Studies on the control of development: Isolation of *Bacillus subtilis* mutants blocked early in sporulation and defective in synthesis of highly phosphorylated nucleotides

[differentiation/initiation of sporulation/regulatory nucleotides/vegetative and sporulation type ribosomes/adenosine 3'(2')-triphosphate 5'-triphosphate synthetase]

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ABSTRACT   To test our model on the mechanism of initiation of differentiation in *Bacillus subtilis*, we tested early blocked (stage 0) sporulation mutants for their ability to synthesize highly phosphorylated nucleotides. We also isolated early blocked asporogenous mutants with the aid of the intercalating drug tilorone. Among all mutants tested we found that the *spo0F*-bearing strain was unable to synthesize adenosine 3'(2')-triphosphate 5'-triphosphate, pppAppp. A revertant of this mutant regained the ability to both sporulate and synthesize pppAppp. Ribosomes of the asporogenous mutant isolated at T_2 (2 hr after the end of logarithmic growth) of sporulation, in contrast to the wild type, do not synthesize adenosine 3'(2')-diphosphate 5'-diphosphate, ppApp, or adenosine 3'(2')-diphosphate 5'-triphosphate, pppApp, but synthesize guanosine 3'(2')-diphosphate 5'-diphosphate, ppGpp, and guanosine 3'(2')-diphosphate 5'-triphosphate, pppGpp. This behavior is characteristic of ribosomes from vegetative, not sporulating, cells. Ribosomes from the sporogenous revertant behave like those of the wild type. The results suggest that the *spo0F* mutation may be a mutation in the structural gene for pppAppp synthetase. The inability to synthesize pppAppp in this strain also prevents the formation of "sporulation-specific ribosomes," i.e., ribosomes that synthesize ppApp and pppApp. The present experiments suggest that the nucleotide pppAppp participates in the initiation of sporulation by triggering a sequence of events required for the production of heat-resistant spores.

In previous communications we have reported that unusual highly phosphorylated nucleotides are found in sporulating, but not in vegetative, cells of *Bacillus subtilis* (1, 2). We were able to correlate the accumulation of these nucleotides with sporulation development (3). Altogether, there are six unusual nucleotides synthesized under different conditions. The "magic spot" nucleotides (4) guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp), which are synthesized by ribosomes isolated from vegetative but not from sporulating cells (for convenience called vegetative or sporulation ribosomes, respectively), are not involved in sporulation (3, 5). The highly phosphorylated nucleotides adenosine 5'-diphosphate 3'-diphosphate and adenosine 5'-triphosphate 3'-diphosphate, which are synthesized by sporulation ribosomes, however, seem to be connected with sporulation (5, 6). There is also evidence that the nucleotide adenosine 5'-triphosphate 3'-triphosphate, pppAppp, which is synthesized by membrane-bound enzymes, is involved in the initiation of sporulation (7). The last of the highly phosphorylated nucleotides, nucleotide III, whose structure is not completely known, also seems to be connected with sporulation development (3).

Data supporting our recently proposed mechanism of initiation of differentiation involving these highly phosphorylated nucleotides (7) can be obtained by the isolation of mutants deficient in enzymes synthesizing either one of the highly phosphorylated nucleotides. As a consequence of such a mutation, sporulation development should be impaired.

In a search for such a mutant, we isolated several asporogenous mutants resistant to the drug tilorone (8) and also tested several early blocked asporogenous mutants (*spo0* mutants; for definition see ref. 9) for their ability to synthesize unusual highly phosphorylated nucleotides. We have found several asporogenous mutants that are unable to synthesize any one of the four nucleotides. Revertants, selected for the ability to sporulate, regained simultaneously the ability to synthesize these nucleotides.

MATERIALS AND METHODS

Bacteria. The transformable Marburg strain *B. subtilis* 60015 (*ind⁻, met⁻*), which is strain SB 26 of Nester, was used in this study. Tilorone-resistant mutants were derived from this strain as described previously (8).

The other mutant strains used in this study are listed in Table 1. A set of isogenic strains was constructed that differ only in the various stage 0 sporulation loci. The genetic locations on the chromosomal map of the mutations *spo0A12*, *spo0B136*, *spo0E11*, and *spo0H81* have been reported previously (10). The *spo0J87* mutation was described by Hranueli et al. (11). The chromosomal location of the *spo0F221* mutation has been recently found near the *ctrA* mutation (Hoch et al., unpublished data). The mutants chosen for this study are representative of the mutations at each locus.

A sporogenous revertant of strain JH649 (see Table 1) was obtained by growing it in a semisynthetic yeast extract medium (12) for 24 hr and heat killing all vegetative cells. After 0.1 ml of the culture had been plated on nutrient agar, a colony was selected and tested for its ability to sporulate normally. This mutant was named 649 R.

Media and Growth Conditions. Growth and sporulation in a semisynthetic yeast extract containing sporulation medium were described earlier (10). Sporulation was induced in vegetative cells by centrifuging cells at an optical density of approximately 1.0 at 600 nm ($OD_{600 nm}$) and resuspending the buffer- (0.1 M Tris·HCl, pH 7.0) washed cells in the above-mentioned medium but without glucose and yeast extract. The yeast extract was substituted by 19 amino acids as described before (10).

Biochemical Methods. Labeling of low-molecular-weight substances and extraction from cells has been described (10). *In vitro* synthesis of unusual nucleotides by ribosome-associated enzymes has also been described before (4), as has the method for synthesizing highly phosphorylated nucleotides by membrane-bound enzymes from *B. subtilis* (6).

Chromatography and Autoradiography. All methods em-

Table 1.   Sporogenous and asporogenous strains
of *B. subtilis*

Strain	Genotype
JH642	*trpC2 phe-1*
JH646	*trpC2 phe-1 spo0A12*
JH647	*trpC2 phe-1 spo0E11*
JH648	*trpC2 phe-1 spo0B136*
JH649	*trpC2 phe-1 spo0F221*
JH651	*trpC2 phe-2 spo0H81*
JH696	*trpC2 phe-1 spo0J87*
60015	*trpC2 ind*
50413	*trpC2 ind*

ployed to detect and separate unusual nucleotides by chromatography and autoradiography have been described before (3, 10).

FIG. 2.   Synthesis of pppAppp by cells of a sporulating strain of *B. subtilis*, JH642 (O); an asporogenous but otherwise isogenic mutant, JH649 (■); and also a revertant of this mutant, 649 R (●). Cells were transferred at $OD_{600\,nm} \approx 1.0$ to a synthetic medium lacking glucose and samples were withdrawn at times up to 90 min and treated as described (12).

RESULTS

Synthesis of Highly Phosphorylated Nucleotides in Wild Type and Asporogenous Mutants of *B. subtilis*.

Strain JH642 (*trpC2 phe-1*) of *B. subtilis*, which was derived from strain 168, and several asporogenous but otherwise isogenic mutants of this strain were tested for their ability to synthesize pppAppp after a shift of late logarithmic phase cells ($OD_{600\,nm} \approx 1.0$) to a medium lacking glucose as described in *Materials and Methods*. An autoradiogram of a polyethyleneimine (PEI)-thin-layer chromatogram of $H_3{}^{32}PO_4$-labeled cells extracted with 2 M formic acid (Fig. 1) shows, as we have shown with strain 60015 (3), that the sporogenous strain JH642 and several asporogenous mutants of this strain (JH646, JH648, JH651, and JH696) synthesize pppAppp at the same or slightly elevated (strain JH646) rates for at least 60 min after a shift to glucose-depleted media. However, one mutant (strain JH649) synthesizes virtually no pppAppp, whereas strain 647, which sporulates at a low frequency (0.1–1%) produces some pppAppp. The other highly phosphorylated nucleotides ppApp, pppApp, and nucleotide III are produced also in different amounts. One-dimensional chromatography, however, is not suitable for a quantitative evaluation of the presence of these nucleotides (see below). The nucleotides ppGpp and pppGpp are also synthesized under these conditions because of the absence of carbon sources in the glucose-deficient medium (3). Mutant JH649, which is asporogenous and apparently deficient in pppAppp synthesis, was investigated more carefully for its ability to synthesize this nucleotide *in vivo* and *in vitro*.

Kinetics of the Accumulation of pppAppp in *B. subtilis* Wild Type and an Asporogenous Mutant. When the wild-type strain JH642 and mutant JH649 were grown in yeast extract medium in the presence of $H_3{}^{32}PO_4$ (0.5 mCi/ml) and transferred at an $OD_{600\,nm} \approx 1.0$ to a yeast extract medium lacking glucose, rather drastic differences in the ability to synthesize pppAppp were observed (Fig. 2). Whereas the sporulating strain JH642 accumulates pppAppp as described previously (3), the asporogenous mutant JH649 accumulates less than one tenth of this nucleotide.

When a sporogenous revertant of mutant JH649, mutant 649 R (isolated as described in *Materials and Methods*) was tested, it was found that this spontaneous revertant had regained the ability to sporulate simultaneously with the ability to synthesize pppAppp, both with almost the same efficiency as the parent wild-type strain (Fig. 2).

This mutant is of the *spo0F* type (10) and maps between the *ctrA* and *narA* markers (J. A. Hoch and P. S. Chen, unpublished data).

Synthesis of pppAppp by Membrane Preparations from *B. subtilis* Wild Type and Asporogenous Mutants.

In order to test whether the inability of mutant JH649 to synthesize pppAppp *in vivo* is due to a structural defect of the membrane-bound enzyme (or enzymes), to which, for convenience, we have assigned the trivial name pppAppp synthetase, or whether the defect results from a pleiotropic sporulation
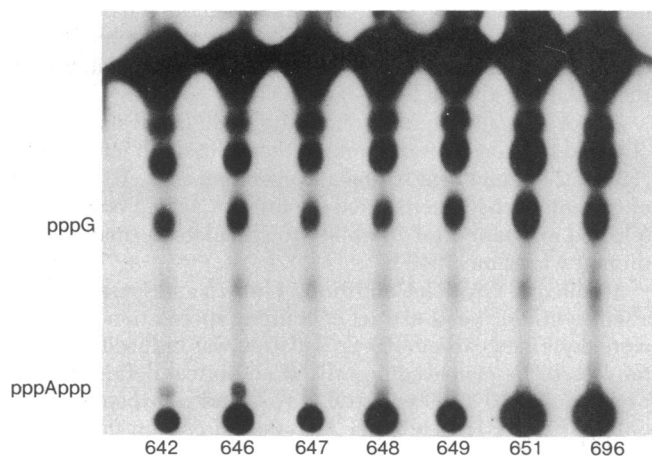

FIG. 1.   Autoradiogram of formic-acid-extracted *B. subtilis* wild-type cells (strain JH642) and *spo0* mutants chromatographed on polyethyleneimine (PEI) impregnated thin-layer plates. Cells were grown in semisynthetic yeast extract medium in the presence of 0.5 mCi of $H_3{}^{32}PO_4$ per ml. After growth to $OD_{600\,nm} \approx 1.0$, cells were transferred to a glucose-deficient medium and further incubated for 60 min. Samples were withdrawn and treated as described previously (10). The genotypes of all asporogenous mutants are listed in Table 1.
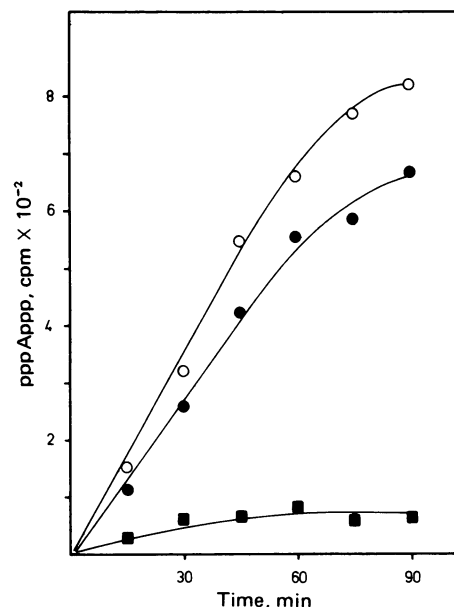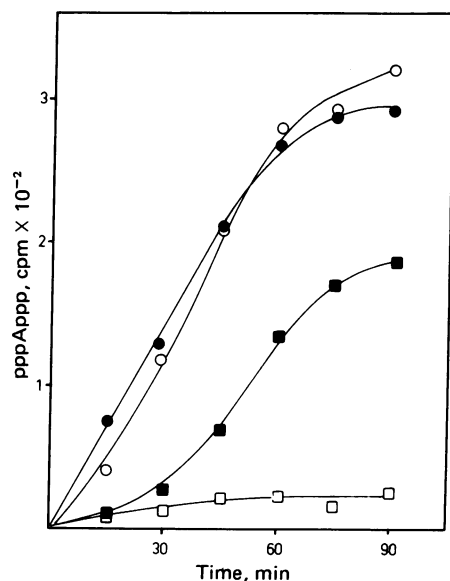
FIG. 3. *In vitro* synthesis of pppAppp by membrane vesicles of *B. subtilis*. The vesicles were isolated and incubated in a standard reaction mixture in the presence of ATP and $H_3{}^{32}PO_4$. After various times of incubation at 37° samples were taken, treated, and chromatographed as described before (7). The pppAppp-synthesizing activity of membrane vesicles isolated from the sporogenous strain JH642 (●) and two asporogenous mutants, JH649 (□) and 50413 (O), as well as the sporogenous revertant strain 649 R (■), was followed for 90 min.

FIG. 4. Synthesis of pppAppp and highly phosphorylated nucleotide III by the sporogenous *B. subtilis* strain 60015 (open symbols) and the asporogenous tilorone-resistant mutant 50413 (filled symbols) after a shift from a glucose-sufficient to a glucose-deficient semi-synthetic yeast extract medium (12). The highly phosphorylated nucleotide III is represented by triangles and pppAppp by squares. Growth was measured spectrophotometrically as $OD_{600\ nm}$ (◐).

mutation, we tested membranes isolated from strains JH642, JH649, and 649 R for their pppAppp-synthesizing ability. Fig. 3 shows that membrane vesicles of the sporogenous strain JH642 synthesize pppAppp in a fashion similar to that shown previously (7) by membranes isolated from strain 60015. On the other hand, membrane vesicles isolated from the asporogenous mutant JH649 are unable to synthesize this nucleotide. Therefore, the apparent inability of the asporogenous mutant JH649 to synthesize pppAppp *in vivo* seems to be due to a defect in the membrane-bound enzyme pppAppp synthetase. Since the pppAppp synthetase is found at all times in membrane vesicles of vegetative as well as sporulating cells of *B. subtilis* (Rhaese and Groscurth, unpublished data), its presence in the cytoplasmic membrane is independent of whether or not sporulation occurs.

Further evidence that sporulation is controlled or rather initiated by pppAppp is obtained from our finding, that a revertant of strain JH649, strain 649 R, selected for its ability to sporulate, has regained a functional membrane-bound pppAppp synthetase (see Fig. 3). This synthetase is also present in the cytoplasmic membrane of both vegetative and sporulating cells of this revertant (Rhaese and Groscurth, unpublished data), although with slightly lower activity. It seems as if this is accompanied by a slightly lower sporulation frequency compared to the parent strain JH642.

In Vivo **Synthesis of Highly Phosphorylated Nucleotides in a Tilorone-Resistant Asporogenous Mutant.** The intercalating drug tilorone does not affect vegetative growth but inhibits the formation of spores in *B. subtilis* (8). Tilorone-resistant mutants were found to be asporogenous. Because tilorone sensitivity has been detected already at T_0, the beginning of the sporulation phase (8), it was anticipated that tilorone-resistant mutants might be blocked early in sporulation. We, therefore, tested tilorone-resistant asporogenous mutants for their ability to synthesize highly phosphorylated nucleotides.
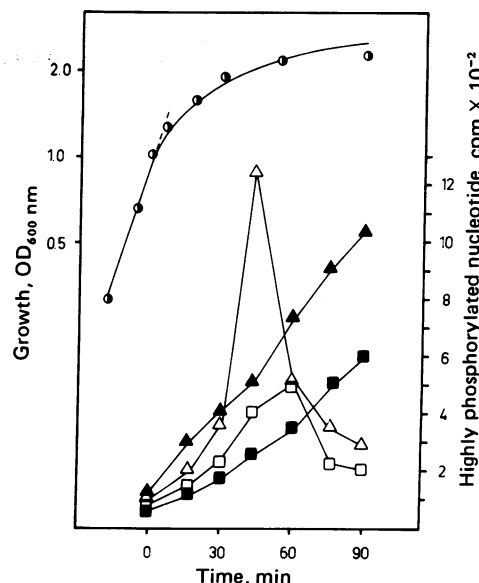
As is shown in Fig. 4, mutant 50413 (met^-, trp^-, tilorone-resistant) accumulates pppAppp and highly phosphorylated nucleotide III at least at the same rate as does the parent strain 60015, when shifted from a glucose-sufficient to a glucose-deficient medium. Accumulation seems to continue even longer than has been observed in strain 60015. This mutant is apparently blocked in a sporulation-specific event occurring after the synthesis of pppAppp. In our model on initiation of sporulation published previously (7) this could mean that the information-processing step (I_p) rather than the information-recognition step (I_R) is not functioning. A genetic defect in either one of these steps could prevent transcription of sporulation-specific genes. To test whether this is indeed the case, we investigated whether or not the transition of ribosomes from a vegetative type (synthesis of substances ppGpp and pppGpp) to a sporulation type (synthesis of highly phosphorylated nucleotides ppApp and pppApp) occurs.

In Vitro **Synthesis of Unusual Nucleotides by Ribosomes from Sporulating Cells and Mutants Blocked in Sporulation.** In all cases investigated, ribosomes isolated from vegetative cells of *B. subtilis* synthesize ppGpp and pppGpp‡. However, when sporulation is initiated, the nucleotide-synthesizing capacity of ribosomes obviously changes. This is reflected by the synthesis of the highly phosphorylated nucleotides ppApp and pppApp by ribosomes isolated from sporulating cells. Synthesis of the substances ppGpp and pppGpp is then no longer observed (5). Ribosomes isolated from cells in which sporulation is inhibited by glucose continue to synthesize ppGpp and pppGpp when isolated at T_2 (2 hr after the end of logarithmic growth) but have not been found to synthesize significant amounts of highly phosphorylated nucleotides (Rhaese and Groscurth, unpublished data). We tested, therefore, whether this apparent correlation in change of unusual-nucleotide-synthesizing ability of ribosomes with sporulation can be further substantiated by

‡ H.-J. Rhaese and R. Groscurth, unpublished data.

Table 2. Synthesis of ppGpp, pppGpp, ppApp, and pppApp by ribosomes isolated at T_2 from sporogenous (Spo⁺) and asporogenous (Spo⁻) strains of *B. subtilis*

Strains	Nucleotide synthesis, cpm			
	ppGpp	pppGpp	ppApp	pppApp
60015 (Spo⁺)	0	0	315	260
JH642 (Spo⁺)	0	0	396	162
JH649 (Spo⁻)	510	96	45	0
649 R (Spo⁺)	0	0	255	126
50413 (Spo⁻)	620	112	32	0
50413 R (Spo⁺)	0	0	295	126

The incubation mixture employed here has been described before (5).

isolating mutants that cannot undergo this change and consequently appear to be asporogenous.

When we isolated ribosomes at T_2 from *B. subtilis* wild type and several asporogenous mutants as well as their respective revertants, we found that ribosomes from all sporulating strains synthesize ppApp and pppApp (see Table 2). The ability to synthesize ppGpp and pppGpp is completely lost.

In contrast, ribosomes of all asporogenous mutants isolated at the same time and by the same procedures synthesize (under identical conditions) ppGpp and pppGpp, but no pppApp and only negligible amounts of ppApp (Table 2).

It is interesting to note that mutant JH649, which is a single site mutation of the *spo0F* type but otherwise isogenic with strain JH642, the sporulating parent, can regain the ability to change ribosomes from a vegetative to a sporulation type when reverted to a sporogenous strain (649 R). The revertant was selected for its ability to sporulate.

We therefore conclude that a change in ribosomal structure, probably ribosomal proteins (13) and ribosomal factors‡ as suggested previously, is necessary for sporulation to occur. We do not know, as yet, whether this change is necessary because the highly phosphorylated nucleotides ppApp and pppApp are necessary to control sporulation or whether the capacity of ribosomes in sporulating cells to synthesize these nucleotides only reflects the necessity of having ribosomes that somehow control translation, for example, by exerting some selectivity to translate sporulation-specific mRNA instead of mRNA needed for vegetative growth (14).

To show clearly that ribosomes isolated at T_2 from sporulating cells synthesize only ppApp and pppApp (strains JH642 and 649 R in Fig. 5) and those from cells unable to sporulate continue to synthesize ppGpp and pppGpp as ribosomes from vegetative cells do (strains JH649 and 50413), we have separated the products of a standard incubation mixture (4) by two-dimensional thin-layer chromatography. As can be seen in Fig. 5, a genetic defect in the ability to synthesize pppAppp (strain JH649) prevents changes in the nucleotide-synthesizing capacity of ribosomes. This means that ribosomes of mutant JH649, even though isolated at T_2, behave like ribosomes isolated from vegetative cells and synthesize ppGpp and pppGpp. The same is observed in another asporogenous mutant, 50413 (Fig. 5).

We conclude, therefore, that a change in the nucleotide-synthesizing character of ribosomes (synthesis of ppApp and pppApp instead of ppGpp and pppGpp) at or shortly after the beginning of sporulation is necessary for completion of sporulation and that this change cannot occur when pppAppp cannot be synthesized by membrane-bound enzymes (see Table 2, strain JH649). This is further substantiated by the observation
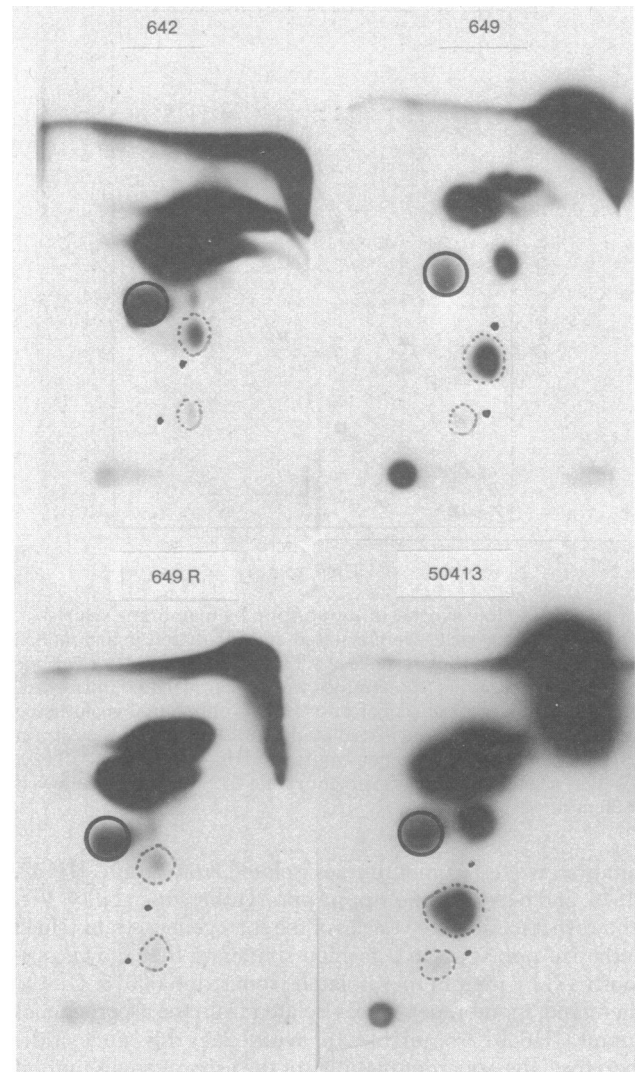

FIG. 5. Synthesis of unusual nucleotides by ribosomal factors in the presence of washed ribosomes (5) isolated at T_2 (2 hr after the end of logarithmic growth) from sporogenous (JH642 and 649 R) and asporogenous (JH649 and 50413) strains of *B. subtilis*. A 20 μl sample of a standard incubation mixture (5) was chromatographed two-dimensionally as described previously (3) and exposed to x-ray films (2). The sporogenous strains JH642 and 649 R synthesize ppApp (upper spots in broken circles) and pppApp (lower spots in broken circles) but no ppGpp and pppGpp (the positions where these substances would migrate are indicated by dots). The asporogenous mutants JH649 and 50413, in contrast, synthesize ppGpp (upper spots in broken circles) and pppGpp (lower spots in broken circles). The nucleotides ppApp and pppApp, if present, would migrate to positions indicated by dots. Nonradioactive GTP was applied to each chromatogram together with the sample and after chromatography was detected under ultraviolet (255 nm) light and circled by a solid line.

that sporulation is prevented in a mutant in which this change cannot occur (see Table 2, strain 50413). A genetic defect preventing synthesis of pppAppp seems to be sufficient to inhibit the above-mentioned ribosomal changes, showing that in a sequence of events leading to sporulation, pppAppp formation is necessary to cause changes in ribosomes from a vegetative to a sporulation type. The sporogenous revertants of strains JH649 and 50413, strains 649 R and 50413 R, which simultaneously regain the ability to sporulate and to change ribosomes to a sporulation type (see also Table 2), seem to underline this con-

clusion. In the case of mutant 649 R the ability to synthesize pppAppp is also regained.

DISCUSSION

A model on the mechanism of initiation of sporulation in *B. subtilis* proposed by us recently (7) suggests that upon carbon or phosphate starvation nucleotides of a new class, named highly phosphorylated nucleotides, are synthesized, which, via several steps, trigger changes in metabolic processes leading to heat-resistant spores. The enzyme (or enzymes) synthesizing one of these nucleotides, pppAppp, was found to be located in the membrane of *B. subtilis* (7). Substances, such as phosphorylated metabolites of glucose or other carbon sources, that inhibit sporulation (15) also inhibit synthesis of this nucleotide. This and further close connections of sporulation and synthesis of this nucleotide as shown previously (3), however, are not sufficient to prove our hypothesis.

Mutants, however, that cannot sporulate owing to a single-site mutation in the structural gene coding for the enzyme pppAppp synthetase would be sufficient evidence that sporulation depends on the prior synthesis of this nucleotide.

It is likely that mutant JH649 described in this communication is mutated in the structural gene for the enzyme pppAppp synthetase. The possibility that both sporulation and synthesis of pppAppp are controlled simultaneously and that therefore the *spo0F* mutation in this mutant is located in a gene coding for a protein that controls both pppAppp synthesis and sporulation is unlikely, because pppAppp synthetase is present in membranes of sporogenous strains of *B. subtilis* (e.g., strains 60015 and JH642) at all times, in vegetative and sporulating cells. Membranes isolated from both vegetative and sporulating cells of the asporogenous mutant JH649 do not have any pppAppp-synthesizing activity. The sporogenous revertant of this mutant, strain 649 R, regained together with the ability to sporulate the ability to synthesize pppAppp both *in vivo* and *in vitro*. Furthermore, inhibition of protein synthesis by chloramphenicol prior to the isolation of pppAppp synthetase from membrane vesicles prepared from vegetative cells has no effect on the *in vitro* synthesis of pppAppp (H. J. Rhaese, R. Grade, and H. Dichtelmüller, unpublished data), indicating that pppAppp synthetase is not produced when sporulation is triggered but rather is a constituent part of the membrane of *B. subtilis* cells regardless of the developmental stage.

We conclude, therefore, that the activity of pppAppp synthetase is repressed in vegetatively growing cells of *B. subtilis* by phosphorylated metabolites of carbon sources, such as glucose 6-phosphate, fructose 1,6-bisphosphate, etc., as long as these substances can be formed in the membrane from phosphate and carbon sources in the medium. As soon as any one of these nutrients is exhausted, pppAppp synthetase is derepressed and pppAppp is produced.

The apparent absence of pppAppp-synthesizing activity in mutant JH649, which is coupled with asporogeny, seems to indicate that pppAppp is necessary for sporulation to occur.

The results reported in this communication can be interpreted as showing that nutrient deficiencies in *B. subtilis* are recognized by synthesis of a substance with informational character. This substance, the unusual nucleotide pppAppp, seems to be responsible for the induction of sporulation because a genetic defect that apparently rests in the structural gene for the enzyme pppAppp synthetase also blocks sporulation. How pppAppp causes changes in transcription from vegetative to sporulation-specific genes is unknown. However, a change in the structure of the ribosomes and the ribosome-associated factors seems to be necessary. Ribosomes from the pppAppp-deficient mutant JH649 retain the vegetative character and therefore cannot synthesize ppApp and pppApp, whereas the revertant 649 R not only sporulates and synthesizes pppAppp but also has sporulation type ribosomes (see Table 2). Mutant 50413 is also unable to sporulate and possesses ribosomes of vegetative character, whereas the revertant 50413 R sporulates normally and ribosomes isolated at T_2 synthesize ppApp and pppApp.

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