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Effects of Temperature-Sensitive Variants of the *Bacillus subtilis* *dnaB* Gene on the Replication of a Low-Copy-Number Plasmid

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The *dnaB* gene of *Bacillus subtilis* is involved in the initiation of DNA replication and also in the binding of the chromosomal origin to the bacterial membrane. We studied the effect of temperature-sensitive *dnaB* mutants (*dnaB1* and *dnaB19*) on the replication and on the DNA-membrane binding of the plasmid pKW1, which was derived from the low-copy-number plasmid pBS2. In the *dnaB19* mutant, pKW1 was not able to replicate at the restrictive temperature. In the *dnaB1* mutant, however, the dimeric form of pKW1 DNA was preferentially produced at the restrictive temperature, but the replication of the monomeric form was totally blocked. We also examined the effects of the *dnaB*(Ts) gene on the DNA-membrane binding of both the double-stranded and single-stranded DNA from pKW1. The single-stranded DNA from pKW1 was prepared from the DNA of the phage M13 mp19, which contained the origin of replication of pKW1. In the *dnaB1* mutant, pKW1 DNA in both the double-stranded and single-stranded form was released from the membrane at the restrictive temperature. On the other hand, in the *dnaB19* mutant, only double-stranded DNA, and not single-stranded DNA, was released from the membrane at the restrictive temperature. These results suggest that the product of the *dnaB* gene has at least two domains which influence the replication of DNA and the binding of DNA to the cell membrane in separate ways.

During the past decade, studies on the replication of DNA have revealed that a variety of mechanisms and enzymes are involved in the replication of DNA in both procaryotes and eucaryotes (7, 13). Very little is known, however, about how the machinery of replication is related to the regulation of cell growth. Jacob et al. (5) proposed a model in which the replication of DNA is correlated with the growth and division of procaryotic cells. They postulated the attachment of the chromosome to the bacterial membrane, and suggested that such attachment might play a key role in the regulation of DNA replication and chromosomal segregation into daughter cells. Since then, evidence has accumulated to support this hypothesis from studies of both *Bacillus subtilis* (11, 15–17, 21) and *Escherichia coli* (2, 12).

In the case of *B. subtilis*, Winston and Sueoka (20) showed that the function of the *dnaB* gene is directly involved in the binding of the chromosomal origin to the bacterial membrane, as well as in the initiation of DNA replication. Their results provided the first evidence that the initiation of DNA replication is related to the association of DNA with the membrane. However, *dnaB* is the only gene, which has been found thus far in procaryotes, that has been shown to be directly involved in the binding of DNA to the cell membrane.

Recently, the *dnaB* gene was cloned; and the complete nucleotide sequence, as well as an open reading frame, was determined (3, 10). The amino acid sequence of this open reading frame contained potential binding sites for both DNA and ATP. Computer analysis of the sequencing data revealed that no homologous nucleotide sequence has been identified in *E. coli*. However, the biological function of the product of the *dnaB* gene is poorly understood.

We have been studying the replication of the plasmid pKW1, which is a useful model system with which to investigate the function of the *dnaB* gene. The plasmid pKW1 carries the origin (2.3 kilobases [kb]) of plasmid pBS2, which is indigenous to *B. subtilis*, has a low copy number (1), and has a chloramphenicol resistance gene derived from plasmid pC194. Recently, we have examined (R. Forough, J. Herrick, and K. Watabe, J. Gen. Microbiol., in press) requirements of host functions for replication of various plasmids in *B. subtilis* by a double-labeling method and found that the plasmid pKW1 requires host *dnaB* (*dnaB19*) *dnaC*, *dnaE*, *dnaF*, *dnaG*, and *dnaH* functions for its replication. Thus, its mode of replication is considered to resemble closely the chromosomal replication of *B. subtilis*.

In the present study, we investigated the effects of two temperature-sensitive (Ts) mutations in the *dnaB* gene, namely, *dnaB1* and *dnaB19*, on the replication of pKW1. Our data suggest that there are at least two important domains in the *dnaB* gene product and that these domains are involved in the replication of DNA and the binding of DNA to the cell membrane in different ways.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. subtilis* 463 (*trp his phe polA*), 463BI (*his phe polA dnaB1*), and 463BII (*his phe polA dnaB19*) and *E. coli* JM103 were from our laboratory stocks. *dnaB1* and *dnaB19* were identical to *dnaB* mutants described previously by White and Sueoka (18) and Karamata and Gross (6), respectively. These genes were introduced into *B. subtilis* 463 by transformation. Plasmids pBS2 (1) and pHV60 (9) were kindly provided by W. Goebel (Universität Würzburg, Würzburg, Federal Republic of Germany) and D. Ehrlich (Institut de Recherche en Biologie Moleculaire, Paris, France). Plasmid pKW1 and M13 mp19-pKW1 were constructed as shown in Fig. 1. The plasmid pKW1 can replicate in both *E. coli* and *B. subtilis*. It expresses chloramphenicol resistance and has a low copy number (ca. ~2 to 5), similar to that of the parent plasmid pBS2 (1).

Assay for plasmid replication. Plasmids pKW1 and pUB110 were introduced into *B. subtilis* 463, 463BI, and

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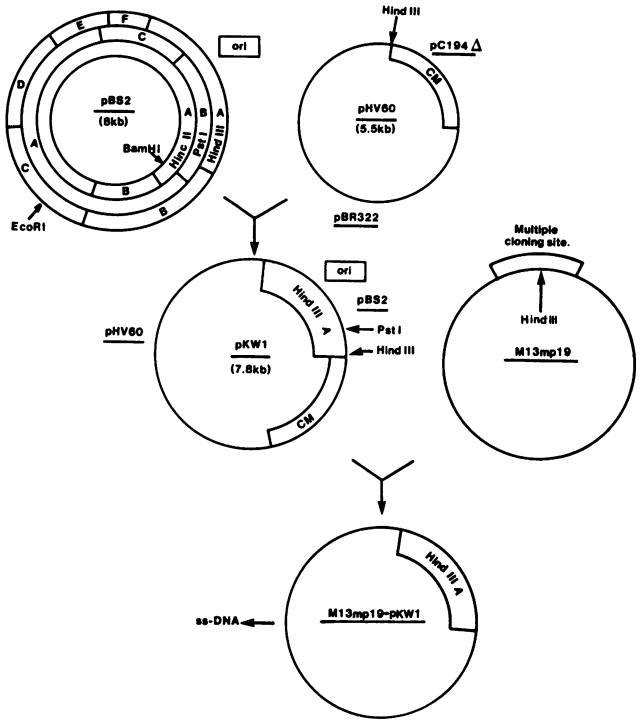

FIG. 1. Physical map of pBS2 and construction of pKW1 and M13 mp19-pKW1. pHV60 is a deletion mutant of a chimeric plasmid between pBR322 and pC194. The plasmid can replicate in *E. coli* but not in *B. subtilis* because of the absence of the region around the origin of replication of pC194. The replication origin of pBS2 (*Hin*dIII A fragment) was inserted into the pHV60 *Hin*dIII site. The resultant plasmid was designated pKW1. The *Hin*dIIIA fragment of pBS2 was also inserted into the *Hin*dIII site of M13 mp19, and the resultant phage was designated M13 mp19-pKW1.

463BII by transformation, as described previously (19). Transformants were grown in L-broth medium at 32°C until the optical density at 600 nm (OD_{600}) reached 0.1. Cultures were divided into two portions (1 ml each) and further incubated at 32 or 46°C for 2 h. Cells were harvested and lysed with lysozyme (100 μg/ml) and sodium dodecyl sulfate (1%). NaCl (1 M) was added to the lysate, and the mixture was centrifuged at 12,000 × *g* for 10 min to sediment the bulk of the chromosomal DNA. The supernatant was extracted with phenol, and the DNA was precipitated with ethanol. The DNA was subjected to electrophoresis on a 0.8% agarose gel, stained, and photographed.

Assay for the binding of DNA to the membrane. *B. subtilis* cells harboring pKW1 were grown in GMI medium (19), which contained 10 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) per ml. Preparation of the DNA-membrane complex was performed in essentially the same way as that described by Sueoka and Hammers (16). Cell lysates were subjected to centrifugation on cesium chloride-sucrose gradients in a rotor (SW50.1; Beckman Instruments, Inc., Fullerton, Calif.) for 40 min at 35,000 rpm. After fractionation of the gradients, each fraction was dialyzed against buffer (0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]) and extracted with phenol, and the DNA was precipitated with ethanol. Cultures of *B. subtilis* 463 were transformed with the DNA from each fraction, and the number of chloramphenicol-resistant colonies was counted for each culture.

The binding of single-stranded DNA (ssDNA) to the host membrane was examined by using ssDNA prepared from M13 mp19 and M13 mp19-pKW1 phages. Samples of the ssDNA were mixed with competent cells of *B. subtilis* 463, 463BI, or 463BII. The mixtures were incubated for 30 min at 32°C and divided into two portions. The cultures were further incubated for 15 min at 32 or 46°C. Cells were harvested and lysed, and the DNA-membrane complex was obtained by fractionation on gradients, as described above. Each fraction was extracted with phenol, and the DNA was precipitated with ethanol. Transforming activities of the phage DNA in each fraction were assayed with *E. coli* JM103, as described previously (8). The number of PFU was counted in each case.

RESULTS

Effects of Ts variants of the *dnaB* gene on the replication of pKW1 and pUB110. Plasmids pKW1 and pUB110 were introduced into *B. subtilis* 463BI (*dnaB1*) and 463BII (*dnaB19*) by transformation and examined for their ability to replicate at the permissive and the nonpermissive temperatures. Replication of pKW1 was blocked at 46°C in the *dnaB19* mutant (Fig. 2A). On the other hand, in the *dnaB1* mutant, plasmid DNA corresponding to the dimeric form was preferentially produced, and synthesis of monomeric DNA was completely shut off at the nonpermissive temperature.

We compared these results with those obtained with another plasmid, pUB110, and found that pUB110 also produced dimeric DNA during replication in the *dnaB1*

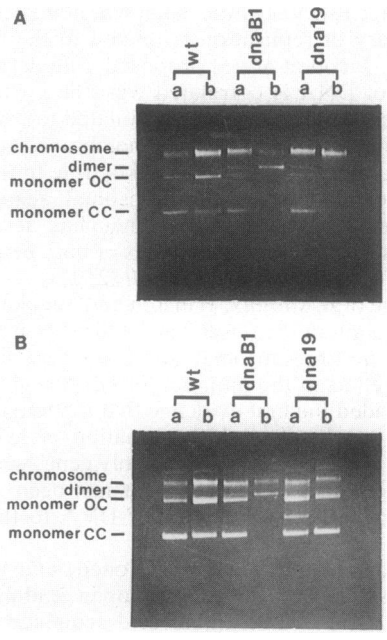

FIG. 2. Effects of *dnaB1* and *dnaB19* on the replication of plasmids pKW1 and pUB110. Plasmids pKW1 (A) and pUB110 (B) were introduced into *B. subtilis* 463 (wild type [wt]), 463BI (*dnaB1*), or 463BII (*dnaB19*) by transformation. Transformants were cultured in L-broth medium at 32°C until the OD_{600} reached 0.1. Cultures were split into two portions (1 ml each) and further incubated at 32°C (lane a) or 46°C (lane b) for 2 h. Plasmid DNA was prepared as described in the text and subjected to electrophoresis on a 0.8% agarose gel. Bars indicate positions of chromosomal DNA, dimeric plasmid DNA, open circular (OC) plasmid DNA, and closed circular (CC) plasmid DNA.

mutant at the restrictive temperature (Fig. 2B). In the *dnaB19* mutant, however, pUB110 replicated normally, as was described previously by others (14).

The kinetics of replication of pKW1 DNA in the *dnaB1* mutant at the permissive and the nonpermissive temperatures is shown in Fig. 3. At the permissive temperature, the monomeric form of the DNA was preferentially produced over the course of incubation, and only a small amount of dimeric DNA was synthesized. At the nonpermissive temperature, however, no monomer DNA was observed at any time during the incubation and the dimeric form of the DNA was produced as the duration of incubation increased.

To confirm that these dimeric forms of DNA are, indeed, pKW1 DNA, plasmid DNA was prepared from *dnaB1* cells grown at the nonpermissive or the permissive temperature. Extracted DNAs were digested with *Eco*RI, which cuts pKW1 DNA at a single site. Samples of DNA prepared from cells grown at the permissive or the nonpermissive temperature and digested with *Eco*RI migrated at the same position as full-length linear DNA (Fig. 4). This result suggests that the DNA, which is produced preferentially at the nonpermissive temperature, is head-to-tail dimeric DNA or a dimeric concatemer of pKW1.

Effects of Ts variants of the *dnaB* gene on the binding of DNA to the cell membrane. As mentioned above, Winston and Sueoka (20) have shown that the *dnaB* gene is directly involved in the binding of the chromosomal origin to the cell membrane. To examine the effects of *dnaB1* and *dnaB19* on the binding of pKW1 DNA to the cell membrane, we first examined whether pKW1 does actually bind to the cell membrane. We used essentially the same methods used to examine binding of the plasmid pUB110 as those described

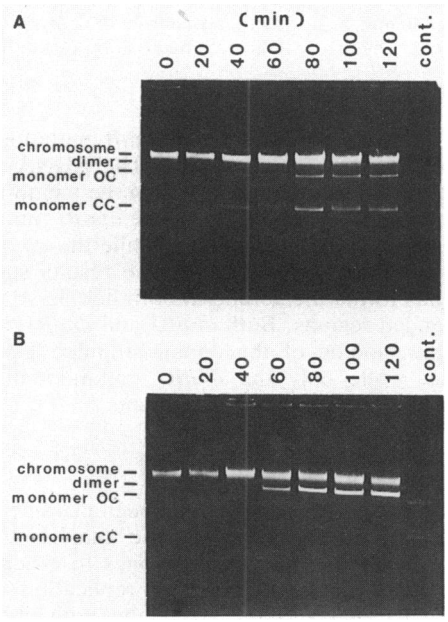

FIG. 3. Kinetics of replication of the plasmid pKW1. *B. subtilis* 463BI(pKW1) was grown in L-broth at 32°C until the OD_{600} reached 0.1. The culture was split into two portions and further incubated at 32°C (A) or 45°C (B). Samples of 1 ml were withdrawn at 0, 20, 40, 60, 80, 100, and 120 min. Plasmid DNAs were prepared as described in the text and were subjected to electrophoresis on a 0.8% agarose gel. The last lane contains pKW1 DNA as a control (cont.). Bars indicate positions of chromosomal DNA, dimeric plasmid DNA, open circular (OC) plasmid DNA, and closed circular (CC) plasmid DNA.

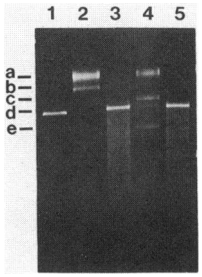

FIG. 4. Digestion of the dimeric DNA with *Eco*RI. Plasmid DNAs were prepared from *B. subtilis* 463BI(pKW1), which was grown at 32 or 45°C. Plasmid DNAs were digested with *Eco*RI, which cuts pKW1 at a single site. The digested DNAs were subjected to electrophoresis on a 0.8% agarose gel. Lane 1, control pKW1 DNA digested with *Eco*RI; lane 2, undigested pKW1 DNA; prepared from cells grown at 46°C; lane 3, plasmid DNA as described for lane 2; digested with *Eco*RI; lane 4, undigested pKW1 DNA; prepared from the cells grown at 32°C; lane 5, DNA described in lane 4; digested with *Eco*RI. Bars indicate positions of chromosomal DNA (a), dimeric plasmid DNA (b), open circular plasmid DNA (c), full-length linear plasmid DNA (d), and closed circular plasmid DNA (e).

by Winston and Sueoka (20). The DNA-membrane complex was prepared from cells which contained pKW1 and which were grown in the presence of [^{3}H]thymidine. The complex was obtained by fractionation on cesium chloride-sucrose density gradient (Fig. 5). The peak of transforming activity of chloramphenicol resistance corresponded to a peak of radioactivity which corresponded to a density of about 1.52. Free DNA, which was run on a parallel gradient, remained close to the top of the gradient. These results are in good agreement with those described by Sueoka and Hammers (16). Therefore, we conclude that most pKW1 DNA binds to the cell membrane of *B. subtilis*, in the same way as the chromosomal origin and pUB110 DNA bind to the membrane (16, 20).

To extend this observation further, we investigated whether pKW1 ssDNA bound to the cell membrane. M13 mp19-pKW1, which consists of the replication origin of pKW1 (*Hin*dIII A fragment) ligated into M13 mp19, was introduced into competent cells of *B. subtilis* 463. Although most of the DNA appeared to be degraded, a small fraction of ssDNA was introduced into the cells. After these cells were incubated at the permissive or the nonpermissive temperature, the DNA-membrane complex was obtained by fractionation on a cesium chloride-sucrose gradient, and each fraction was assayed for its ability to form plaques. The ssDNA that contained the origin of replication of pKW1 sedimented as a DNA-membrane complex, while the ssDNA that did not contain the origin of pKW1 sedimented at the same position as free DNA (Fig. 6). These results suggest that the ssDNA region of pKW1 can bind to the cell membrane just as the double-stranded DNA can.

Using these systems, we examined the effects of the *dnaB1* and *dnaB19* mutations on the binding of pKW1 DNA to the cell membrane. Double-stranded pKW1 DNA was released from the membrane at the restrictive temperature in both the *dnaB1* and *dnaB19* mutants (Fig. 7). As a control, we also examined the effect of a Ts variant of the *dnaH* gene on the formation of the DNA-membrane complex. The *dnaH* gene is one of the genes required for the replication of pKW1 (Forough et al., in press). Figure 7C shows that the *dnaH*(Ts) mutation did not affect the binding of the DNA to the membrane at the nonpermissive temperature.

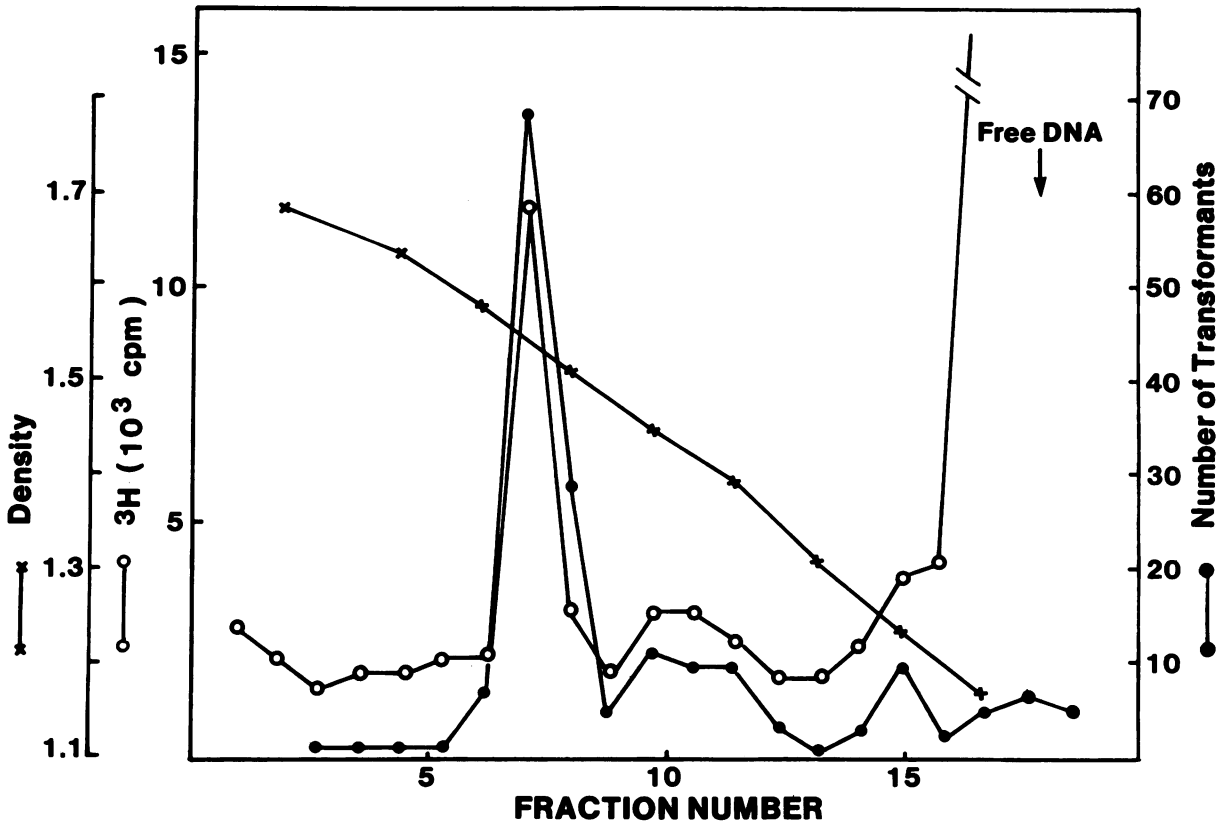

FIG. 5. Plasmid pKW1 binds to the cell membrane. *B. subtilis* 463(pKW1) was grown in GMI medium in the presence of 10 μCi of [^{3}H]thymidine per ml until the OD_{600} reached 0.5. Cells were harvested and lysed as described previously (17). The lysate was centrifuged in a cesium chloride-sucrose gradient in a rotor (SW50.1; Beckman) at 35,000 rpm for 40 min. After fractionation from the bottom of the tube, radioactivity (○) and transforming activity (●) were assayed as described in the text. The arrow indicates the position of the free form of pKW1 DNA run in a parallel gradient as a control.

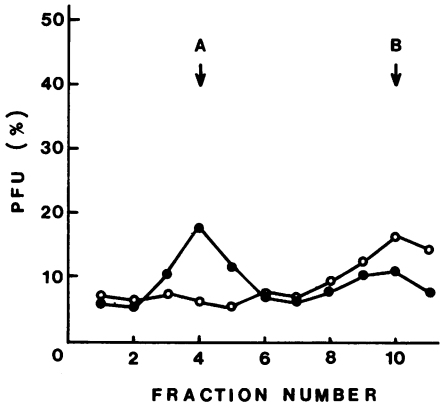

FIG. 6. ss-DNA from M13 mp19 and M13 mp19-pKW1 binds to the cell membrane. Samples of the ssDNA of M13 mp19 and M13 mp19-pKW1 were prepared from phages. Competent *B. subtilis* 463 cells were mixed with either M13 mp19 (○) or M13 mp19-pKW1 (●) and incubated at 32°C for 30 min. Cells were harvested and lysed as described in the text. Lysates were centrifuged as described in the legend to Fig. 5. DNA from each fraction was extracted and examined for transforming activity in *E. coli* JM103 by measuring the PFU. The ratio (percent) of PFU in each fraction to the total number of PFU (1,200) was plotted. Arrows indicate the position of the DNA-membrane complex (A) and the free form of M13 ssDNA (B).

Figure 8 shows the effects of *dnaB* mutations on the binding of ssDNA to the membrane. The ssDNA containing the origin of pKW1 was released from the membrane of the complex prepared from cultures of the *dnaB1* mutant grown at the nonpermissive temperature, while the *dnaB19* mutation did not affect the binding. These results suggest that pKW1 binds to the membrane in both double-stranded and single-stranded regions. Both *dnaB1* and *dnaB19* mutations affected the binding of the double-stranded DNA to the membrane, while only the *dnaB1* mutation affected the binding of the ssDNA to the membrane.

DISCUSSION

Mutations in the *dnaB* gene have been classified into two groups, groups I and II, by genetic analysis (4). *dnaB1* belongs to group I and *dnaB19* belongs to group II. Both mutations affect the initiation of DNA replication (4). Recent DNA sequence analysis has revealed that both mutations are in the same open reading frame, which could encode a protein with a molecular weight of 56,000 (3, 10). As described by Winston and Sueoka (20), however, these groups of mutations have different phenotypes with respect to their effects on the replication of DNA and on the binding of plasmid pUB110 DNA to the cell membrane. The *dnaB1* mutation affected both the replication of DNA and the binding of pUB110 DNA to the cell membrane, while *dnaB19* did not affect either of these events. In this report,

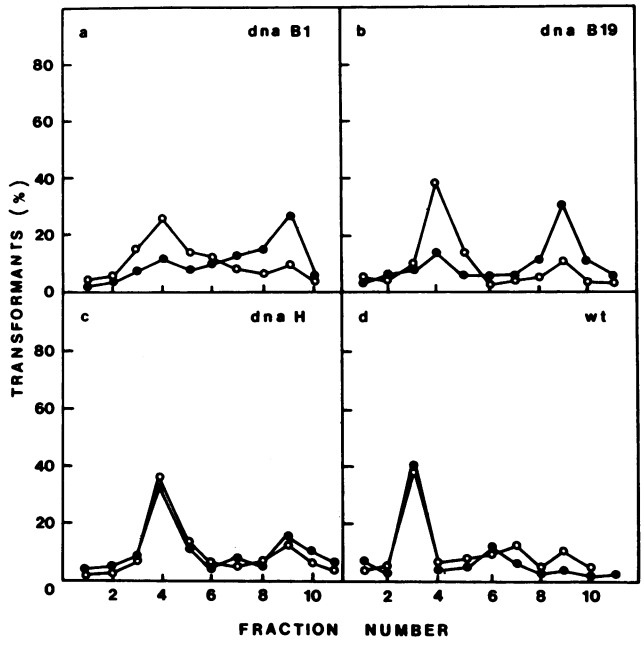

FIG. 7. Effects of Ts variants of the *dnaB* gene on the binding of DNA to the cell membrane. Cultures of *B. subtilis* 463BI(pKW1) (a), 463BII(pKW1) (b), 463 *dnaH*(pKW1) (c), and 463(pKW1) (d) were grown at 32°C until the OD_{600} reached 0.1. Each culture was split into two portions and further incubated at 32°C (○) or 46°C (●) for 20 min. Cells were harvested and lysed, and the DNA-membrane complexes were analyzed as described in the legend to Fig. 5.

we have described that both *dnaB1* and *dnaB19* mutations affected the replication of DNA and the binding to the cell membrane of pKW1 DNA. There are clear differences, however, between the effects of the *dnaB1* and *dnaB19* mutations. In the case of *dnaB1*, pKW1 produced preferentially the dimeric form of DNA at the nonpermissive temperature, while the *dnaB19* mutation blocked the total replication of the DNA. This exclusive production of the dimeric form was also observed in the case of plasmid pUB110 in the *dnaB1* mutant. By contrast to pKW1, however, pUB110 replicated normally in the *dnaB19* mutant. This sharp difference between pKW1 and pUB110 is probably due to differences in the modes of replication between these plasmids. pKW1 has a low copy number, while pUB110 has a high copy number. Generally, a low-copy-number plasmid requires rigid control of replication and segregation of the DNA to prevent a loss of plasmids during cell proliferation (13). Therefore, it is tempting to assume that the *dnaB19* domain is responsible for such control. It should be noted that the *dnaB19* domain is not necessary for the binding of the ssDNA of pKW1 to the cell membrane.

From the results of DNA-membrane binding of the ssDNA, it is likely that one domain in the product of the *dnaB* gene, which includes the site of the *dnaB1* mutation, is responsible for binding to the membrane of the region of ssDNA which is probably actively undergoing replication. If this binding is impaired by a mutation such as *dnaB1*, the plasmid is released from the membrane and a dimeric form of DNA may be produced. Alternatively, the dimeric form of DNA may not need to bind to the membrane via a region of ssDNA and may be used preferably as a template.

We do not know the exact shape of the dimeric form. However, the dimeric DNA, when digested with *Eco*RI, migrated at the same position as a full-length linear DNA. This result suggests that the DNA is either a head-to-tail dimeric form of DNA or a dimeric concatemer. In either case, this intriguing phenomenon of the dimeric formation should provide an important clue to the function of the *dnaB* product.

Since the *dnaB*(Ts) mutation has a phenotype of deficiency in initiation, it is possible that when the product of the *dnaB* gene is inactivated, the chromosomal DNA, which binds to the membrane via double-stranded and single-stranded regions of DNA, is released from the cell membrane and continues to replicate until the termination site is reached, but the daughter strand cannot segregate. As a result a concatemer is formed, and consequently, the chromosome cannot reinitiate a round of replication. Since the association of DNA with the membrane offers a mechanism for segregation of the chromosomes, this hypothesis is quite attractive. To prove this hypothesis, it will be essential to isolate the product of the *dnaB* gene and to characterize it biochemically. Thus far, very little is known about the biochemical nature of the product of the *dnaB* gene. It is, however, noteworthy that the nucleotide sequence of the *dnaB* gene contains consensus amino acid sequences for the binding of DNA and ATP (3, 10). In fact, recently we identified the *dnaB* gene product in the cell extract of *B. subtilis* and found that the gene product has DNA-binding

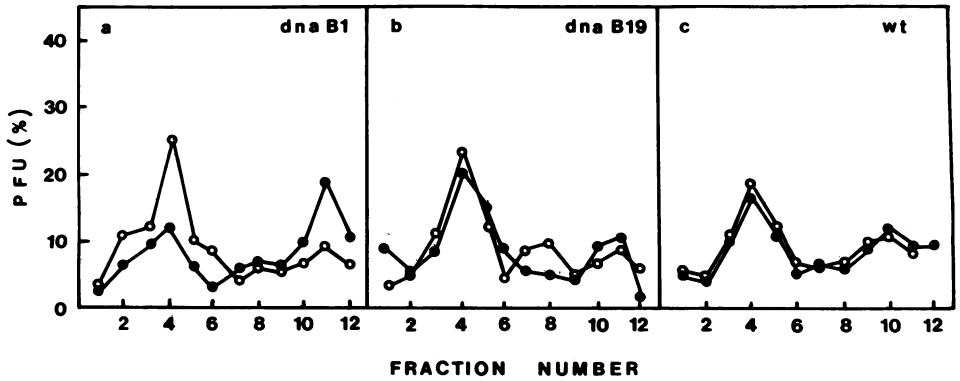

FIG. 8. Effects of Ts variants of the *dnaB* gene on the binding of ssDNAs to the cell membrane. Competent cells were prepared from *B. subtilis* 463BI (a), 463BII (b), or 463 (c). Samples of 400 μl of competent cells were mixed with 5 μg of ssDNA prepared from phage M13 mp19 or M13 mp19-pKW1. The mixtures were incubated at 32°C for 30 min, split into two portions, and further incubated at 32°C (○) or 46°C (●) for 15 min. Cells were harvested and lysed, and the DNA-membrane complexes were analyzed as described in the legend to Fig. 6.

activity (K. Watabe and D. Forough, Biochem. Biophys. Res. Commun., in press).

It is still unknown whether a counterpart of the *dnaB* gene exists in *E. coli*. The DNA sequence of the *dnaB* gene does not have significant homology with any other *E. coli* genes that have been sequenced thus far (3, 10). In *E. coli*, *dnaA* appears to be the only gene that is required for the initiation of DNA replication (22). The counterpart of the *dnaA* gene of *E. coli* has already been identified in *B. subtilis* and does not correspond to *dnaB* (10). Therefore, *B. subtilis* has at least two genes which are required for the initiation of replication. It is clear that the *dnaB* gene is involved in the initiation and, perhaps, in the regulation of DNA replication. The most interesting question to be answered is whether the same gene is also involved in the regulatory machinery which links the replication of DNA to cell growth and to the partitioning of chromosomes to daughter cells, since *dnaB* is required for the binding of the DNA to the cell membrane.

Plasmids pKW1 and pUB110 offer excellent models with which to investigate the function of the *dnaB* gene.

ACKNOWLEDGMENT

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