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A Role for Fructose 1,6-Diphosphate in the ATPase-Mediated Energy-Spilling Reaction of *Streptococcus bovis*

DANIEL R. BOND[1] AND JAMES B. RUSSELL[1,2]*

Section of Microbiology, Cornell University,[1] and Agricultural Research Service, U.S. Department of Agriculture,[2] Ithaca, New York 14853

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The amount of ATP produced by *Streptococcus bovis* was larger than the amount that could be attributed to growth and maintenance, and even glucose-limited continuous cultures used ATP inefficiently (spilled ATP). Rapid-dilution-rate cultures always spilled more ATP than those growing at slow dilution rates, but rates of ATP spilling could also be enhanced by amino acid deprivation (with only ammonia as a nitrogen source). Energy spilling and intracellular ATP were not correlated, but energy spilling was always greatest when the rate of lactate production was high. The relationship between lactate production and energy spilling was supported by the observation that amino acid deprivation increased lactate production and ATP spilling. The lactate production rate of nongrowing (energy-spilling) *S. bovis* cells was fructose 1,6-diphosphate (FDP) dependent, and previous work showed that the lactate dehydrogenase of *S. bovis* was activated by FDP (M. J. Wolin, Science 146:775–777, 1964). The role of FDP in energy spilling was supported by the observation that the membrane-bound ATPase of *S. bovis* could be stimulated by FDP. FDP decreased the K_m for ATP by as much as fivefold. Other glycolytic intermediates could not stimulate the ATPase of washed membrane preparations, and FDP had no effect on soluble ATPase activity.

In 1960, Bauchop and Elsden (2) studied the growth of bacteria in batch cultures and concluded that bacterial dry weight was always "proportional to the concentration of energy source in the medium." Individual bacteria sometimes had different yield values for the same energy source, but yield based on the "amount of ATP synthesized" (Y_{ATP}) remained more or less constant. Their value of 10.5 g of cells per mol of ATP was soon treated as a biological constant and was even used as a method for estimating the ATP production in catabolic schemes (9, 17).

By the 1970s, the constancy of Y_{ATP} was being questioned (23). When Stouthamer (21) estimated the amount of ATP that would be needed to produce bacterial biomass, the theoretical Y_{ATP} was approximately threefold higher than the one determined by Bauchop and Elsden (2). Some variation could be explained by maintenance energy (14, 23), but the experiments of Neijssel and Tempest (13) indicated that maintenance energy per se could not account for the very low yields of carbon- and energy-sufficient cultures. The terms "uncoupled growth," "overflow metabolism," "futile cycles," "slip reactions," "wastage," and "energy spilling" were all used to justify variations in yield, but the mechanism of nongrowth energy dissipation was not defined (4, 12, 20, 22, 25).

In many cases, bacterial energetics has been an exercise in feeding and weighing bacteria. The amount of the energy source used for cell carbon was often not accounted for, and ATP production in many cases was merely a theoretical estimate (17). Because pathways and stoichiometries of respiration-dependent ATP production are not constant, substrate-level phosphorylation is the only well-defined mechanism of ATP generation (9, 17). The fermentative bacterium *Streptococcus bovis* utilizes substrate-level phosphorylation only for ATP generation, does not store glycogen or other reserve materials (15), and can use ammonia as the sole source of nitrogen (28).

Nongrowing *S. bovis* cells consumed glucose and hydrolyzed ATP at a very rapid rate (19). Because this nongrowth energy dissipation was stimulated by the protonophore 3,3′,4′,5-tetrachlorosalicylanilide and inhibited by the F_1F_0 ATPase inhibitor *N*,*N*-dicyclohexylcarbodiimide (DCCD), it appeared that ATP was being spilled by the F_1F_0 ATPase and a futile cycle of protons through the cell membrane (6, 19). Energy-limited, slow-dilution-rate continuous cultures spilled little energy (15), but the regulation of energy spilling was not precisely defined. The following experiments sought to identify the intracellular signal of energy spilling.

MATERIALS AND METHODS

Cell growth. *S. bovis* JB1 cells were grown anaerobically at 39°C in medium containing the following (per liter): K_2HPO_4, 292 mg; $(NH_4)_2SO_4$, 480 mg; NaCl, 480 mg; $MgSO_4 \cdot 7H_2O$, 100 mg; $CaCl_2 \cdot 2H_2O$, 64 mg; hemin, 1 mg; pyridoxamine dihydrochloride, 2 mg; riboflavin, 2 mg; thiamine hydrochloride, 2 mg; nicotinamide, 2 mg; calcium pantothenate, 2 mg; lipoic acid, 1 mg; *para*-aminobenzoic acid, 0.1 mg; folic acid, 0.05 mg; biotin, 0.05 mg; coenzyme B_{12}, 0.05 mg; cysteine hydrochloride, 600 mg; and glucose, 4 g. Amino acid-containing medium was supplemented with 3 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.) per liter. The medium was adjusted to pH 6.5. *S. bovis* was also grown in glucose-limited continuous culture (22 mM glucose in the medium reservoir) under O_2-free CO_2 (360-ml culture vessel, 39°C, 0.1 to 1.55 h^{-1} dilution rates). At least 98% of the medium turned over between sampling (approximately 4 culture vessel volumes).

Biomass. Cultures were harvested by centrifugation (10,000 × *g*, 5°C, 5 min), washed in 0.9% NaCl, and stored at −15°C. Cell protein from NaOH-hydrolyzed cells (0.2 M NaOH, 100°C, 15 min) was assayed by the method of Lowry et al. (10). Cellular polysaccharide was assayed by the anthrone method (1).

Fermentation products. Glucose was analyzed by an enzymatic method, with hexokinase and glucose-6-phosphate dehydrogenase (3). Lactate, ethanol, and fermentation acids in cell supernatant samples were analyzed by high-pressure liquid chromatography with a Beckman 334 liquid chromatograph equipped with a model 156 refractive index detector and a Bio-Rad HPX-87H organic acid column. The sample size was 20 μl, the eluant was 0.0065 M H_2SO_4, the flow rate was 0.5 ml/min, and the column temperature was 50°C.

Fermentation rate. Bacterial heat production was measured as described previously (15, 16, 19) with an LKB 2277 bioactivity monitor equipped with semiconducting Peltier elements as thermopiles and gold flow cells. The instrument was calibrated with an internal heat source, and the flow cell used was sterilized

* Corresponding author. Mailing address: Wing Hall, Cornell University, Ithaca, NY 14853. Phone: (607) 255-4508. Fax: (607) 255-3904. Electronic mail address: jbr8@cornell.edu.

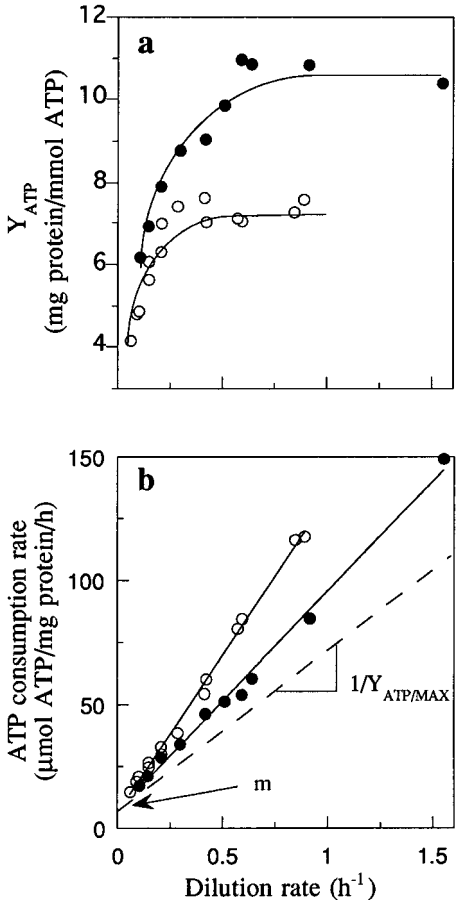

FIG. 1. Effect of dilution rate on the Y_{ATP} (a) and ATP consumption rate (b) of *S. bovis* in glucose-limited continuous cultures with either ammonia (○) or amino acids (●) as a nitrogen source. The dotted line in panel b represents the theoretical ATP consumption rate based on the calculations of Stouthamer (23).

with 95% ethanol and 1 N HCl. Medium was pumped through the flow cell at 40 ml/h, creating an effective flow cell volume of 0.678 ml. The flow cell temperature was set at 39.00°C. Total transit time through the flow cell and back to the culture vessel was approximately 4 min, and because most of this time was spent in a 39°C equilibration coil, the reading from the bioactivity monitor corresponded to the activity of the culture. The lactate production rate was estimated from the enthalpy (ΔH) of glucose conversion to lactate (22 cal [88 J]/mmol of glucose) and the conversion factor 1.16 mW/cal/h.

Intracellular ATP. One milliliter of culture was rapidly mixed with 0.5 ml of ice-cold 14% (vol/vol) perchloric acid, which contained 9 mM EDTA. Samples were extracted on ice for 20 min and centrifuged to remove cell debris (13,000 × *g*, 5 min, 22°C). One milliliter of supernatant was neutralized with 0.5 ml of KOH-$KHCO_3$ (1 M each) and frozen at −15°C. Prior to analysis, samples were thawed, centrifuged to remove potassium perchlorate (13,000 × *g*, 5 min, 22°C), and diluted 50-fold with 40 mM Tris-SO_4 containing 2 mM EDTA, 10 mM $MgCl_2$, and 0.1% bovine serum albumin (pH 7.75). The luciferase reaction was initiated by addition of 100 μl of a purified luciferine-luciferase mix to 100 μl of diluted extract according to the supplier's recommendations (Sigma Chemical Co., St. Louis, Mo.). Light output was immediately measured with a luminometer (model 1250; LKB instruments, Inc., Gaithersburg, Md.) with ATP as a standard (11). All samples were corrected for extracellular adenine nucleotides. The cytoplasm volume (4.3 μl/mg of protein) was estimated from the difference between ^3H_2O and [^{14}C]polyethylene glycol uptake. Cell volume was not affected by medium or growth rate conditions.

Intracellular FDP. *S. bovis* cultures were washed and concentrated 10-fold in 200 mM K-MES buffer (pH 6.7) containing 4 mM $MgCl_2$ and 1 mM dithiothreitol (DTT). Concentrated cell suspensions (approximately 1.6 mg of protein per ml) were incubated at 39°C with glucose. Samples were placed in microcentrifuge tubes containing silicon oil (an equal-parts mixture of Dexter Hysol 550 and 560) layered on top of 100 μl of 14% perchloric acid plus 9 mM EDTA. After centrifugation (13,000 × *g*, 1 min), the cell supernatant and silicon layer were removed. The cell pellet was vortexed and left on ice for 20 min. After centrifugation to remove cell debris (13,000 × *g*, 5 min), 80 μl of this extract was added to 28 μl of 50% K_2CO_3. After centrifugation to remove the precipitate, the samples were analyzed for fructose 1,6-diphosphate (FDP). The FDP assay contained (per ml): 5 U of FDPase, 500 U of phosphoglucose isomerase, 250 U of glucose-6-phosphate dehydrogenase, 0.5 μmol of NADP, 100 μM triethanolamine, and 4 μmol of $MgCl_2$ (pH 8.5). Corrections were made for intracellular glucose-6-phosphate, fructose-6-phosphate, and NADH or NADPH by incubation of the samples in mixtures lacking FDPase.

ATPase activity. Cells were harvested by centrifugation, washed twice (ice-cold 50 mM triethanolamine, 2 mM $MgCl_2$, 1 mM DTT [pH 7.0]), concentrated (approximately 1.6 mg of protein per ml), and placed on ice. Cells were permeabilized by addition of 28 μl of a 50:50 toluene-ethanol solution to 1 ml of concentrated cell suspension. The cells were mixed vigorously on a Vortex mixer for 1 min. Toluene-treated cells were immediately assayed for ATPase activity by monitoring the ADP production. The ADP assay contained the following (per ml): 50 μmol of triethanolamine, 2 μmol of $MgCl_2$, 0.16 μmol of NADH, 0.83 μmol of phosphoenolpyruvate, and 5 U (each) of pyruvate kinase and lactate dehydrogenase (pH 7.0, 22°C). The initial concentration of ATP was never greater than 4 mM. The ATPase activity was 30% higher at 39°C, but assays were typically performed at 22°C. DCCD and iodoacetate were added to whole cells or extracts (described below) to a final concentration of 100 μM and incubated on ice for 1 h before being assayed. Activities were expressed as micromoles of ATP hydrolyzed per milligram of cell protein per hour.

French pressure cell extracts and solubilized enzymes were prepared by a procedure similar to the method of Sturr and Marquis (24) from 2 liters of cells grown in anaerobic medium supplemented with 20 mM DL-threonine. Cultures were harvested (11,000 × *g*, 10 min) during exponential growth. Cells were washed twice with 100 mM potassium phosphate buffer (pH 7.0) and suspended in buffer containing the following (per ml): 20 μmol of sodium maleate, 20 μmol of $MgCl_2$, 0.7 mmol of lactose, 4 mg of lysozyme, and 0.1 mg of mutanolysin (pH 6.5). After 45 min of incubation at 39°C, a mixture of protease inhibitors (40 mM ε-amino-*n*-caproic acid, 6 mM 6-aminobenzamidine, and 1 mM phenylmethylsulfonyl fluoride [Sigma Chemical Co., St. Louis, Mo.]), along with 10 μg (each) of DNase and RNase per ml, was added. Protoplasts were passed repeatedly (more than three times) through a precooled (4°C) French pressure cell at 20,000 lb/in^2. Unbroken cells and large debris were removed by centrifugation (18,000 × *g*, 30 min), and cell membranes were separated from the supernatant by centrifugation (45,000 × *g*, 30 min, 4°C). The membranes were washed three times in 20 mM Tris-HCl (pH 7.0) containing 0.5 mM DTT, 10% glycerol, 2 mM $MgCl_2$, and protease inhibitors (described above). Membrane proteins were solubilized in detergent (<1% Triton X-100, detergent/protein ratio of 5:1; 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 0.5 mM DTT, 20% glycerol, 30 min, 0°C, pH 7.0). Extracted membranes were removed by ultracentrifugation, and detergent-protein complexes were stabilized with 1 mg of azolecithin (crude soybean phospholipids) per ml.

Materials. All chemicals were analytical reagent grade. All enzymes and cofactors were purchased from Sigma. [1,2-^{14}C]polyethylene glycol (60 μCi/μmol) and ^3H_2O (3.6 μCi/μmol) were from Amersham International (Amersham Laboratories, Rockford, Ill.).

Statistics. All of the experiments were performed two or more times, and the measurements were highly reproducible. The coefficient of variation (standard deviation/mean) was always less than 10%.

RESULTS

S. bovis cells grew faster (1.8 versus 0.9 h^{-1}) and more efficiently when they were provided with amino acids. Continuous cultures that were forced to use ammonia (amino acid deprivation) always had lower Y_{ATP} values, but this difference was most dramatic at high dilution rates (Fig. 1a). When the Y_{ATP} values were transformed ($1/Y_{ATP}$ × dilution rate) to determine ATP consumption rates, the maintenance coefficients (intercepts) of ammonia- and amino acid-grown cultures were similar (Fig. 1b). On the basis of the $Y_{ATP/MAX}$ of Stouthamer (21), the cultures using ammonia were always spilling ATP twice as fast as the cultures using amino acids (Fig. 2a). The energy-spilling rate and amounts of intracellular ATP of *S. bovis* cultures growing in continuous culture were not correlated (Fig. 2a and b), but the rate of energy spilling was related to the rate of lactate production (Fig. 2c). The rate of energy spilling was greatest when the lactate production rate was high, and amino acid deprivation increased both energy spilling and lactate production at slow dilution rates.

When exponentially growing cells were washed and resuspended in nitrogen-free medium with 10 mM glucose, lactate was the primary end product and the intracellular FDP con-

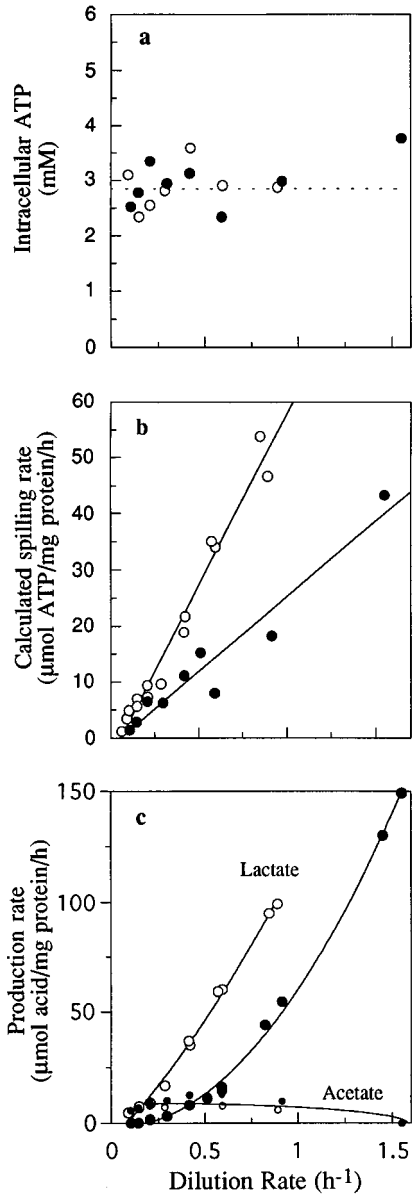

FIG. 2. Effect of dilution rate on intracellular ATP (a), ATP-spilling rate (b), and acid production rate (c) of *S. bovis* in glucose-limited continuous cultures with either ammonia (○) or amino acids (●) as a nitrogen source.

centration was very high (Fig. 3a). There was little decrease in intracellular FDP until most of the glucose was depleted. The relationship between the lactate production rate and FDP was not linear (Fig. 3b), and high concentrations of FDP were only observed at lactate production rates greater than 40 μmol of lactate per mg of protein per h. Resting cells that were permeabilized with toluene hydrolyzed exogenous ATP, and most of this ATP hydrolysis could be prevented by the F_1F_0 ATPase inhibitor DCCD (Fig. 4a). FDP addition increased the rate of ATP hydrolysis, and this effect was mediated by a decrease in the K_m for ATP (Fig. 4b). No ADP formation was detected if only FDP was added, and the FDP-dependent increase in ATP hydrolysis was sensitive to DCCD. The ATPase activity of cells treated with iodoacetate was also stimulated by FDP. ATP hydrolysis was not stimulated by additional sodium, potassium, or magnesium.

Toluene-treated cells still had phosphoglucose isomerase and phosphofructokinase activities and converted fructose-6-phosphate or glucose-6-phosphate to FDP. French pressure cell membrane preparations that had been washed in triethanolamine buffer had ATPase activity but no detectable glycolytic capacity. The membrane-bound ATPase was still sensitive to DCCD, and FDP was the only glycolytic intermediate that caused an increase in activity (Table 1). Soluble ATPase activity was also detected, but this activity was not inhibited by DCCD or stimulated by FDP. The membrane-bound ATPase could be solubilized with Triton X-100, but the activity was labile. Stability was improved by the addition of soybean phospholipid. FDP still stimulated the ATPase activity of detergent-soluble extracts, but the magnitude of the stimulation varied. The K_m of the detergent-soluble extracts for ATP was highly variable (dependent upon the detergent and/or phospholipid used).

DISCUSSION

Previous work indicated that *S. bovis* could spill energy when glucose was in excess, and it appeared that energy spilling might be a threshold phenomenon that only dissipated "excess" ATP (e.g., a large imbalance of catabolic and anabolic

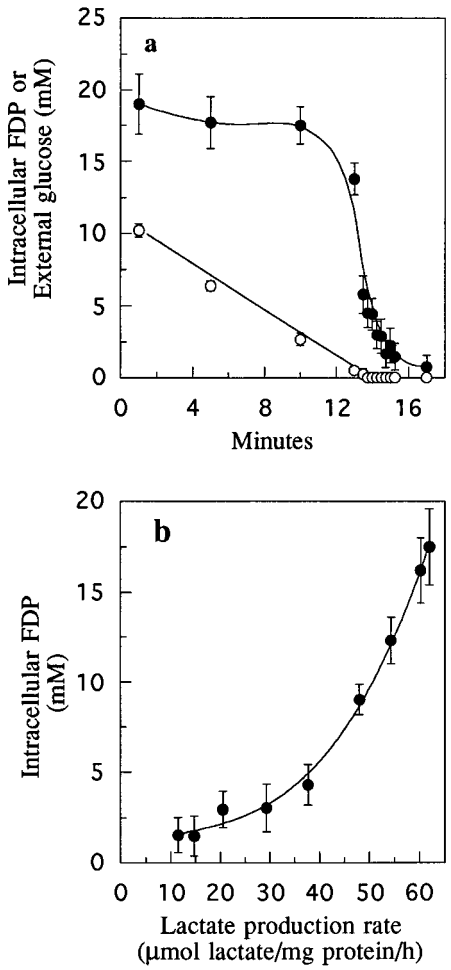

FIG. 3. Relationship between extracellular glucose (○) and intracellular FDP (● [a]) or lactate production rate (● [b]) in nongrowing, energy-spilling *S. bovis* cells.

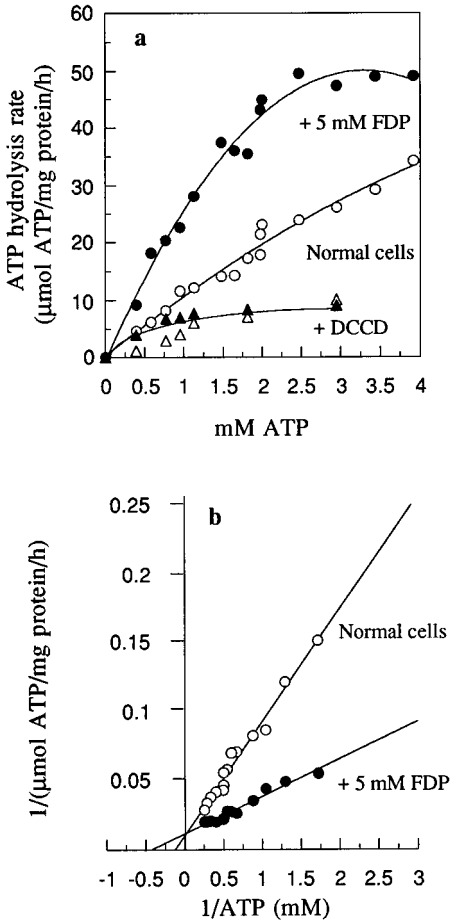

FIG. 4. Effect of FDP (5 mM) and DCCD (100 μM) on ATP hydrolysis by toluene-treated cells of *S. bovis.*

capacity) (16). This assumption was supported by the observation that a glycolytic inhibitor (iodoacetate) decreased the rate of energy spilling (6), but the present work indicated that even glucose-limited continuous cultures (with nitrogen in excess) could spill energy. Because intracellular ATP was not influenced by either nitrogen source or growth rate, it appeared that some other aspect of metabolism (e.g., lactate production) was acting as the regulator of energy spilling.

S. bovis continuous cultures did not spill large amounts of ATP until the lactate production rate was high, and ammonia utilization promoted lactate production and energy spilling. On the basis of this result, there appeared to be a correlation between energy spilling and lactate production. More than 30 years ago, Wolin (27) noted that the lactate dehydrogenase of *S. bovis* required FDP as an allosteric activator, and this regulation is a common feature of lactic acid bacteria (8). Bacteria with FDP-activated lactate dehydrogenases regulate lactate production in a fermentation rate-dependent fashion (18, 26). When the fermentation rate is slow, cells have little FDP, lactate dehydrogenase is not activated, and glucose is converted to acetate, formate, and ethanol. An increase in the fermentation rate causes an increase in FDP, the lactate dehydrogenase is activated, and more of the glucose is then converted to lactate. In effect, lactate production serves as an indicator of intracellular FDP.

When Thomas et al. (26) measured the FDP content of

TABLE 1. Effect of FDP and DCCD on ATPase activity of French pressure extracts of *S. bovis* cells

Addition	ATPase activity (μmol of ATP hydrolyzed/mg of protein/h)	
	Membranes	Cytoplasm
Control (1 mM ATP)	6.2	3.0
+DCCD (100 μM)	1.1	2.9
+FDP (5 mM)	15.3	3.2
+FDP and DCCD	1.6	2.8
+Fructose-6-phosphate (5 mM)[a]	6.5	ND[b]

[a] Glucose-6-phosphate, dihydroxyacetone phosphate, glucose-3-phosphate, and phosphoenolpyruvate gave similar results.
[b] ND, not determined. Fructose-6-phosphate-dependent ATPase (phosphofructokinase) activity interfered with the ATPase assay.

Lactococcus lactis cells, rapid sampling was critical, especially in slow-dilution-rate cultures. Our spectrophotometric method for measuring FDP required dense cell suspensions (>10 optical density units). Such cultures could not be grown continuously or prepared rapidly from normal cell concentrations. However, nongrowing *S. bovis* cells had the same rate of energy spilling as rapidly growing continuous cultures. Rapidly glycolyzing, nongrowing *S. bovis* cells had approximately 20 mM intracellular FDP, and a similar value was reported by Thomas et al. (26) for exponentially growing *L. lactis* cells. As the lactate production rate of *S. bovis* declined, the amount of intracellular FDP decreased, and this result supported the idea that FDP was required for high rates of ATP production.

A direct impact of FDP on membrane-associated ATP turnover was demonstrated with permeabilized cells and cell extracts as follows: (i) FDP stimulated the membrane-bound, DCCD-sensitive ATPase activity of toluene-treated cells and decreased the K_m for ATP, (ii) FDP had no effect on soluble ATPase activity, and (iii) other glycolytic intermediates did not mimic the effect of FDP on the membrane-bound ATPase.

L. lactis has an FDP-activated protein kinase that uses ATP to phosphorylate the phosphotransferase protein, HPr, at serine 46, and this derivative [HPr(ser-P)] activates a sugar phosphate phosphatase that triggers inducer expulsion (29). *S. bovis* has an FDP-activated inducer expulsion mechanism for thiomethylgalactoside (5), but there was little evidence that an FDP-activated protein kinase was stimulating the membrane-bound ATPase of *S. bovis*. The FDP-activated protein kinase is removed from membranes by washing (7), and the substrate of this protein kinase (HPr) is a soluble protein (5). FDP was able to stimulate the membrane-bound ATPase of *S. bovis* even after cytoplasmic components were removed.

It had generally been accepted that the ATPase activity of lactic acid bacteria was regulated by gene expression, proton motive force, and cytoplasmic pH (17). To our knowledge, this is the first report of an FDP-stimulated ATPase, but it should be noted that even this ATPase did not have an obligate requirement for FDP. Previous work indicated that a pulse dose of glucose into a slow-dilution-rate, glucose-limited continuous culture caused an almost immediate increase in glucose consumption, energy spilling, and proton motive force (6). These effects can be explained by glucose-dependent increases in FDP and FDP stimulation of a membrane-bound, proton-pumping ATPase.

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