SPERMIDINE LEVELS AND ITS RELATIONSHIP TO DNA SYNTHESIS IN OUTGROWING SPORES AND VEGETATIVE CELLS OF *BACILLUS SUBTILIS*

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Received 2 December 1981

1. Introduction

The naturally occurring diamine putrescine $NH_2(CH_2)_4NH_2$ and the triamine spermidine $NH_2(CH_2)_3NH(CH_2)_4NH_2$ are widely distributed in nature and occur in all bacteria so far analyzed [1,2]. Even though their biological functions are not clearly understood, they have been implicated in cell division [3] and in the synthesis of cellular macromolecules, mainly DNA [4,5]. More recently, evidence accumulated that spermidine serves as a cofactor for DNA gyrase [6,7]. The occurrence and synthesis of spermidine during the germination of *Bacillus megaterium* has been described in [8,9], and the activation of ornithine decarboxylase (EC 4.1.1.17), the rate-limiting enzyme in the biosynthesis of putrescine in *Bacillus subtilis*, has also been reported [10].

The induction of sporulation in *Bacillus* sp. may be linked to DNA replication [11,12]. However, spore outgrowth occurs in the absence of DNA synthesis [11,13,14], but requires the activation of DNA gyrase [15]. After germination, namely, after the conversion of the spore into the vegetative cell, RNA and protein synthesis begin immediately but net DNA synthesis occurs only after 45 min. Because of the involvement of spermidine in the activation of DNA gyrase, it was of interest to find out whether this polyamine is synthesized and accumulates in outgrowing spores when the activity of DNA gyrase increases. If spermidine is only related to DNA replication, it should be synthesized and should accumulate only in the germinating cells. If spermidine synthesis is a prerequisite for the activation of DNA gyrase and for DNA replication, it should accumulate in the respective cells even if DNA replication or the activation of DNA gyrase are arrested.

To clarify this point, we used 6-(*p*-hydroxyphenylazo)-uracil (HPUra) to inhibit DNA synthesis [16] and a temperature-sensitive mutant of *B. subtilis* MB65 (temperature sensitive for the initiation of chromosome replication). Here, we demonstrate that both in spore outgrowth and in vegetative cells, spermidine is synthesized in the absence of DNA replication.

2. Materials and methods

2.1. *Bacteria and growth conditions*

Bacillus subtilis 168 trp C_2 and its mutant MB65 temperature-sensitive for the initiation of chromosome replication, isolated in [17] and obtained from Joel Mandelstam, were used throughout.

Spores were prepared and purified as in [18]. For outgrowth studies, spores were heat-activated by incubation for 30 min at 70°C. The activated spores were suspended in semi-synthetic medium as in [18]. For vegetative growth, activated spores were grown overnight at 37°C with agitation in semi-synthetic medium [18], and then diluted in the same fresh medium to $A_{600} \simeq 0.2$–0.3. Vegetative growth and outgrowth were followed by measurement of A_{600} with a Spectronic 21 model MV spectrophotometer (Bausch and Lomb).

DNA synthesis was arrested during vegetative growth and spore outgrowth either by addition of HPUra (a gift from N. Brown), at final conc. 20 μg/ml, or by growing the *ts* mutant MB65 in restrictive temperature (44°C). The inhibition of DNA synthesis in these strains was verified as in [18].

2.2. *Polyamine assay*

Bacteria and outgrowing spores were sedimented by

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

centrifugation at 4000 rev./min for 10 min and then washed once in salt solution identical to the salt concentration in the growth medium, followed by shaking with 3% perchloric acid. The precipitate formed was sedimented by centrifugation at 5000 rev./min for 10 min. To 0.2 ml aliquots of the supernatant fluid, 20 mg sodium carbonate and 0.4 ml dansyl-chloride (30 mg/ml in acetone) were added. After keeping the preparation in the dark for 15–20 h, dansyl derivatives were extracted with 0.5 ml quantities of benzene and analyzed by thin-layer chromatography [19] on Silica G plates, using ethylacetate: cyclohexane (2:3) as solvent. Fluorescence was determined as in [19].

3. Results

3.1. *Spermidine synthesis during spore outgrowth of* Bacillus subtilis

In order to test the timing and the rate of spermidine synthesis during outgrowth, heat activated spores of *B. subtilis* were suspended in semi-synthetic medium and shaken in a 37°C water bath (section 2). Samples were withdrawn at 30 min intervals, and their absorbance and spermidine content were measured.

Results are given in fig.1A which shows that outgrowth starts with the increase of absorbance at ~30 min. Spermidine starts to accumulate at a slow rate with the beginning of the experiment and continues to accumulate at an increased rate during outgrowth. Putrescine was detected in these cells only at trace amounts. It should be remembered that the synthesis of DNA starts in this strain and under identical conditions at ~45 min [11] and the first cell division occurs at ~110 min [11,18]. Thus, it appears that the accumulation of spermidine precedes the initiation of DNA synthesis.

3.2. *Effect of HPUra on spermidine accumulation during spore outgrowth*

To determine whether spermidine accumulation in

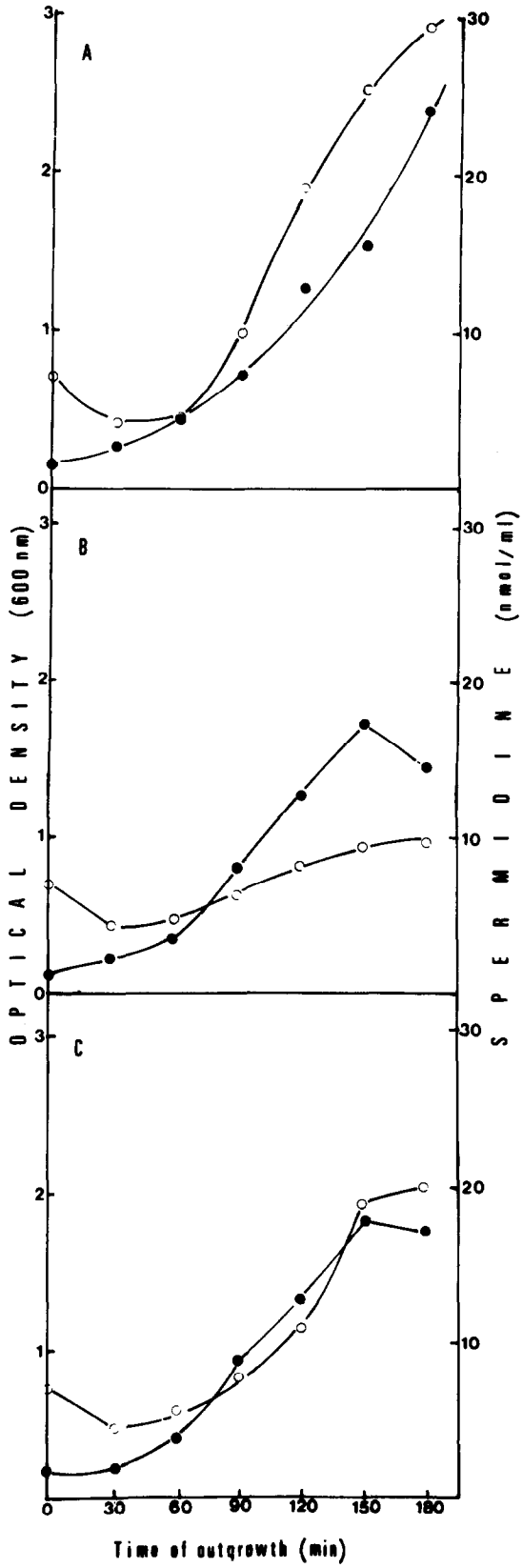

Fig.1. Spermidine levels during outgrowth of *B. subtilis* spores. Heat-activated spores of *B. subtilis* 168 or MB65 were introduced into semi-synthetic medium. Absorbance (o——o) and spermidine contents (•——•) followed for *B. subtilis* 168 outgrown in the absence (A) and presence (B) of HPUra at 37°C, and for *B. subtilis* MB65 at 44°C (C).

the spores during outgrowth is linked to DNA synthesis, activated spores were outgrown in the presence of the DNA synthesis inhibitor (HPUra) and spermidine levels were determined (section 2). The results are given in fig.1B which shows that in the presence of HPUra, which completely blocks DNA synthesis, the increase in absorbance is arrested after 120 min but the initial increase in cellular spermidine level is not affected significantly by the inhibitor. The decrease in cellular spermidine observed at the end of the experiment may be explained by a partial lysis of the cells in the presence of the inhibitor.

It may be concluded from this experiment that the increase in cellular spermidine content during spore outgrowth is not linked to DNA synthesis.

3.3. *Accumulation of spermidine during outgrowth of spores of a temperature-sensitive mutant*

To ascertain further that spermidine accumulation in outgrowing spores is not linked to DNA synthesis, a temperature-sensitive mutant of *B. subtilis* (MB65) was employed. This mutant is temperature sensitive for the initiation of chromosome replication under restrictive conditions (44°C). When spores of this mutant were incubated under permissive conditions (37°C), outgrowth and DNA synthesis proceed normally, and changes in absorbance and spermidine level are similar to those observed with *B. subtilis* 168 (not shown).

When spores of the temperature-sensitive mutant were incubated at 44°C (fig.1C), the increase in cellular spermidine levels resembles that observed in the wild strain, with or without the presence of HPUra. These findings thus confirm the notion that the early accumulation of spermidine in spores during outgrowth is not related to DNA synthesis.

3.4. *Effect of inhibition of DNA synthesis on cellular spermidine levels during growth of vegetative cells of* B. subtilis

To determine whether spermidine accumulation in vegetative cells of *B. subtilis* is or is not linked to

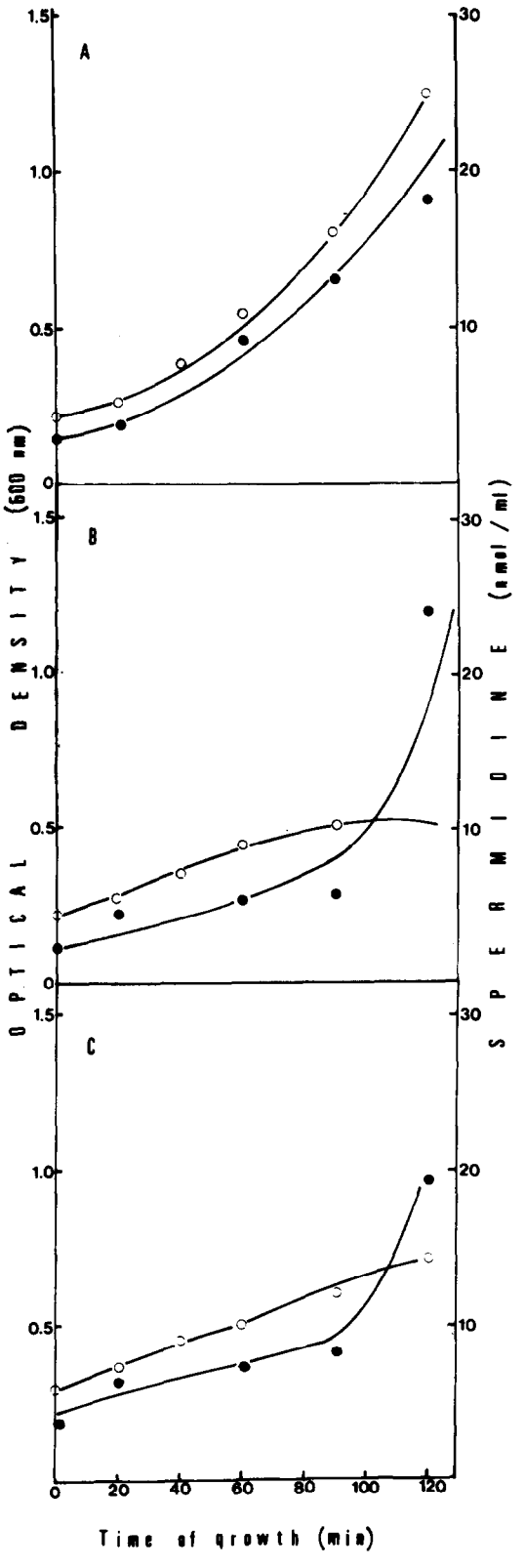

Fig.2. Spermidine levels during vegetative growth of *B. subtilis*. Vegetative growth was started by dilution of an overnight culture by fresh semi-synthetic medium. Absorbance (o——o) and spermidine level (•——•) were detected (section 2) for *B. subtilis* 168 in the absence (A) and presence (B) of HPUra at 37°C, and for *B. subtilis* MB65 at 44°C (C).

DNA synthesis, experiments similar to those with outgrowing spores were performed with vegetative cells.

A culture of *B. subtilis* 168, in the stationary phase, was diluted into fresh medium and cultures were grown in the absence or presence of HPUra. Spermidine levels and growth were followed as in section 2. In growing vegetative cells of *B. subtilis* 168 the cellular spermidine content rises progressively throughout the incubation period (fig.2A). The same is observed when vegetative cells are treated with HPUra and DNA synthesis is blocked (fig.2B).

The same pattern of spermidine accumulation was found when measured during vegetative growth of a *ts* mutant (MB65) of *B. subtilis* 168 under restrictive conditions (44°C) (fig.2C), confirming that, as in outgrowing spores, spermidine synthesis in vegetative cells was also not DNA synthesis-dependent.

4. Discussion

These experiments show that spermidine accumulation during the outgrowth of *B. subtilis* spores and during the growth of its vegetative cells is not dependent on DNA synthesis. During spore outgrowth a consistent increase in cellular spermidine is observed, regardless of DNA synthesis. This has been demonstrated by 2 distinct experimental approaches: the use of the inhibitor of DNA synthesis HPUra; and the growth of the temperature-sensitive mutant of *B. subtilis* MB65 under restrictive conditions. The same situation exists in vegetative cells: spermidine accumulation appears not to be linked to DNA synthesis.

The rate of DNA synthesis in *E. coli* may depend on polyamine concentration [4]. However, these experiments indicate that spermidine synthesis, at least under our conditions, is not linked to DNA synthesis and that spermidine is actively synthesized, even when the synthesis of DNA is inhibited.

The accumulation of spermidine in outgrowing spores in the absence of DNA synthesis deserves special discussion. *Bacillus subtilis* contains DNA gyrase and this enzyme requires spermidine for its optimal activity [7]. In this respect DNA gyrase of *B. subtilis* resembles that of *E. coli* which also requires spermidine [6].

According to [20], DNA gyrase fulfils an essential role in bacterial physiology, and its activity is regulated by spermidine concentration. DNA gyrase induces DNA supercoiling and is a required active enzyme necessary for DNA replication [21,22]. DNA gyrase has a function in selective gene expression in bacteria [23–25].

We had shown that spore outgrowth is most probably DNA gyrase-dependent [15]. Therefore, we assumed that spermidine must be present during the outgrowth of spores. This assumption has now been confirmed.

According to this model, spores committed to outgrowth will activate the key enzyme, DNA gyrase, thereby initiating RNA and protein synthesis. This activation of DNA gyrase takes place only when spermidine levels are optimal [20]. In our experiments it was shown that spermidine synthesis starts immediately with the beginning of outgrowth and is present in the outgrowing spores at adequate levels. Arresting DNA synthesis by HPUra or by growing a temperature-sensitive mutant at restrictive temperatures, has no effect on this early event of spermidine accumulation.

These findings are consistent with the notion that spermidine synthesis might be a prerequisite for the activation of the DNA gyrase. DNA gyrase activity has been suggested to constitute an essential step in the mechanism leading to outgrowth of germinated spores.

Acknowledgement

This work was supported by the Stiftung Volkswagenwerk.

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