Effects on *Bacillus subtilis* of a Conditional Lethal Mutation in the Essential GTP-Binding Protein Obg†

JAN KOK, KATHLEEN A. TRACH, AND JAMES A. HOCH*

Division of Cellular Biology, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

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The *obg* gene is part of the *spo0B* sporulation operon and codes for a GTP-binding protein which is essential for growth. A temperature-sensitive mutant in the *obg* gene was isolated and found to be the result of two closely linked missense mutations in the amino domain of Obg. Temperature shift experiments revealed that the mutant was able to continue cell division for 2 to 3 generations at the nonpermissive temperature. Such experiments carried out during sporulation showed that Obg was necessary for the transition from vegetative growth to stage 0 or stage II of sporulation, but sporulation subsequent to these stages was unaffected at the nonpermissive temperature. Spores of the temperature-sensitive mutant germinated normally at the nonpermissive temperature but failed to outgrow. The primary consequence of the *obg* mutation may be an alteration in initiation of chromosome replication.

Sporulation in *Bacillus subtilis* is under control of a large number of genes. Mutations that block the onset of sporulation are designated stage 0, or *spo0*, mutations. Stage 0 mutations have many pleiotropic effects, but all *spo0* mutants are characterized by their inability to form the asymmetric septum, the first morphological difference between normal cell division and sporulation initiation. The products of the *spo0* genes are involved in the transduction of the metabolic signals that induce sporulation to the transcription machinery. The main transcription factor in sporulation is SpooA, a protein homologous to several response regulators of two-component regulatory systems (8, 23). The SpooA protein is a transcription regulator repressing certain genes and activating others (19, 24, 25). For SpooA to carry out its regulatory functions, it needs to be activated by phosphorylation, which results in an apparent higher affinity for its binding site in vitro (25).

Phosphorylation of SpooA is the final step in the signal transduction pathway that controls transcription of the genes involved in the initiation of sporulation. This pathway includes KinA and KinB, kinases with homology to the transmitter kinases of two-component regulatory systems (2, 17), and the products of the *spo0B* and *spo0F* genes. KinA is capable of phosphorylating SpooF in vitro (17), and that phosphate is transferred via SpooB to SpooA (5). In this series of phosphotransfer reactions, which has been termed a phosphorelay (5), SpooB acts as a protein phosphotransferase catalyzing the transfer of phosphate from SpooF–P to SpooA. The SpooB protein phosphotransferase has no primary sequence similarity to transmitter kinases but resembles this class of proteins in the ultimate location and nature of the phosphate group transferred to SpooA. As outlined by Burbulys et al. (5), the fact that two proteins, SpooF and SpooB, are present between the transmitter kinase KinA and the response regulator SpooA has two important regulatory implications. First, SpooF could be the receiver of phosphate groups from different kinases and, thus, act as an accumulation point for various stress inputs. Second, SpooB could serve not only to couple this input to SpooA, but at the same time to provide an additional control point for regulation by cell cycle signals.

The *spo0B* gene is present in an operon structure together with a second gene, *obg* (26). The *obg* gene product, Obg, is a GTP-binding protein that is indispensable for the cell. Obg is a protein with similarity to proteins in the GTPase superfamily (3, 13). The similarity to this large class of proteins is confined to a small portion of the protein that constitutes a consensus GTP-binding sequence (26). Fluctuations in the GTP content of the cell are thought to be the primary cause of the initiation of sporulation in bacteria and yeasts (15, 27), and the presence of the gene for a GTP-binding protein in an operon structure with a *spo0* gene seems more than just a coincidence. In the model presented by Burbulys et al. (5), SpooB is thought to be a critical control point of the phosphorelay, and its activity may be subject to external control. Perhaps Obg functions to communicate the intracellular GTP levels to the sporulation initiation machinery. It is conceivable that Obg, in its GTP-bound form, stimulates growth and prevents sporulation by activating an intrinsic phosphatase activity of SpooB. In this scheme, lowered GTP levels would deactivate Obg.

Until now, the function of Obg in the cell and, if any, in sporulation was unclear. The study of its role in the cell cycle was hampered by the fact that Obg is essential for growth and that viable *obg* mutants were not available. As a first step in the elucidation of the role(s) Obg plays in the cell, a temperature-sensitive (Ts) *obg* mutant was isolated and characterized at the nucleotide level. The mutant was used to analyze the effect of *obg* (Ts) on growth and sporulation.

**MATERIALS AND METHODS**

**Bacterial strains, media, and transformation.** *Escherichia coli* strains were maintained on Luria-Bertani medium and agar (14) to which, when appropriate, chloramphenicol or ampicillin was added to 30 or 100 μg/ml, respectively. *E. coli* DH5α competent cells were obtained from Bethesda Research Laboratories, Inc., and transformed as specified by the manufacturer.

*B. subtilis* 1H642 (*trpC2* *phe*-1) and its derivatives and BR151
Tris-HCl–1 mM EDTA, pH 7.4. Two micrograms of denatured plasmid DNA was treated with o-methylhydroxylamine as described elsewhere (28). The DNA was used to transform B. subtilis JH642 to phenylalanine proficiency. The Phe' transformants were tested for their ability to grow at 45°C by picking them onto two minimal agar plates supplemented with trypto- 

one plate was incubated at 45°C, and the other was incubated at 30°C. Putative candidates were colony purified and tested again in liquid medium and as single colonies on 

agar plates for their ability to grow at 45°C.

Construction of pJH4222 deletion derivatives and complementation analysis. All plasmids were made in E. coli JM109 with selection for ampicillin resistance. The relevant part of each plasmid is shown in Fig. 1. Plasmids pJH4222-1, pJH4222-3, and pJH4222-5 were made by digestion of pJH4222 with EcoRI, HindIII, and EcoRV, respectively, and self ligation of the vector fragments. Plasmid pJH4222, isolated from E. coli GM48, was digested with Bsp106 and BamHI and treated with the Klenow fragment of E. coli DNA polymerase I, and the vector fragment was ligated. The resulting plasmid pJH4222-2. Plasmid pJH4222-4 was constructed from pJH4222-2 by deletion of the 1,566-bp AatII fragment. The 1,332-bp BglII-AatII fragment from pJH4222-2 was cloned in pJH101 (6) digested with BamHI and AatII, resulting in plasmid pJH4222-6. Plasmids pJH4222-7 and pJH4222-8 were made by insertion into pJH101, cut with BamHI and EcoRV, of the 1,703-bp BglII-Aval fragment and the 1,966-bp BglII- 

Ndel fragment, both from pJH4222-2, respectively. Plasmid pJH4222-9 is pJH101 carrying the 2,202-bp BglII-EcoRI fragment of pJH4222-2 into its BamHI and EcoRI sites.

Cloning of the obr(Ts) gene and overproduction of Ob(Ts) in E. coli. Chromosomal DNA of B. subtilis JH642:42-3, with pJH4222-3 inserted immediately upstream of the spoO operon via a single crossover event, was digested with BamHI and Clal and subsequently treated with the Klenow fragment of E. coli DNA polymerase I. After agarose gel electrophoresis, fragments larger than 7 kb were isolated, ligated, and used to transform E. coli DH5α competent cells. Among the Amp' transformants, a colony carrying a plasmid, pJH4423, with the expected size and restriction enzyme map was found.

Isolation of suppressors of the obr(Ts) mutations. B. subtilis JH642-42 carrying the obr(Ts) mutations was subjected to in vivo mutagenesis with nitrosoguanidine in a modification of the procedure described in reference 28. The incubation temperature during NTG (N-methyl-N'-nitro-N-nitrosoguanidine) treatment was 30°C. Aliquots of the mutagenized culture were plated on Schaefer's medium and incubated at 45°C to select for those cells which had regained the ability to grow at the nonpermissive temperature.

RESULTS

Isolation of a Ts mutation in the spoOB operon. The obr gene is located in an operon with the early sporulation gene spoOB (4, 7). Upstream of the spoOB operon is the gene for the

FIG. 1. Restriction map of the spoOB chromosomal region (arrows indicate directions of transcription) and the extents of chromosomal DNA (shown below) contained in pJH101-derived plasmids used in this study.

(lys met trpC2) were routinely maintained on Luria-Bertani medium. For sporulation assays, the cells were grown on Schaeffer sporulation medium (20). Spizizen’s salts medium was used as a minimal growth medium (21). When needed, chloramphenicol was used at 5 μg/ml. B. subtilis strains were transformed by the method of Anagnostopoulos and Spizizen (1).

Enzymes and reagents. Restriction enzymes were purchased from New England BioLabs, Inc., or Bethesda Research Laboratories and were used as recommended by the supplier. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim Biochemicals. Bacteriophage T4 polynucleotide kinase was from United States Biochemical Corp. Bacteriophage T4 DNA ligase, E. coli DNA polymerase I large fragment, and the 1-kb DNA ladder were obtained from Bethesda Research Laboratories. Acrylamel and bisacyramel were purchased from National Diagnostics.

DNA sequence analysis, primers, and PCR. DNA sequence analysis was done on double-stranded plasmid DNA by the dideoxy chain termination technique (18) with the Sequenase 2.0 kit from United States Biochemical. PCR-generated DNA was sequenced with the Amplicore cycle sequencing kit according to protocols of Perkin-Elmer Cetus. DNA amplification by PCR was done according to the GENE AMP kit and protocol of Perkin-Elmer Cetus on an Ericomp Inc. (San Diego, California), cyclor (4 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 48°C, and 50 s at 72°C, and 3 min at 72°C).

In vitro DNA mutagenesis and selection for a Ts mutation in obr. Plasmid pJH4222 was denatured by incubation for 5 min in 0.1 M sodium hydroxide–0.2 M EDTA, after which the DNA was precipitated with ethanol and resuspended in 10 mM

FIG. 2. Chromosomal structure of the integrant used to isolate the obr(Ts) mutation. Not all the restriction sites are shown. Arrows indicate directions of transcription. The extent of pJH4223 is shown below.
ribosomal protein L27, while downstream of the operon two genes involved in phenylalanine biosynthesis, pheA and pheB, are located (26). The region encompassing all these genes is present in plasmid pJH4222 (26) and is shown in Fig. 1. In order to isolate mutants in \textit{obg}, plasmid pJH4222 was treated with methyloxalate in vitro and the DNA was used to transform \textit{B. subtilis} JH642; Phe+ transformants were selected. More than 99% of the Phe+ transformants were sensitive to chloramphenicol and were thus the result of a double crossover event. Approximately 0.5% of the colonies obtained were apparently Spo−, as evidenced by their transparent appearance, and had probably acquired a mutation in the \textit{spoOB} gene. About 1,000 transformants were transferred to replicate plates and incubated at 30 or 45°C. One strain, JH642-4, did not grow at 45°C, either on minimal agar or on Schaeffer's agar plates. The strain did grow at 30°C and produced large transparent colonies at this temperature, indicating that it was unable to sporulate. As JH642-4 was sensitive to chloramphenicol, the strain was the result of a double crossover event.

The \textit{Spo−} and Ts phenotypes of JH642-4 are separable. Since \textit{SpoOB} is essential for sporulation but dispensable for growth, it seemed likely that the \textit{Spo−} and Ts of JH642-4 were due to different mutations. To try to separate these mutations, chromosomal DNA from JH642-4 was used to transform strain JH642 to Phe+. Both \textit{Spo−} and \textit{Spo+} colonies were obtained. All \textit{Spo−} colonies tested were unable to grow at 45°C. More importantly, some of the \textit{Spo−} colonies tested did not grow at 45°C, showing that the \textit{Spo−} phenotype and the Ts trait were separable. One of the \textit{Spo−} Phe+ colonies that did not grow at 45°C was purified and designated JH642-42.

Localization by single crossover recombination of the \textit{Spo−} and Ts mutations in the \textit{obg} operon. Figure 1 shows the maps of nine deletion derivatives of the integrative plasmid pJH4222 used to localize the mutations in JH642-4 by single crossover integration. Plasmid pJH4222-1 did not restore the strain's ability to grow at 45°C or to sporulate, indicating that both mutations are upstream of the \textit{EcoRI} site located in \textit{pheA}. Strains carrying plasmids pJH4222-3, pJH4222-5, and pJH4222-6 were unable to grow at 45°C, showing that the Ts mutation is downstream of the \textit{AattII} site in the \textit{S′} end of the \textit{obg} gene. This conclusion is in agreement with the results of transformations made with pJH4222-4, which carries the \textit{obg} gene sequences downstream of the \textit{AattII} site. Integrants of this plasmid form large \textit{Spo−} colonies at 45°C. To further pinpoint the Ts mutation, plasmids pJH4222-7, pJH4222-8, and pJH4222-9 were constructed. These plasmids carry the \textit{spoOB} gene and its promoter and progressively more of the \textit{obg} gene. Strain JH642-42 was used as a recipient in transformation in this case, and the Cm' colonies were tested for growth at 45°C. The transformants obtained with the three plasmids were all able to grow at 45°C, indicating that the Ts mutation is located in a 371-bp DNA sequence between the \textit{AattII} and \textit{Aval} sites in the \textit{S′} end of \textit{obg}.

Cloning of the mutated \textit{spoOB} operon. The Ts mutation in \textit{obg} was retrieved from the chromosome of JH642-4 by using the single crossover integrant of this strain carrying pJH4222-3 (Fig. 2). DNA from this integrant was digested with \textit{BamHI} and \textit{ClaI}, treated with Klenow polymerase, ligated, and used to transform competent cells of \textit{E. coli} JM109. The DNA from a colony carrying a plasmid with the proper restriction enzyme map was isolated and used to transform \textit{B. subtilis} JH642 to phenylalanine proficiency. Both \textit{Spo+} and \textit{Spo−} transformants were obtained. Most \textit{Spo−} colonies did not grow at 45°C, showing that both mutations present in strain JH642-4 were located on the isolated plasmid, pJH4223. As expected, both Ts and Ts+ colonies were found among \textit{Spo−} transformants.

The Ts \textit{obg} gene carries two mutations. Two primers flanking the \textit{AattII}-\textit{Aval} region of \textit{obg} were used to sequence this region from plasmids carrying the mutated \textit{obg} gene and the wild-type gene. The sequence obtained with pJH4222 was identical to that published (26). Two changes in this part of the nucleotide sequence were present in \textit{obg(Ts)}. Mutation of G to A at position 905 changed the glycine at codon 79 in \textit{obg} to a glutamate codon, and a G-to-A change at position 919 (codon 84) resulted in a change from Asp in \textit{Obg} to Asn in \textit{Obg(Ts)} (Fig. 3). In the nucleotide sequence of \textit{obg(Ts)} downstream of the \textit{Aval} site, up to codon 275, no further alterations were found (not shown).

Suppressors of the \textit{Obg(Ts)} mutations. By the NTG treatment described in Materials and Methods, four independent derivatives of JH642-42 that had regained the ability to grow at 45°C were isolated. Chromosomal DNA of each of the four

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Codon and amino acid changes in \textit{obg(Ts)} and revertants.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Growth curves for JH642 (○ and ●) and JH642-42 (□ and ■) in Luria-Bertani medium at 30°C (○ and □) and after shift to 45°C (● and ■). Cultures were shifted after 3 h.}
\end{figure}
suppressor mutants, carrying the sog-1, sog-2, sog-4, and sog-5 mutations, was isolated, and sog-1 was shown by transformation of JH642 to be very closely linked to obg(Ts) (results not shown). Two primers, one encompassing codons 35 to 40 of obg and the other complementary to codons 278 to 286, were used to amplify the intervening nucleotide sequences from the chromosomes of the four suppressor mutants. The PCR fragments were sequenced partially with an internal primer. The results are presented in Fig. 3 and show that all four suppressor mutations still carry the mutant Asn codon at position 84. In sog-2, sog-4, and sog-5, the Glu codon at position 79 in obg(Ts) had reverted to that for Gly, as in wild-type obg. In sog-1, this codon was changed to an Ala codon by an A-to-C mutation in the second position. The D84N mutation by itself is apparently not enough to elicit Ts. At the moment, it is not known whether the Ts phenotype of Obg(Ts) can be attributed solely to the Gly-to-Glu change or whether both mutations in Obg(Ts) are necessary for the Ts of the protein.

Growth characteristics of JH642-42 and effect of obg(Ts) on sporulation. Overnight cultures at 30°C of strains JH642 and JH642-42 in Luria-Bertani broth were diluted 1,000-fold and incubated at 30°C. After 3 h, half of each culture was transferred to 45°C. The Ts strain (doubling time = 54 min) grew more slowly at 30°C than did JH642 (doubling time = 36 min), but both strains reached comparable densities at the end of the growth phase (Fig. 4). Upon shifting to 45°C, JH642 grew at a faster rate and underwent eight doublings before reaching stationary phase 3 h after the temperature shift. Although strain JH642-42 initially grew faster after the temperature shift, the growth rate gradually decreased and the strain ultimately stopped growing after approximately three doublings (about 3 h).

To examine the relationship of Obg to the sporulation process, the effects on sporulation of a temperature shift at various times during growth of strains JH642 and JH642-42 obg(Ts) in Schaeffer’s medium were compared (Fig. 5). The strains were grown at 30°C and, at the times indicated, a sample of each culture was shifted to 45°C and allowed to grow for 12 to 24 h, after which the spores were counted.

Shifting the JH642 culture to 45°C during exponential growth or early stationary phase resulted in about 10^7 spores per ml. After this time, the yield of spores jumped to about 10^9/ml. In contrast, the ability of strain JH642-42 to produce spores after the shift from exponential growth was much lower but the capacity for sporulation under these conditions increased rapidly after the onset of stationary phase. In order to more precisely determine the requirement for Obg in sporulation, experiments with the Sterlini-Mandelstam downshift in defined media were undertaken (22). At intervals after the initiation of sporulation occasioned by the downshift, samples of the culture were placed at 45°C and allowed to sporulate overnight (Fig. 6). Under these conditions, strain JH642 produced about 10^8 spores per ml regardless of the time of shift after initiation. Although a small proportion of the population of JH642-42 cells was temperature resistant at the time of nutritional downshift, temperature resistance was gradually gained over the course of the next 3 h. The levels of spores at 30°C were identical in both cultures, but JH642 appeared to sporulate about 2 h earlier.

Germination properties of the obg(Ts) mutant. Germination and outgrowth studies were carried out on enriched media supplemented with glucose and l-alanine. Both strains, JH642 and JH642-42, germinated normally at 30 and 45°C with an expected drop in optical density for the first hour of incubation (Fig. 7). Subsequently to this, the JH642 culture began to show a rise in optical density at both 30 and 45°C, as did the JH642-42 culture at 30°C. Microscopically, the spores in these cultures germinated to a phase-dark form and grew out to normal-sized bacillary forms. Strain JH642-42 at 45°C showed the normal decrease in optical density, but no rise in optical density was observed in the following hours. Microscopically, the phase-dark spores were not observed to outgrow to rods. After 6 h, the optical density began to rise because of Ts revertants in the culture. The scatter in the data for JH642-42 at 45°C is due to clumping of the germinated spores that fail to outgrow.

DISCUSSION

The Obg protein belongs to the GTPase superfamily of proteins that likely carry out essential roles in all cells (3). In bacteria, several of these proteins that differ from the classical translation initiation and elongation factors or putative protein secretion factors have recently been recognized (13). The best studied of these is the Era protein of E. coli. Ts mutations in Era are lethal at high temperature, and the mutant protein exhibits Ts GTP binding (10) and GTPase activity (12) in vitro. This suggests that both GTP binding and GTPase are essential to the functioning of Era. Depleting the cells of Era activity by growth at a nonpermissive temperature causes pleiotropic effects, including derepressed synthesis of heat shock genes, lower ppGpp pools, and altered carbon metabolism (11). There are conflicting results for the effect of Era depletion on cellular morphology. The Ts mutant maintained normal cellular morphology at the nonpermissive temperature (11), whereas depletion of Era by reduction of transcription of its gene resulted in filamentous cells lacking septa but with normal DNA synthesis and segregation (9).

The Ts Obg mutant shares some of the properties of the Era
mutants, although Obg and Era are not structurally related. In culture, the Ts Obg mutant continues to grow for two to three generations after shift to the nonpermissive temperature. The Ts Era mutant grows for six to eight generations after a similar shift (11). These data suggest that in both cases some dilution of the proteins or their ultimate products or effects must occur before cessation of cell division. When growth ceases in the Ts Obg mutant, the cells are of normal size with no hint of filamentation. Since cells continue to divide without filamentation for about two generations after shift of exponential phase cultures to 45°C, Obg does not seem to affect septation and the Ts mutation cannot be seriously impairing the basic processes of RNA and protein synthesis. The properties of the Ts Obg mutant are those that would be expected of a mutant blocked in the initiation of chromosome replication. More than one generation after temperature shift may be needed to resolve the multikary chromosome replication of exponential cells in rich media (16) and reach a point at which initiation becomes limiting for growth. This is in contrast to the conclusion that Era is involved in the cell cycle and required for growth but cannot be affecting DNA replication since DNA continues to be produced in cells depleted of Era (9). It seems possible that both proteins are involved in signal transduction systems that control the cell cycle but each has specific targets.

Temperature shift experiments with the Ts Obg mutant established that Obg was essential during exponential growth and during the first stages of sporulation. In a rich sporulation medium, the capacity for sporulation at the nonpermissive temperature was gained rapidly after the end of log-phase growth and reached its maximal value 4 to 5 h after the end of log-phase growth. In Sterlini-Mandelstam resuspension me-


