Evidence that *Bacillus subtilis* Sporulation Induced by the Stringent Response Is Caused by the Decrease in GTP or GDP

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Partial amino acid deprivation of *Bacillus subtilis*, which evokes the stringent response, initiates sporulation not because the highly phosphorylated guanine nucleotides guanosine-5′-diphosphate-3′-diphosphate (ppGpp) and guanosine-5′-triphosphate-3′-diphosphate (pppGpp) increase but because GTP decreases. This was shown with a mutant (Myc) partially resistant to mycophenolate, an inhibitor of IMP dehydrogenase. Upon amino acid deprivation, the Myc mutant (62032) showed the usual increase in ppGpp and pppGpp but a reduced decrease in GTP, and only few cells sporulated. Extensive sporulation was restored by the addition of mycophenolate or decoyinine, an inhibitor of GMP synthetase, which caused a further decrease in GTP.

Several publications from this laboratory have shown that *Bacillus subtilis* sporulation can be initiated by the partial or transient deprivation of one or more amino acids. This deprivation causes a stringent response, including the transient increase in guanosine-5′-diphosphate-3′-diphosphate (ppGpp) and guanosine-5′-triphosphate-3′-diphosphate (pppGpp) (3, 4, 7), hereafter called (p)ppGpp. In vivo experiments have also shown that the stringent response causes the inhibition of IMP dehydrogenase, the first enzyme specifically needed for the synthesis of guanine nucleotides (4). In fact, the increase in (p)ppGpp was always accompanied by a decrease in GTP and GDP (3, 4, 6, 7). In relaxed (relA) mutants the (p)ppGpp increase was almost completely abolished, the GTP decrease was greatly diminished, and massive sporulation was absent. However, in both stringent (rel-†) and relaxed cells, the decrease in intracellular guanine nucleotides alone, resulting from guanine deprivation of a guanine auxotroph or from the addition of a specific inhibitor of GMP synthesis, initiated extensive sporulation without a concomitant increase in (p)ppGpp. Consequently, it had been argued that the decrease in GTP (or GDP) rather than the increase in (p)ppGpp was probably responsible for the initiation of sporulation in all cases (3, 4, 6, 7). However, it was still conceivable that (p)ppGpp, which inhibit many metabolic reactions in addition to that controlled by IMP dehydrogenase, might by themselves induce sporulation so that the decrease in GTP was not necessary when sporulation was initiated by the stringent response. This paper excludes this possibility.

IMP dehydrogenase is the specific target of mycophenolic acid (8), an antibiotic that inhibits GMP synthesis. Therefore, we reasoned that some mutants resistant to mycophenolic acid might be altered in IMP dehydrogenase so that they would be less sensitive to the inhibition by (p)ppGpp or that they might be altered in the feedback control of purine synthesis, producing increased amounts of IMP which would overcome the inhibition of IMP dehydrogenase by (p)ppGpp. To isolate mycophenolic acid-resistant mutants, we plated cells of *B. subtilis* 61886 (ΔilvB1 kauA1) on plates containing synthetic medium (7), 0.5 mM each isoleucine and valine (ilv-synthetic medium), and 1 mM mycophenolic acid. After 1 to 2 weeks at 37°C in a humid incubator, we isolated cells from the distinctly larger colonies. One of these mutants (strain 62032 = ΔilvB1 kauA1 Myc-6; Myc = mycophenolate resistant) was used for this paper. The mutant produced 36% spores in nutrient sporulation medium (1) and 56% spores in synthetic medium after the induction by decoyinine (0.5 mM), an inhibitor of GMP synthetase; this compares well with the parent (61886), which produced 71% spores in nutrient sporulation medium and 62% spores in synthetic medium after induction by decoyinine. The mutant strain retained the original genetic properties *ilv* (requirement for isoleucine and valine) and *kau* (no active transport for the oxo acid precursors) and grew at a higher rate (doubling time, 55 min) than did the parent (doubling time, 71 min) in ilv-

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FIG. 1. Induction of sporulation by mycophenolic acid. The parent strain 61886 and the Myc mutant strain 62032 were grown at 37°C in ilv-synthetic medium. When the OD$_{600}$ was 0.5, samples of the culture were distributed to 10 flasks containing mycophenolic acid (producing the final concentrations indicated on the abscissa). The titer of heat-resistant (20 min at 75°C) spores was determined after 10 h of further shaking.

FIG. 2. Induction of sporulation by the stringent response to partial deprivation of intracellular isoleucine. Cells were grown as in Fig. 1 to an OD$_{600}$ of 0.5. They were then washed with synthetic medium on a membrane filter and suspended in synthetic medium containing 0.5 mM valine and the stated concentrations of DL-oxomethylvalerate. The spore titers were determined 10 h later. The total viable cell titers ranged from $4 \times 10^7$ ml for very low oxomethylvalerate concentrations to $7 \times 10^9$/ml for high oxomethylvalerate concentrations. 6188b, Parent strain; 62032, Myc mutant strain.
mM valine and different concentrations of oxomethylvalerate. The parent (61886) showed a sporulation optimum at about 0.45 mM oxomethylvalerate, in agreement with previous data (7) (Fig. 2). In contrast, the Myc mutant (62032) sporulated much less, having an optimum of $10^4$ spores per ml at a concentration of 0.2 mM oxomethylvalerate.

However, the mutant still responded to amino acid deprivation by producing the typical stringent response of increasing the concentrations of (p)ppGpp. To measure these and other nucleotides, we grew both the mutant strain and the parent strain in jlv-synthetic medium to an $OD_{600}$ of 0.5, washed the cells in synthetic medium, and transferred them to synthetic medium containing 0.5 mM valine and 0.4 mM oxomethylvalerate. Just before and at different times after the transfer, we rapidly collected the cells of 100-ml samples of culture on a membrane filter and extracted them with 0.5 M formic acid. After rapid lyophilization and suspension in water, we chromatographed the nucleotides by high-pressure liquid chromatography on a Partisil PXS 10/25 SAX column (Whatman, Inc.) in the $SO_4^{2-}$ form as described earlier (7). Figure 3 shows the typical transient increase in ppGpp and pppGpp in the parent strain, and it demonstrates that the mutant strain produced the same increases. However, in contrast to the parent strain in which GTP decreased to 80 pmol per AM$_{600}$ unit, GTP decreased in the mutant strain only to 140 pmol per AM$_{600}$ unit. (One AM$_{600}$ unit is the amount of cells that produces an OD$_{600}$ of 1 if contained in 1 ml of solution.) The decrease in GTP to a lower concentration is apparently critical for the efficient initiation of sporulation, as earlier studies with decoyinine have also shown (5, 7). The addition of decoyinine (0.2 mM) or mycophenolate (0.1 mM) produced a more extensive and longer lasting decrease in GTP, and it induced sporulation (spore titer, $>10^7$/ml); the decrease

![Diagram](http://jb.asm.org/)

**FIG. 3.** Increase in (p)ppGpp in response to partial isoleucine deprivation. Cells were grown and transferred to medium containing DL-oxomethylvalerate (0.4 mM) as in Fig. 2. Just before cell transfer and at the indicated times thereafter, the cells of 100 ml samples were rapidly collected on a membrane filter (pore size, 0.45 μm; diameter, 10 cm) and extracted by formic acid; the concentration of extracted nucleotides was determined by high-pressure chromatography (7). (— — —) Parent strain (61886); (—— —) Myc mutant strain (62032).

**FIG. 4.** Changes in GTP and IMP during partial isoleucine deprivation. Cells were grown and extracted, and the nucleotides were chromatographed as in Fig. 3. At the time of cell transfer, an additional culture of the Myc mutant contained mycophenolate (100 μM). (A) GTP; (B) IMP. (— — —) Parent strain (61886); (—— —) Myc mutant strain (62032) without (●) and with (▲) mycophenolate. The vertical bars represent standard deviations of three separate experiments.
in GTP after mycophenolic acid addition is shown in Fig. 4A. The concentration of IMP was about 40% higher in the Myc mutant than in the parent strain, and it increased upon amino acid deprivation transiently to a value two to three times higher than that observed in the parent strain (Fig. 4B). In both strains the addition of decoyinine or mycophenolic acid did not cause an increase in (p)ppGpp (concentration, <3 pmol per AM600 unit; measured at the same times as GTP in Fig. 4A).

To determine whether the Myc mutant (62032) harbors a mutation in the guaA gene (or the neighboring region) which is responsible for the synthesis of IMP dehydrogenase (2), we transformed a guaA mutant (strain 62018 = Δilyl kauA1 guaA1) with DNA of the Myc mutant (62032). All 10 gua A" transformants examined produced a high spore titer (10^7/ml) after oxomethylvalerate starvation (0.4 mM). This result indicates that the inability of the Myc mutant (62032) to sporulate well after oxomethylvalerate starvation was not due to a mutation of IMP dehydrogenase. Presumably, the Myc mutant is changed in the regulatory feedback mechanisms of purine biosynthesis. This is indicated by the increased level of IMP (Fig. 4B) which may be responsible for the lesser decrease in GTP because it may enable IMP dehydrogenase to synthesize more XMP despite the presence of (p)ppGpp. Regardless of the detailed genetic alteration of the Myc mutant, our results show that sporulation is not induced directly by (p)ppGpp but is an indirect consequence of the stringent response. In agreement with all other studies concerning the initiation of sporulation, this indirect effect apparently is due to the decrease in GTP, caused by the inhibition of IMP dehydrogenase by (p)ppGpp.

LITERATURE CITED