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Apparent dependence of sporulation on synthesis of highly phosphorylated nucleotides in *Bacillus subtilis*

[adenosine-5′,3′(2′)-bis(triphosphate) synthetase/inhibition by phosphorylated carbon metabolites/temperature-sensitive mutants/electron microscopy/sensor model of initiation of differentiation]

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ABSTRACT ***Bacillus subtilis*** **contains an enzyme that synthesizes the highly phosphorylated nucleotide adenosine 5′,3′(2′)-bis(triphosphate), abbreviated p_3Ap_3. This enzyme can be demonstrated to be present in the cytoplasmic membrane of *B. subtilis* at all stages of growth and development. During vegetative growth its enzymatic activity is inhibited by phosphorylated metabolites of energy-rich carbon sources, as was shown with the aid of conditional sporulation mutants. The finding that a temperature-sensitive sporulation mutant with a mutation in the *spoOF* gene, an early sporulation gene, shows a temperature-sensitive activity of p_3Ap_3-synthetase indicates that p_3Ap_3 plays an important role in sporulation, probably initiation of sporulation.**

Spores are formed by healthy cells facing starvation. This classical description of sporulation by Knaysi (1) obviously implies, among other things, that bacilli starved (for example, for energy sources) can sporulate only if enough energy is stored intracellularly to ensure the highly energy-consuming process of sporulation. Whether poly(β-hydroxybutyrate), which is found in the form of inclusions in spore formers (2, 3) and which is usually completely utilized during sporulation (4), or any other polymer, such as glycogen, etc., serves as energy source after depletion of exogenous energy supply is rather unimportant as far as sporulation is concerned. As long as enough ATP or other energy-rich molecules can be generated [for example, by a functioning citric acid cycle (5)] by utilizing these energy reserves, sporulation takes place.

It is obvious that not only growth but also sporulation depends on the constant availability of energy. Yet, deprivation of external energy supply in the presence of sufficient internal energy sources induces sporulation, which means induction of drastic changes in transcription (for review see ref. 6). Several genes active during vegetative growth are turned off, others active during sporulation are turned on.

Because cells induced to sporulate by extracellular carbon deprivation never faced starvation intracellularly, changes in transcription are unlikely to be induced by the well-known repression–derepression mechanisms controlling gene activity.

We have postulated (7) that a system must exist that is capable of recognizing changes in energy sources outside of a cell. This system should be insensitive to the presence of intracellular energy sources. In a series of experiments we have shown (8–10) that unusual highly phosphorylated nucleotides are synthesized in response to nutrient deprivation, which simultaneously induces sporulation. The correlation of synthesis of adenosine 5′,3′(2′)-bis(triphosphate), p_3Ap_3, and sporulation led us to conclude that this substance may trigger sporulation (11, 12). The enzyme system synthesizing this compound is located in the cytoplasmic membrane. It may create the signal for nutrient insufficiencies in the medium and thus may cause initiation of sporulation.

This assumption depends on the validity of three conditions: (*i*) The p_3Ap_3-synthesizing enzyme must be present at all times in vegetative, stationary phase nonsporulating, and sporulating cells. (*ii*) The enzyme must be sensitive to inhibition by energy sources or metabolites derived from them. (*iii*) Mutations in the gene coding for this enzyme must influence sporulation.

In this communication we intend to show that these conditions indeed exist by using conditional sporulation mutants of *Bacillus subtilis*.

MATERIALS AND METHODS

Bacteria and Growth Conditions. *B. subtilis* strain 60015 and a set of isogenic stage 0 sporulation mutants described previously were used (11). The following mutants blocked at various steps in the catabolism of carbon sources were kindly provided by E. Freese (13): strains 61447 deficient in phosphoglucoisomerase (PGI; EC 5.3.1.9) and phosphofructokinase (PFK; EC 2.7.1.11), 61402 [deficient in NAD-independent glycerol-phosphate dehydrogenase (GlpD)], 61364 [PGI, also deficient in glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and phosphoglucomutase (PGM; EC 2.7.5.1)], and 61106 (glycerol-requiring).

The temperature-sensitive conditional sporulation mutant, strain JH 756b with a mutation in the *spoOF* gene was isolated by J. Hoch. Other mutants of this type were isolated by transforming strain QB123 (*ctrA;* obtained from P. Piggot) with hydroxylamine-treated 60015 DNA and selecting for temperature-sensitive conditional asporogenous cytidine-independent cotransformants (unpublished data).

Growth conditions in semisynthetic yeast extract and in sporulation medium have been described (10, 12). Nutrient sporulation medium buffered with Tris (NSMT) is described by Fortnagel and Freese (14) with the exception that phosphate buffer is replaced by Tris·HCl, 0.5 M, pH 7.0.

Biochemical Methods. All methods concerning labeling, analysis, and quantitative measurements of highly phosphorylated nucleotides have been described in detail (9, 10, 12).

Electron Microscopy. Thin sections of vegetative, sporulating, and stationary-phase nonsporulating cells were obtained by following published procedures (15). A Hitachi H500 electron microscope was used. Cells were fixed with 2.5% (wt/vol) glutaraldehyde in phosphate buffer, stained with uranyl acetate, and osmium tetroxide, embedded in Epon, and cut with an ultramicrotome (LKB).

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Abbreviations: p_3Ap_3-synthetase, adenosine-5′,3′(2′)-bis(triphosphate) synthetase; PGI, phosphoglucoisomerase; PFK, phosphofructokinase; GlpD, glycerol-phosphate dehydrogenase, G6PD, glucose-6-phosphate dehydrogenase; PGM, phosphoglucomutase; NSMT, nutrient sporulation medium buffered with Tris; PTS, phospho*enol*-pyruvate:sugar phosphotransferase system.

Table 1. p_3Ap_3-synthesizing activity during the life cycle of *B. subtilis*

Strain	Medium	Activity of p_3Ap_3-synthetase: Exponential phase Early	Mid	Late	Sporulation phase* T_0	T_1	T_2	T_4
60015 (*spo*$^+$)	Sporulation	+	+	+	+	+	+	+
60015 (*spo*$^+$)	Nonsporulation†	+	+	+	+	+	+	+
JH649 (*spoOF*)	Sporulation	−	−	−	−	−	−	−
JH642 (*spo*$^+$)	Sporulation	+	+	+	+	+	+	+

* T_0, T_1, etc., end of logarithmic phase, 1 hr, 2 hr, etc., after end of logarithmic growth.
† Sporulation suppressed by excess glucose (10 mM).

Electron micrographs of thin sections at ×30,000 magnification were used to screen large numbers of longitudinally sectioned *B. subtilis* cells for the presence of asymmetric septa.

Assay of Adenosine-Bis(triphosphate) Synthetase (p_3Ap_3-Synthetase). The assay of p_3Ap_3-synthetase in membrane vesicles has been described in detail (7).

RESULTS

p_3Ap_3-Synthesizing Activity Is Found in *B. subtilis* Cells at All Times during Growth and Development. If initiation of sporulation requires synthesis of a trigger substance by an enzyme activated when nutrients are removed from the medium, we should expect this enzyme to be a constitutive part of *B. subtilis*, present at all times. This would enable vegetatively growing cells to react immediately to starvation by inducing sporulation.

To support this hypothesis we isolated membrane vesicles from the sporulating strain 60015 at different times of growth and sporulation. The vesicles ability to synthesize p_3Ap_3 was then investigated (7). As is shown in Table 1, p_3Ap_3-synthesizing activity can be detected in strain 60015 in early-, mid-, and late-exponential-phase cells, at the end of growth (T_0), and any time thereafter, regardless of whether the medium favors sporulation at the end of growth or whether sporulation is prevented by excess glucose (Table 1). Similar observations were made with another sporogenous strain of *B. subtilis* (Table 1, JH642). The p_3Ap_3-synthesizing activity did not vary significantly in all experiments using sporogenous strains. On the other hand, the activity cannot be detected in the asporogenous strain JH649 (11) at any time of isolation (Table 1).

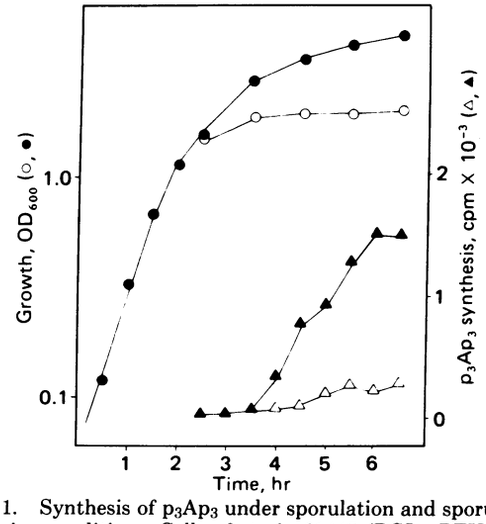

FIG. 1. Synthesis of p_3Ap_3 under sporulation and sporulation-suppressing conditions. Cells of strain 61447 (PGI^-, PFK^-) were grown in NSMT with 0.2% glycerol to $OD_{600} \approx 1.2$ (●). One half of the culture was centrifuged, washed with saline, and resuspended in NSMT containing 11 mM mannose (sporulation-suppressing medium) (○). Synthesis of p_3Ap_3 was measured in the cultures permitting sporulation (▲) and suppressing it (△).

From this we conclude that the p_3Ap_3-synthesizing enzyme (p_3Ap_3-synthetase) is an integral part of *B. subtilis* cells independent of sporulation. Its location in the cytoplasmic membrane underlines its close connection or identity with the postulated "detector" or "sensor" for nutrient deficiencies outside of the bacterial cell.

The enzyme p_3Ap_3-synthetase was always found to be associated with the cytoplasmic membrane. We were able to solubilize this enzyme by the use of the detergent Genapol X100 (12). From preliminary experiments we conclude that two enzymes are involved in synthesis of p_3Ap_3 according to the reaction mechanism proposed previously (7). Because the membrane-bound enzyme missing in the *spoOF* mutant (JH649) is also able to synthesize p_2Ap_2 when ADP (p_2A) is present in the reaction mixture, we conclude that the second enzyme in the synthesis of p_3Ap_3, a pyrophosphoryltransferase adding a pyrophosphate to the hypothetical intermediate adenosine 5′-triphosphate 3′(2′)-phosphate (p_3Ap), is coded for by the *spoOF* gene.

Inhibition of p_3Ap_3-Synthetase by Phosphorylated Metabolites of Carbon Sources. *In vitro* studies (7) indicated that p_3Ap_3-synthetase is inhibited by phosphorylated metabolites of carbon sources. However, *in vivo* studies using mutants blocked in the catabolism of carbon sources, which accumulate phosphorylated metabolic intermediates when grown under the proper conditions (that is, in the simultaneous presence of a utilizable carbon source and a second sugar that is accumulated intracellularly as a phosphorylated metabolite owing to a mutation in its catabolism) should show more clearly that p_3Ap_3-synthetase is inhibited as long as carbon sources can be transported through the cytoplasmic membrane. This transport is mediated by phosphorylation with the aid of the phospho*enol*pyruvate:sugar phosphotransferase system also located in the cytoplasmic membrane. Several mutants isolated by Freese and coworkers (13) grow and sporulate normally in nutrient sporulation medium. However, when a carbon source is added that cannot be utilized because of a genetic block but is rather accumulated as phosphorylated metabolite, sporulation is prevented (13). Freese *et al.* have termed these substances (13) "repressors" of sporulation.

We have analyzed four of these mutants (blocked at different stages in the catabolism of carbon sources) for their ability to synthesize p_3Ap_3 under conditions allowing or preventing sporulation. As can be seen in Fig. 1, mutant 61447, which is deficient in the enzymes PGI and PFK, synthesizes amounts of p_3Ap_3 similar to those of the wild-type strain 60015 when grown in nutrient sporulation medium to OD_{600} of 1.2 in the presence of 0.5 mCi of $H_3{}^{32}PO_4$ per ml and then resuspended in a "sporulation medium" containing only salts, 0.5 mCi of $H_3{}^{32}PO_4$ per ml, and amino acids as described (12). (One Ci = 3.7×10^{10} becquerels). However, when this medium contains mannose, leading to accumulation of fructose 6-phosphate (13), no p_3Ap_3 synthesis ensues (see also Table 2). Sporulation is also

Table 2. Conditions for accumulation of metabolites of carbon sources, for synthesis of highly phosphorylated nucleotides, and for sporulation

Strain	Deficient in	Addition to NSMT	Accumulation of	p_3Ap_3 synthesis	Sporulation
61447	PGI, PFK	Glycerol	—	+	+
		Mannose	Fructose-6-*P*	−	−
61402	GlpD	Glycerol + malate	—	+	+
		Glycerol	Glycerol-*P*	−	−
61364	PGI, G6PD, PGM	None	—	+	+
		Mannose	—	+	+
		Glucose	Glucose-6-*P*	−	−
61106	Glycerol metabolism	Malate*	—	+	+
		Glycerol	Glycerol-*P*	−	−

* For conditions of growth see text.

inhibited. Apparently, accumulation of fructose 6-phosphate prevents p_3Ap_3 synthesis. Lack of synthesis of p_3Ap_3 in turn appears to prevent sporulation.

Another mutant, strain 61402, deficient in GlpD, grows and sporulates normally in nutrient sporulation medium containing malate and low amounts of glycerol. Synthesis of p_3Ap_3 is observed (Fig. 2). However, when malate is removed from the medium, neither sporulation nor p_3Ap_3 synthesis can be detected (Table 2). Glycerol phosphate is accumulated under these conditions (13). This accumulation seems to prevent p_3Ap_3 synthesis, and therefore sporulation is apparently not triggered.

Strain 61346, which is deficient in the enzymes PGI, G6PD, and PGM, synthesizes p_3Ap_3 and sporulates normally (13) when grown in nutrient sporulation medium (Fig. 3). The same is true when 0.2% mannose is added to this medium. However, when glucose (0.2%) is added to nutrient sporulation medium, glucose 6-phosphate is accumulated and sporulation is prevented (13). Synthesis of p_3Ap_3 is also not observed under these conditions (see Fig. 3 and Table 2).

A fourth mutant, strain 61106 (Fig. 4), requires glycerol for growth. If this mutant grows on nutrient sporulation medium in the presence of 10 mM each of glycerol and malate, synthesis of p_3Ap_3 and sporulation are normal. However, when malate is omitted and the glycerol concentration is increased to 0.45 M, glycerol phosphate is accumulated (13). Synthesis of p_3Ap_3 and sporulation are inhibited (Fig. 4 and Table 2).

The results described above are summarized in Table 2. All mutants investigated do not synthesize p_3Ap_3 when a phosphorylated metabolite of a given carbon source is accumulated because of a genetic block in its catabolism. We, therefore, conclude that the enzyme synthesizing p_3Ap_3 is inhibited by different types of phosphates of carbon sources. Release from inhibition occurs when these carbon sources are removed from the growth medium and formation of the metabolite is no longer possible (13).

Accumulation of sugar phosphates in these mutants has apparently no toxic effect, because vegetative growth is hardly affected (13) by the presence of the indicated rather low concentrations of carbon sources leading to intracellular accumulation of phosphorylated metabolites.

To show that in the mutants described above the p_3Ap_3-synthetase has the same properties as the wild-type enzyme and can be inhibited by phosphorylated metabolites of carbon sources *in vitro* too, we isolated membrane vesicles from wild type and mutants and tested their *in vitro* synthesizing properties as described (7).

As shown in Table 3, membrane vesicles from all mutants synthesize p_3Ap_3 similarly to the wild-type strain 60015. Glucose 6-phosphate, glycerol phosphate, and fructose 6-phosphate inhibit p_3Ap_3 synthesis in wild type and mutants between 35%

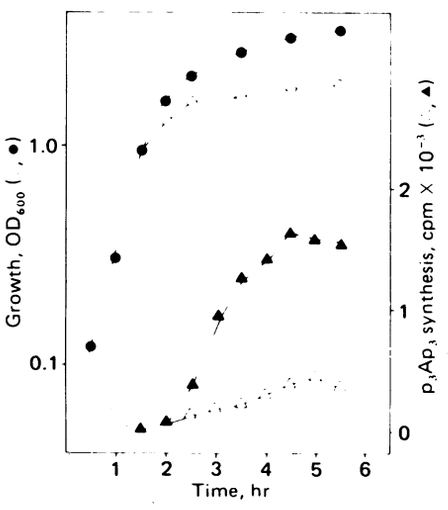

FIG. 2. Strain 61402 (GlpD$^-$) was grown under conditions similar to those described for Fig. 1, but NSMT + 0.45 M glycerol and 40 mM malate (●) was used for growth and NSMT + 0.45 M glycerol (○) was used to inhibit sporulation. Synthesis of p_3Ap_3 was measured under conditions inhibiting (△) and permitting (▲) sporulation.

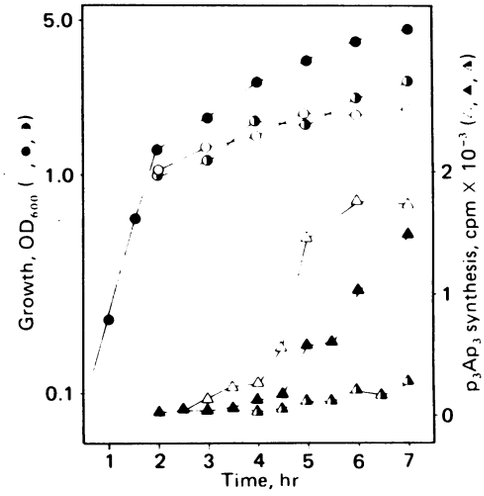

FIG. 3. Strain 61364 (PGI$^-$, G6PD$^-$, PGM$^-$) was grown under conditions similar to those described for Fig. 1, but NSMT + 0.2% mannose (●) or NSMT (○) was used for growth and NSMT + 0.2% glucose (◑) was used to inhibit sporulation. Synthesis of p_3Ap_3 was measured in the media supporting (△, ▲) and inhibiting (▲) sporulation.

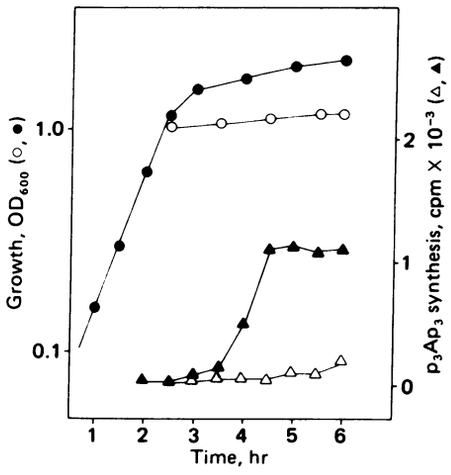

FIG. 4. Strain 61106 (glycerol-requiring) was grown under conditions described for Fig. 1, except that NSMT + 10 mM glycerol + 10 mM malate (●) was used. Sporulation-suppressing medium, NSMT + 0.45 M glycerol, was also used (○). Synthesis of p_3Ap_3 was measured in the media supporting (▲) and suppressing (△) sporulation.

and 53%. The p_3Ap_3-synthesizing enzyme, therefore, appears to be "normal" and unaffected by mutations in the catabolism of carbon sources.

Mutations in the *spoOF* Gene Are Mutations in the Structural Gene for p_3Ap_3-Synthetase. Mutations in the *spoOF* gene lead to asporogeny. In those mutants p_3Ap_3 is not synthesized (11). In collaboration with J. Hoch, we have isolated several temperature-sensitive sporulation mutants, mutated in the *spoOF* gene. All mutants tested so far are also temperature sensitive in p_3Ap_3-synthesizing activity. An example is given in Fig. 5 *A* and *B*. At the permissive temperature (30°C), sporulation frequencies of 50–70% are obtained after 24 hr. Synthesis of p_3Ap_3 does not differ significantly from that in the wild type (Fig. 5*A*). However, at the nonpermissive temperature (42°C), sporulation is not observed (<1% after 24 hr) and p_3Ap_3 synthesis is practically abolished (Fig. 5*B*).

When membrane vesicles were isolated (7) and p_3Ap_3 was allowed to be synthesized at the nonpermissive temperature for 60 min, some p_3Ap_3-synthesizing activity was observed (Fig. 6). One half of the reaction mixture was then quickly cooled to 30°C while the other half was kept at 42°C. Fig. 6 shows that p_3Ap_3 synthesis began to increase as soon as the reaction mixture was adjusted to the permissive temperature. At the nonpermissive temperature, p_3Ap_3 synthesis continued at the previously observed low rate.

Table 3. Synthesis of p_3Ap_3 by membrane vesicles and its inhibition by metabolites of carbon sources *in vitro*

Strain	Inhibition of p_3Ap_3 synthesis, %		
	Glucose-6-*P*	Glycerol-*P*	Fructose-6-*P*
60015	40	53	51
61402	32	35	48
61364	48	36	38
61447	35	43	42
61106	38	46	33

Radioactivity incorporated into p_3Ap_3 in the standard reaction mixture varies among different preparations. Inhibition studies were performed only if at least 10^3 cpm/10 μl was incorporated into p_3Ap_3.

Even though p_3Ap_3 synthesis is not equally inhibited *in vitro* and *in vivo* at the nonpermissive temperature in this temperature-sensitive sporulation mutant, there is a dramatic difference in the activity of p_3Ap_3-synthetase at the permissive temperature as compared to the nonpermissive temperature. This leads us to propose that the mutation in this strain may be located in the structural gene for p_3Ap_3-synthetase (*spoOF* gene). Consequently, a mutation in the structural gene for abt-synthetase clearly affects sporulation.

Because p_3Ap_3 synthesis *in vivo* is influenced by a great number of factors not present in a cell-free (membrane vesicle-containing) *in vitro* system, it is not surprising that there is a difference in the temperature sensitivity of the p_3Ap_3-synthetase between *in vivo* and *in vitro* experiments. The reason for this difference, however, has not been determined.

Electron Microscopy. Electron micrographs of thin sections at ×30,000 magnification of mutant JH765b grown at the permissive temperature revealed that asymmetric septa were formed in this mutant, as was seen in the wild-type strain. However, at the nonpermissive temperature no asymmetric septa have been observed even though 10^3 longitudinal sections have been screened. This confirms that any mutation in *spoOF* (including temperature-sensitive mutations) blocks sporulation at stage 0—i.e., prevents asymmetric septum formation.

Using the same technique, we could also confirm results described by Freese *et al.* (13) that accumulation of metabolites of carbon sources prevents the development of prespore septa. Inability to synthesize p_3Ap_3 seems to be the reason for this observation.

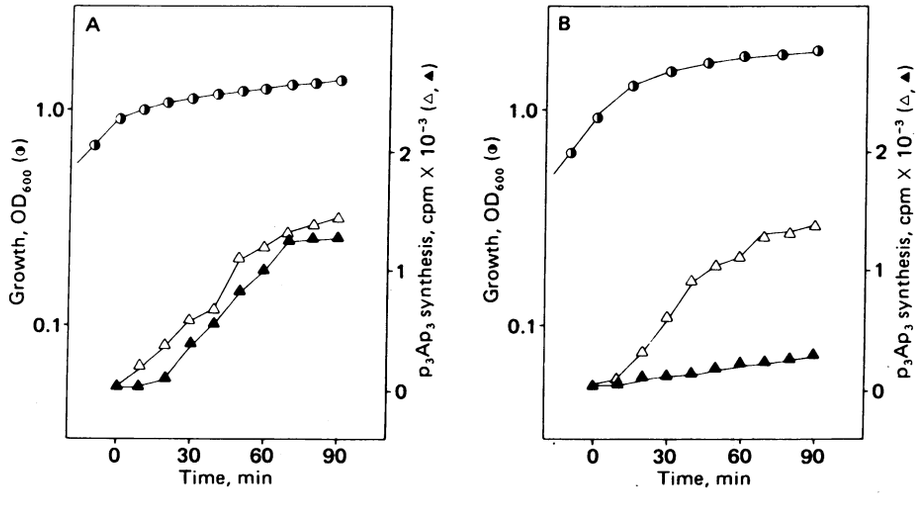

FIG. 5. Growth of *B. subtilis* strains JH642 and JH756b in synthetic yeast extract medium (◑). Synthesis of p_3Ap_3 at (*A*) the permissive (30°C) and (*B*) the nonpermissive (42°C) temperature was measured in the sporogenous strain JH642 (△) and the temperature-sensitive conditional sporulation strain JH756b (▲).

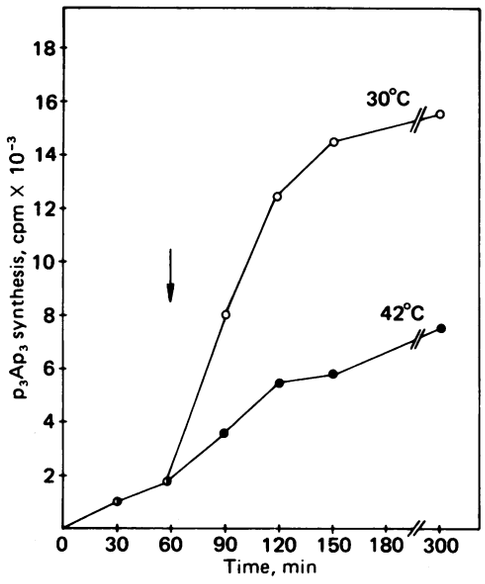

FIG. 6. *In vitro* synthesis of p_3Ap_3 by membrane vesicles from *B. subtilis* JH765b at 42°C (●). At the time indicated (arrow), one half of the reaction mixture was shifted from 42°C to 30°C (○).

DISCUSSION

The experiments described lead us to suggest that the membrane-bound enzyme p_3Ap_3-synthetase is involved in synthesis of p_3Ap_3, which functions as a "sensor" or "detector" for changes in the energy supply outside of a cell. This enzyme thus may be responsible for the synthesis of a trigger for sporulation, because it meets three conditions: (*i*) It is constitutively synthesized and present in the cytoplasmic membrane independent of growth and development. (*ii*) It is inhibited by metabolites of carbon sources. Release from inhibition occurs as soon as rapidly metabolizable carbon sources are removed from the medium. (*iii*) Mutations in the *spoOF* gene, which codes for one enzyme apparently involved in p_3Ap_3 synthesis, which we have called p_3Ap_3-synthetase, abolish sporulation.

From the facts known so far we derive the following model, which we have termed the "sensor model of initiation of differentiation": As long as carbon sources are present in the medium, p_3Ap_3 synthesis is inhibited and growth occurs. Lack of carbon sources releases p_3Ap_3-synthetase from inhibition, thus triggering production of p_3Ap_3. This small molecular effector now triggers sporulation by a yet-unknown mechanism. RNA polymerase may be involved in this latter mechanism, because we have found (12) that RNA polymerase is strongly inhibited by this regulatory nucleotide.

Even though phosphorylation and transport of sugars through the cytoplasmic membrane with the aid of the phospho*enol*-pyruvate:sugar phosphotransferase system (PTS) is normal in the asporogenous mutant that cannot synthesize p_3Ap_3, the possibility of a relationship between this system and p_3Ap_3-synthetase should be discussed. Both p_3Ap_3-synthetase and PTS are localized in the cytoplasmic membrane and have phosphorylating capabilities. The synthetase is inhibited as long as PTS functions and vice versa. It is, therefore, possible that p_3Ap_3-synthetase is one or belongs to one of the enzymes of PTS. Alternatively, one of the enzymes or compounds of PTS, enzyme I, enzyme II, or the energy-coupling proteins HPr~*P*, could activate p_3Ap_3-synthetase when no longer engaged in phosphorylation and transport of sugars, similarly to the proposed activation of cyclic AMP in *Escherichia coli* (16). If this latter case is true, sugar transport would be normal in *spoOF* mutants, as observed, but p_3Ap_3 synthesis could not occur in spite of activation because of a genetic defect in the structural gene for p_3Ap_3-synthetase.

Our sensor model of initiation of differentiation presented here, in addition to describing initiation of sporulation after starvation, is also able to explain the observation that a rather high level of spores is present in cultures of bacilli growing vegetatively in a chemostat in a poor medium, whereas this level is markedly reduced in rich medium. The availability of nutrients for individual cells follows a Gaussian distribution. In a rich medium the number of individuals lacking sufficient nutrients will be smaller than in a poor medium. Consequently, according to the sensor model, the number of starved cells in which p_3Ap_3 is synthesized, and sporulation is therefore induced, is larger in poor than in rich media.

Induction of sporulation as described by the sensor model is obviously a type of regulation of gene activity quite different from the positive or negative regulation systems known so far. It is only comparable to the activation of adenylate cyclase in the case of catabolite repression and similar systems involving cyclic AMP.

The enzyme or enzymes synthesizing p_3Ap_3 play a key role in this new type of regulation. Enzyme activity is inhibited by molecules (phosphorylated metabolites of carbon sources) totally unrelated to the synthesizing activity (p_3Ap_3). In addition, the inhibitor acceptor site must be quite unspecific, because many kinds of phosphorylated carbon sources are effective.

Investigations of the properties of purified p_3Ap_3-synthetase will give further insight into the regulation of this interesting enzyme.

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