Hausser chamber. Hence, higher dilutions into fresh medium can be used wihout sacrificing the ability to detect committed cells. This also has the advantage that the second round of sporulation is more delayed because it takes a longer time for the cells to grow up and exhaust the medium. In the absence of methyl anthranilate, the number of octanol-resistant particles detected in a culture diluted 50-fold in nutrient sporulation medium + casein hydrolysate increased and then decreased (Fig. 2), similar to the change of phase-bright particles (Fig. 1). Again, addition of methyl anthranilate to the medium prevented the decrease almost completely. The effect of methyl anthranilate on the appearance of heat-resistant particles was even more dramatic. In the presence of the germination inhibitor, committed cells formed the same titer of heat-resistant as octanol-resistant particles; no decline due to germination was observed. Without methyl anthranilate, however, the maximum number of heat-resistant particles detected at any time was only 25% of that seen in its presence. Therefore, in such diluted cultures either octanol-resistant spores germinate before becoming heat resistant (3), or heat-resistant spores germinate so rapidly that they remain undetected. In either case,

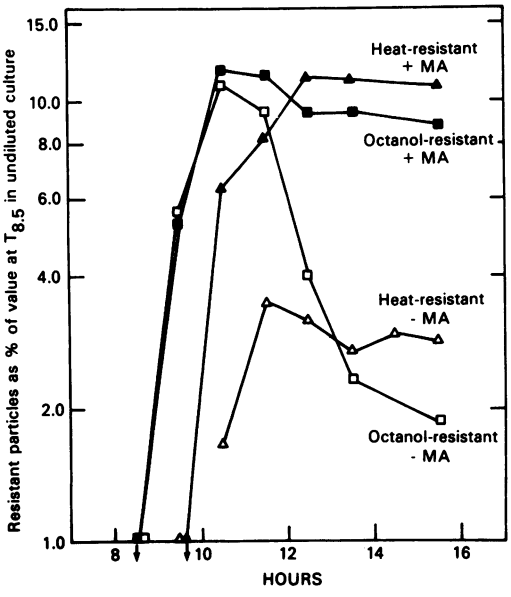

FIG. 2. *Change in the titer of octanol- and heat-resistant spores in a diluted culture in the presence and absence of methyl anthranilate. At t_2, cells were diluted 50-fold into nutrient sporulation medium + casein hydrolysate with (solid symbols) and without (open symbols) 1 mM methyl anthranilate. Octanol (□, ●) and heat-resistant (△, ▲) particles were determined at different times thereafter. The values at times marked by an arrow were less than the baseline value of 1%.*

most committed cells would remain undetected as heat-resistant particles.

Dependence of commitment time on the type of nutrient added. To determine the timing of commitment with respect to different nutrients, aspartic acid, casein hydrolysate, glucose, glycerol, or malic acid was added at a 5-mg/ml final concentration (neutralized by KOH where needed) to samples withdrawn from a culture in nutrient sporulation medium at different times near and after t_0. Strain 61501 was used since, in addition to the other carbon sources, it grew well on aspartate as sole carbon source (Diesterhaft and Freese, in preparation). When any one of the above single compounds was used as sole carbon source in a synthetic medium, the doubling time was approximately 1 h, whereas that using casein hydrolysate was about 0.5 h.

In addition to the above nutrients, methyl anthranilate was also added. Commitment occurred at distinct times for different compounds, earliest with respect to glucose, as already reported earlier (5), next with respect to aspartate, malate, or glycerol, and latest with respect to casein hydrolysate (Fig. 3). The 50% value of the curve characterizing the commitment with respect to casein hydrolysate was at about t_2, much earlier than the 50% value of the curve characterizing the appearance of phase-bright spores in the original culture, which was at about $t_{7.5}$.

Changes in oxygen consumption. The rate of oxygen consumption per A_{600} slowly decreased after the culture (of 60015) had reached an A_{600} value of about 1.5 to 2.0 (Fig. 4A). Since this decrease could reflect either the availability of nutrients or the capacity of the electron transport system to pass electrons to oxygen, portions of a culture were starved (see Materials and Methods) so that their basal rate of oxygen consumption was less than 5% of that in the original culture. The greatly increased rate of oxygen consumption observed upon addition of glycerophosphate (20 mM) served to measure the overall rate of oxygen consumption at a constant supply of energy source. This rate (per A_{600}) decreased similar to that in the original culture, indicating a decrease in the capacity of the cells to pass electrons from glycerophosphate to oxygen. A similar decrease was observed with NADH as energy source, but the

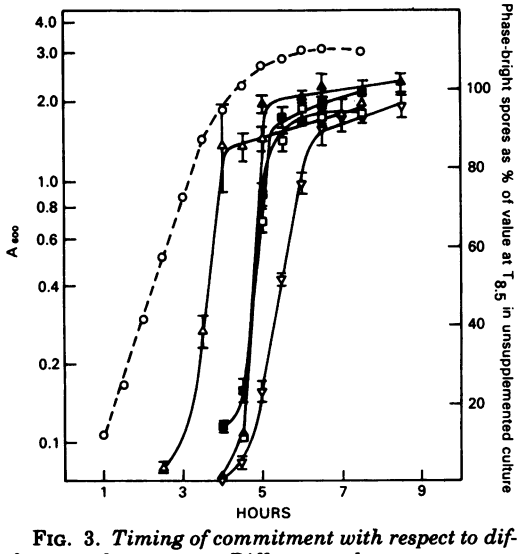

FIG. 3. *Timing of commitment with respect to different carbon sources. Different carbon sources were added (5 mg/ml, final concentration) to samples of a culture of strain 61501 near and after the end of exponential growth. Methyl anthranilate was always present at a concentration of 1 mM. Samples of the cultures and the control were frozen at $t_{8.5}$ of the unsupplemented (control) culture. After thawing, the titer of phase-bright spores was determined and recorded as percentage of the control culture. A_{600} (dashed lines, ○); commitment with respect to glucose (△), glycerol (□), aspartate (▲), malate (■), and casein hydrolysate (▽).*

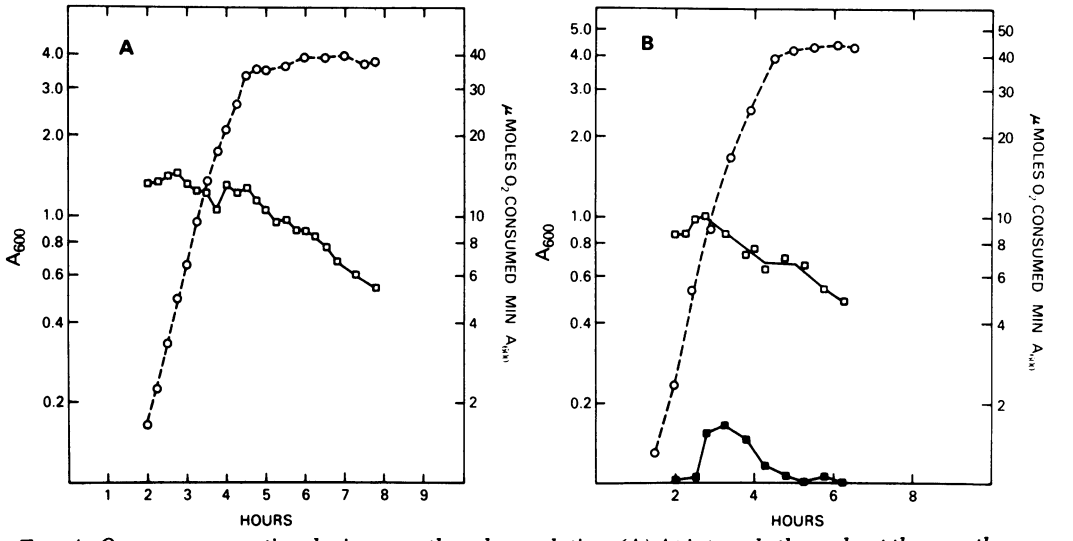

FIG. 4. *Oxygen consumption during growth and sporulation. (A) At intervals throughout the growth curve (dashed lines, ○), the rate of oxygen consumption (□) was measured with a Clark oxygen electrode. When necessary, samples were diluted into analogous supernatants from a parallel culture. (B) Oxygen consumption was also measured throughout growth (dashed lines, ○) in starved cells using either 20 mM* DL-α-*glycerophosphate (□) or 10 mM NADH (■) as energy source.*

rate of NADH oxidation was only about three times the basal level, not enough to allow accurate quantitative measurements (Fig. 4B).

Transport changes during growth and sporulation. To determine whether commitment was correlated with transport changes, the uptakes of the amino acid analogues AIBA and THA, of malic acid; and of an amino acid mixture were measured. The results of competition experiments indicated tha AIBA is mainly an analogue of L-alanine because alanine inhibited AIBA uptake by 99% (when present in a 100-fold excess). AIBA was not appreciably incorporated into protein since only 5% of the total taken up after 5 min was found in trichloroacetic acid-precipitable material. Decarboxylation was also minimal since less than 0.3% of the radioactivity of [1-^{14}C]AIBA had evolved as $^{14}CO_2$ after 5 min. THA is an analogue of aspartic acid and is transported by the aspartate/glutamate active transport system in *B. subtilis* (Diesterhaft and Freese, in preparation). No significant incorporation of this compound into acid-precipitable material was detected when measured for a period of 20 min after addition of ^{14}C-labeled substrate.

Each of the compounds studied showed a similar pattern for the change in the rate of uptake during growth and sporulation. During early exponential growth the rate of uptake was low. The rate increased toward the end of growth and subsequently declined. The time at which the decline occurred was earlier for AIBA and malate than for THA (Fig. 5). The decline occurred slowly and to a similar extent for the three compounds (Fig. 5 and 6). A mixture of amino acids showed a later decline in the rate of uptake than any of the individual compounds studied (Fig. 6). The experiments presented in Fig. 5 and 6 were performed by measurement of uptake at a low substrate concentration (0.1 or 0.2 mM). Other experiments (not shown) demonstrated that a similar pattern of increase and decrease was obtained when a concentration of 10 mM was used (similar to the range of concentrations at which commitment was measured).

The uptake of THA was also measured in a nonsporulating (stage 0) mutant, strain 60622, which does not produce protease or antibiotic activities. The maximum rate of uptake (9.07 nmol/A_{600} per min) occurred at a similar time in the growth curve and subsequently declined at the same rate and to a similar extent as in the sporulating strain 60015. Therefore, the change in uptake rates during growth and the stationary period does not depend on specific sporulation events.

DISCUSSION

Our results demonstrate how two difficulties encountered in the measurement of commitment in *B. subtilis* can be avoided. First, the titer of committed cells can be falsely increased by the contribution of the second round of sporulation, which follows the exponential growth of a stationary culture diluted in fresh medium

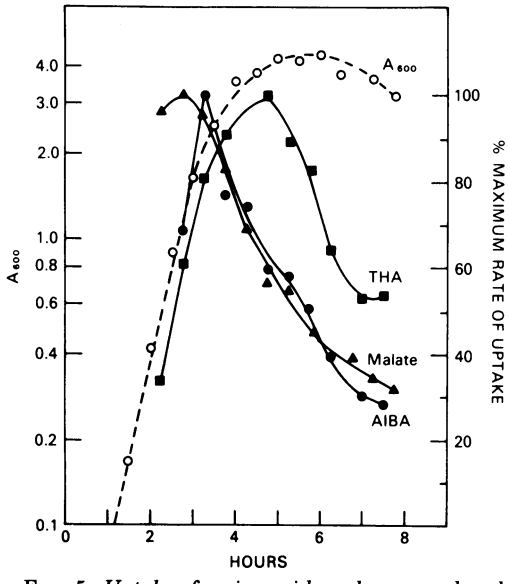

FIG. 5. *Uptake of amino acid analogues and malate during growth and sporulation. At different times during growth and sporulation, the rates of uptake of 0.1 mM threo-β-hydroxy-DL-[3,4-^{14}C]aspartic acid (THA, ■), α-[3-^{14}C]aminoiso-butyric acid (AIBA, ●), and L-[^{14}C]malate (▲) were measured. A_{600} (dashed lines, ○). The maximum rate of uptake for THA was 8.53 nmol/min per A_{600}; for AIBA, 3.17 nmol/min per A_{600}; and for malate, 5.68 nmol/min per A_{600}.*

(Fig. 1). This problem can be minimized if the time course of sporulation identifying the timing of the first and second round of spore appearance is determined and the sporulation of committed cells is measured before the second round begins. The greater the dilution the longer will be the time delay between first- and second-round sporulation. Second, the titer of committed cells can be falsely decreased owing to the germination of spores in the replenished medium (Fig. 1 and 2). If only phase-bright spores were monitored, a time could be chosen before this decrease in titer occurred (Fig. 1). But since the acceptable time period is short (1 h or less) and the timing (determination of t_0) of sporulation varies somewhat, it would be experimentally impractical to rely on such a method. The development of heat-resistant spores could clearly not be determined in this way. The use of the germination inhibitor methylanthranilate prevents this problem. Commitment with respect to a given medium change (e.g., addition of casein hydrolysate) occurs at the same time irrespective of how commitment is measured (i.e., phase-bright or octanol- or heat-resistant spore titer). Methyl anthranilate, which does not interfere with growth or sporulation at the concentration used (1 mM), may also be useful in assessing the sporulation capability of mutants that stop their growth at a low A_{600} or of compounds that might initiate sporulation while the cell titer (or A_{600}) is still low, i.e., before nutrients have been depleted. But note that methyl anthranilate cannot prevent all potential problems with germination. Although it prevents germination by alanine, it does not interfere with germination initiated by a combination of fructose, glucose, and an amino acid (e.g., alanine or aspartate) (12); in fact, spores can germinate and start growth in any rich medium within a few hours in the presence of methyl anthranilate.

Using the mentioned precautions, we have shown that commitment with respect to different carbon sources occurs at different times during the developmental period of *B. subtilis*, in agreement with observations in *B. megaterium* (2, 6). Moreover, there exists a stage of development after which even the dilution into a rich growth medium can no longer reverse sporulation. Results in *B. megaterium* have shown this to be the stage at which the prespore is completely engulfed by a double membrane (2, 6). In *B. subtilis*, the latest time of commit-

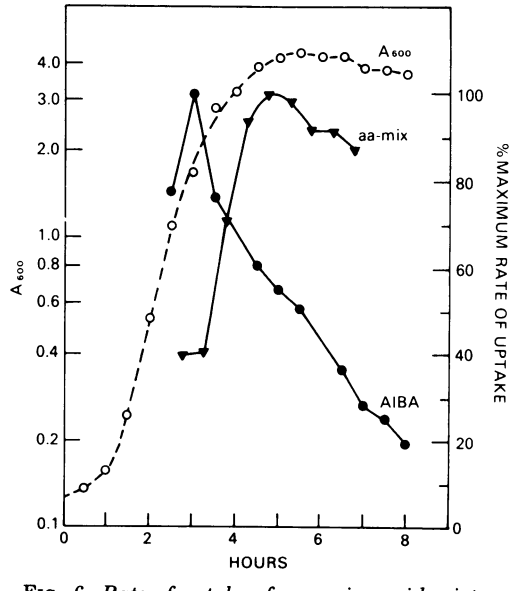

FIG. 6. *Rate of uptake of an amino acid mixture and AIBA. At different times during growth and sporulation, the rates of uptake of an amino acid mixture (0.2 mM of each amino acid except tryptophan; (aa-mix, ▼) and α-[3-^{14}C]aminoisobutyric acid (0.2 mM; AIBA, ●) were measured as described in Materials and Methods. A_{600} (dashed lines, ○). The maximum rate of uptake for AIBA was in this case 4.85 nmol/min per A_{600}.*

ment observed by us was about $t_{2.5}$, and (statistically not evaluated) results of electron microscope examinations suggest that the commitment with respect to a glucose-containing nutrient sporulation medium coincides with the complete engulfment of the prespore (7).

Glucose transport decreases rapidly enough and at the right time that it may alone ensure commitment with respect to glucose in both *B. subtilis* (5) and *B. megaterium* (Cooney and Freese, in press). However, the transport rates of amino acids and malate decreased neither as much nor as rapidly. This decrease does not seem sufficient to explain commitment. More likely, commitment reflects a decrease in the cellular metabolism of these compounds (11).

It is interesting to note that the uptake rate of non-metabolizable amino acids increased toward the end of exponential growth even while the rate of oxygen consumption had already started to decrease in starved cells. Both parameters were measured using glycerophosphate as energy source. Although the rate of oxygen consumption is not a direct measure of the proton-motive force, our results suggest that the increase in amino acid transport does not result from an increase in the rate at which the electron transport system can pass electrons to oxygen. This conclusion disagrees with the interpretation of results obtained with membrane vesicles using a different strain and different media (1). The reaction step limiting the rate of transport in our cells is not known; it could involve, for example, the number of carrier molecules or some change in the membrane affecting transport of many different molecules. The decrease in oxygen consumption cannot be responsible for the decrease of amino acid transport after the end of exponential growth because it starts 2 h earlier than the decrease of THA transport and about 4 h earlier than the decrease in the transport of some amino acids in the amino acid mixture (Fig. 5 and 6). At the time at which the decrease of the amino acid or malate transport begins, the rate of oxygen consumption has already decreased two- to threefold (Fig. 4), but subsequently the amino acid transport declines much more rapidly. Therefore, the decrease in transport seems to result from a more specific effect (inactivation) of individual transport systems.

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