Relationship Between Glucose Utilization and Growth Rate in *Bacillus subtilis*

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The effect of growth rate on the rate of glucose utilization has been examined with a sporogenic and a weakly sporogenic strain of *Bacillus subtilis* by means of the continuous culture technique. Cultures were grown aerobically on a mineral salts medium with glucose as the carbon and energy source. During both nitrogen and L-tryptophan limitation, the rate of glucose consumption (milligrams of glucose per hour per milligrams of cells) decreased when the growth rate was decreased. The coupling between the rate of glucose disappearance and the growth rate was estimated as 76 to 86% during nitrogen limitation and as 60 to 78% during tryptophan limitation. Sporulation had no detectable influence on the coupling.

Little is known about the regulation in bacteria of reactions which supply the biosynthetic machinery of the cell with precursors, energy, and reducing power. It has been established that the activity of isolated enzymes is modulated (2), but it is still not certain that the products of catabolism are supplied only as rapidly as required to meet the biosynthetic demands of the cell.

Literature concerning the regulation of catabolic pathways has been reviewed by Gunsalus and Schuster (5) and by Senez (11). They concluded that energy-yielding metabolism is not controlled by the rate of cell synthesis. Rosenberger and Elsden (10) grew *Streptococcus faecalis* anaerobically on rich medium in a chemostat and found that decreasing the growth rate depressed the growth yields and that it did not affect the rate of glucose utilization.

On the other hand, Ierusalmisky (8) observed that the rate of sucrose consumption in pH-limited continuous cultures of *Azotobacter vine-

landii* decreased when the growth rate was reduced. Neidhardt (9) has demonstrated, with continuous cultures of *Aerobacter aerogenes* grown aerobically on a minimal medium, that the rate of utilization of histidine or of glucose as the sole carbon and energy source was determined by how fast the cells were permitted to grow. A 70% coupling was reported. Neidhardt also presented evidence that the observed coordination of biosynthesis and utilization of histidine resulted from a catabolite repression of histidase at slow growth rates.

It became necessary in our studies of sporo-

lating organisms to determine the rate of glucose utilization by continuous cultures of *Bacillus subtilis*. Our results are further evidence that, given the appropriate growth conditions, some bacteria do restrict the rate of substrate consumption when biosynthesis is restricted.

**MATERIALS AND METHODS**

**Bacterial strains.** The microorganisms used in this investigation were tryptophan auxotrophic mutants of *B. subtilis* strains Marburg, SB 168, and T44. *B. subtilis* T44 was derived from SB 168 and bears a double block in the tryptophan pathway. Continuous cultures of *B. subtilis* SB 168 had a variable sporulation frequency (<0.1 to 8%, depending on growth rate), whereas the proportion of thermoresistant spores in continuous cultures of *B. subtilis* T44 was always <.001%, under the growth conditions employed. Both mutants were kindly supplied by Eugene Nester (Univ. of Washington, Seattle).

**Growth medium.** The minimal salts medium used was similar to that described by Anagnostopoulos and Spizizen (1) except that it contained only 0.15% glucose and was adjusted to pH 7.4 prior to autoclaving. This medium was also supplemented with 10 μg of L-tryptophan per ml. For growth limitation, either the L-tryptophan or the (NH₄)₂SO₄ was reduced to a concentration sufficient to yield a culture with an absorbance of 0.6 or 0.45, respectively, at 525 nm. The stock solutions of the limiting nutrients and glucose were filter-sterilized. The population density was shown to be dependent on the concentration of L-tryptophan or of (NH₄)₂SO₄.

**Batch cultivation.** Batch cultures, used for comparison with chemostat cultures, were grown in a medium containing 2 g of (NH₄)₂SO₄ per liter and 10 mg of tryptophan per liter. A loopful of culture was transferred to 10 ml of medium in a 125-ml
Erlenmeyer flask and incubated on a rotary shaker at 35 C until an absorbance of 0.4 was obtained. This culture was then transferred for at least eight generations of logarithmic growth below an absorbance of 0.6 before sampling. This procedure was carried out to assure that the maximal adjustment of the organisms had been obtained. For comparison with nitrogen-limited chemostats, the samples were taken when the culture absorbance reached 0.45. For comparison with tryptophan-limited cultures, samples were taken when the absorbance reached 0.6.

Inocula for continuous cultures were grown at 35 C on a rotary shaker to an absorbance of 0.4 in a liquid medium of the same composition as that to be used in the chemostat.

Continuous cultivation. The chemostat apparatus employed has been described in detail elsewhere (J. B. DePamphilis, M.S. thesis, Univ. of Wisconsin, Madison, 1968). It consisted of a water-jacketed flask fitted with a top containing a port for medium entry and a sintered-glass dispersion tube, which supplied sterile air to the culture. The entrance to the overflow duct of the flask was shielded by a glass baffle, which helped keep the volume in the chamber constant by preventing the slight amount of foam produced by sparging from leaving the overflow. The temperature of the culture was maintained at 35 ± .01 C by a constant-temperature water pump (Thermomix II, B. Braun, Mulsengen, Germany). The air was passed through a solution of dilute mercuric chloride and sterile water at 35 C before entering the culture vessel. Only 7.5 ± 0.5% of the dissolved oxygen supplied to the culture was utilized, as indicated by an Oxygen Analyzer Beckman Instruments, Inc., Fullerton, Calif. equipped with a polarographic sensor.

The medium was pumped to the growth chamber by a Sigmamotor model AL-2 peristaltic pump or a Beckman Accuo-Flo-Pump (piston type). The flow rate for a given setting varied less than 0.8%. The Beckman pump was preferred for slower flow rates. After delivery to the growth flask, the medium was dispersed in the culture in less than one second by the mixing provided by the sparging of air and the use of a magnetic bar. Changes in pH of the effluent medium were at a maximum of ± 0.5 units.

The inoculum (5% v/v) was transferred to a prewarmed growth flask and allowed to reach a steady state arbitrarily defined by the maintenance of a constant population density and a constant glucose concentration. Cultures were checked periodically for purity by standard microscopic and plating methods. No culture was maintained longer than 2 weeks to avoid significant genetic alteration of the organisms.

Measurement of growth. The specific growth rate of the chemostat culture, μ, is expressed as the ratio of the volume (milliliters) of medium flowing into the flask per hour to the total volume of medium contained within the growth flask (7). Volumes of the continuous culture vessels used varied between 150 and 160 ml. The volume of medium flowing in was assumed to be equal to the amount flowing out and was measured in a graduated cylinder. Thus, μ = milliliters of input per hour/milliliters in growth flask = ω/V. The absorbance of the culture was measured periodically and at each sampling time. At an optical density (OD) of 1.00, 1 liter of culture contained 320 mg of cells (dry weight).

Estimation of glucose utilization. Samples of culture and medium from the chemostat were obtained directly from the overflow duct of the growth flask. When the concentration of glucose in the growth flask had maintained a steady state for at least 12 hr, 1.5- to 3.0-ml samples were collected in test tubes suspended in crushed ice directly below the overflow duct. Each sample was immediately filtered through a membrane filter (0.45 μm pore size; Millipore Corp., Bedford, Mass.) and the filtrate was frozen and stored at −20 C until assays could be performed.

Sampling was continued at 2- to 8-hr intervals for several days. Three samples were collected at each sampling time so that a total of 30 to 60 samples were collected for each growth rate. Two 5-ml samples of the medium in the reservoir were collected at the termination of each experiment for comparison with the effluent medium. At each sampling time, the flow rate and the absorbance of the culture were recorded.

The rate of glucose utilization by batch cultures was determined by collecting and filtering two 5-ml samples just after the final transfer of a batch culture to fresh medium and when that culture had reached absorbancies of 0.45 and 0.6. Glucose concentration was determined by the Glucostat method (Worthington Biochemical Corp., Freehold, N.J.). A commercial standard (Sigma Chemical Co. St. Louis, Mo.) was diluted and used for comparison with unknown samples.

The rate of glucose utilization (G_o) by 1 mg (dry weight) of cells can be calculated as described by Neidhardt (9) with the formula G_o = [ω/V G_in - G_out/C], where 1n 2 = ω/V is substituted in the formula to calculate the rate of glucose utilization in batch culture. G_in is equal to the concentration of glucose in the medium entering the chemostat, and G_out is equal to the concentration in the effluent. C represents the cell density in milligrams (dry weight) per milliliter.

G_o values for a given chemostat agreed within ± 5%. G_o values for different chemostats at the same growth rate agreed within ± 7%. The range in culture absorbance for a single chemostat at a given growth rate was ± 0.01. The maximal range of absorbance for separate chemostats at different growth rates during nitrogen limitation was ± 0.13, whereas during tryptophan limitation it was ± 0.07.

RESULTS

If growth rate does not affect the rate of glucose utilization, then a plot of rate of substrate utilization, G_u, as a function of growth rate, would yield a line parallel to the abscissa. Data which described a straight line that extrapolated to zero would indicate a complete coupling of the rate of substrate utilization to the rate of growth. Figures 1 and 2 illustrate the results obtained when continuous cultures of _B. subtilis_...
T44 were maintained during limited growth. The value for rate of glucose utilization by batch cultures of the organisms is considered here as the value for a culture in completely balanced growth, at the maximal growth rate, in medium containing excess tryptophan and (NH₄)₂SO₄. There is a direct relationship between the rate of glucose utilization and the growth rate in both strains of B. subtilis during nitrogen limitation (see Table 1).

It was necessary to show that the observed relationship was not caused by a special effect of nitrogen limitation on glucose consumption. Consequently, the same strains were employed to study the effect of L-tryptophan-limited growth on the rate of glucose utilization (Fig. 2).

The results obtained with both types of limitation demonstrate a coupling of the rates of growth and glucose consumption. The coupling, however, is not absolute since the rate of substrate utilization as growth rate decreases does not extrapolate to zero.

The growth yield constants, Y(glucose), calculated from these experiments afford a similar indication of the degree of coupling. Y(glucose) values for B. subtilis SB 168 are presented, as an example, in Table 1. A nonproportional decrease in growth yield with decreasing growth rate was

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**Fig. 1.** Effect of growth rate on the rate of glucose utilization by B. subtilis T44 during (NH₄)₂SO₄-limited growth. The line drawn parallel to the abscissa indicates the results expected for complete uncoupling of glucose utilization and growth rate. The results expected if the two processes were completely coupled are indicated by the line which extrapolates to the origin. Symbols: ▲, chemostat cultures; ▼, batch culture.

**Fig. 2.** Effect of growth rate on the rate of glucose utilization by B. subtilis T44 during L-tryptophan-limited growth. See Fig. 1 for explanation.

**Table 1.** Growth yield constants for B. subtilis SB 168 during continuous culture

<table>
<thead>
<tr>
<th>Tryptophan limitation (μ)</th>
<th>Y(glucose)</th>
<th>(NH₄)₂SO₄ limitation (μ)</th>
<th>Y(glucose)</th>
</tr>
</thead>
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<tr>
<td>0.13</td>
<td>39.4</td>
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<td>70.2</td>
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</tr>
<tr>
<td>0.76</td>
<td>100.8</td>
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</table>

*a* Specific growth rate constant, equal to ω/V for continuous culture.

*b* Expressed as milligrams of cells per millimole of glucose.

*c* Batch culture.
observed, indicating that whereas there is partial coupling of the rate of glucose utilization to the rate of growth, it is not absolute. The looseness of coupling appears to be more dramatic during tryptophan limitation than during nitrogen-limited growth of *B. subtilis* SB 168.

**DISCUSSION**

Our results indicate that when *B. subtilis* is grown aerobically on a minimal medium, there is partial coupling between the rate of biosynthesis and the steady-state rate of glucose utilization.

Because the cells can never be adjusted to a zero-growth rate, the $G_o$ value for resting cells can only be approximated. It is not clear from the experimental data whether $G_o$ versus $\omega/V$ is a linear function for all values of $\omega/V$. However, if a straight line is drawn through the experimental values in Figures 1 and 2, the coupling can be estimated. For both strains, during nitrogen-limited growth, a $G_o$ value of 0.2 to 0.3 mg of glucose hr$^{-1}$ (mg of cells$)^{-1}$ can be obtained at $\omega/V = 0$ or from 76 to 84% coupling for *B. subtilis* T44 and 78 to 86% for *B. subtilis* SB 168. During tryptophan limitation, the coupling is less and is estimated at from 63 to 70% for strain SB 168 and 60 to 78% for strain T44.

A comparison of $Y$(glucose) values for *B. subtilis* SB 168 reveals approximately a threefold reduction of growth yield over a sixfold decrease in growth rate during tryptophan limitation, whereas during nitrogen-limited growth there is approximately a 1.5-fold decrease in growth yield over a sevenfold decrease in growth rate.

Belaich et al. (3) have shown that the rate of glucose utilization by *Zymomonas mobilis* and a petite strain of *Saccharomyces cerevisiae* is proportional to the glucose concentration at low glucose concentrations. The steady-state glucose concentration in the effluent medium of our cultures was several times higher than the range reported in which the rate of utilization depended on the concentration.

The data presented here are at least superficially contradictory to the demonstrations by Rosenberger and Eldsen (10), Forrest (4), and others that the rate of catabolism is not tightly coupled to the rate of anabolism. These workers examined the coupling of energy production to the rate of energy utilization by employing anaerobic conditions and a nutritionally rich medium so that glucose was used solely as an energy source. Neidhardt (9) has suggested that the requirement for adenosine triphosphate (ATP) during growth on a nutritionally complete medium has necessitated the observed looseness of coupling. Our experiments, on the other hand, required *B. subtilis* to use glucose aerobically as both a carbon and an energy source.

*B. subtilis* behaves similarly to *A. aerogenes* when it is cultured aerobically on a minimal medium (9). Neidhardt (9) found evidence for excreted polysaccharide at slow growth rates and postulated that carbon assimilation or the formation of inactive byproducts could account for the 30% uncoupling he observed. The 70% coupling he reported for *A. aerogenes* during growth on arginine- or sulfate-limiting medium is less than we have found with *B. subtilis*, especially during nitrogen limitation. We have made no attempt to analyze the growth medium for by-product accumulation or the cells for storage products. It should be pointed out, however, that *B. subtilis*, unlike many other *Bacillus* species, does not assimilate carbon as poly-$\beta$-hydroxybutyric acid.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**