Characterization of two *Bacillus subtilis* proteins required for the initiation, restart, and control of DNA replication

by

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ABSTRACT

DnaB and DnaD are essential proteins that function in the initiation and control of DNA replication in *Bacillus subtilis*. I found that DnaB and DnaD are required to load the replicative helicase onto chromosomal origins during replication initiation. DnaB and DnaD are also involved in loading helicase during replication restart at sites of stalled replication forks.

Despite the fact that DnaB and DnaD are thought to work together to load helicase, DnaB and DnaD are found in separate subcellular compartments. I showed that DnaB is found in the membrane fraction of cells, and DnaD is found in the cytoplasmic fraction. This separation could prevent helicase loading during the majority of the cell cycle. I isolated a missense mutation in *dnaB, dnaBS371P*, that disrupts the spatial separation of DnaB and DnaD. I isolated *dnaBS371P* as a suppressor of the temperature sensitivity of *dnaBts* cells and *dnaDts* cells. *dnaBS371P* also suppresses the growth defects of *ApriA* cells, which cannot restart replication at stalled forks. I found that a significant fraction of DnaD is found in the membrane fraction of *dnaBS371P* cells. In addition, I observed a direct interaction between DnaBS371P and DnaD that is not observed between the wild-type proteins. I hypothesize that the DnaB-DnaD interaction is regulated, thereby controlling when these two proteins converge at the membrane to coordinate helicase loading.

*dnaBS371P* cells lack proper control of replication, suggesting that the spatial separation of DnaB and DnaD is an important mechanism of replication control in *B. subtilis*. I showed that *dnaBS371P* cells over-initiate replication when grown slowly in minimal medium, but contain decreased DNA content when grown faster, in rich medium. This is consistent with an inability of *dnaBS371P* cells to adjust the frequency of initiation according to growth rate. I also found that cells over-producing DnaBS371P are filamentous, contain decreased DNA contents, and are hypersensitive to DNA-damaging agents. These abnormalities may result from a defect in replication restart at damaged or stalled replication forks. Thus, whereas *dnaBS371P* suppresses the defects of mutant cells that cannot initiate or restart replication, expressing DnaBS371P in wild-type cells causes defects in initiation and restart.

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Chapter 1:

Introduction
All cells must replicate their genomes prior to cell division in order to pass on a complete genome to each newborn cell. DNA replication is highly regulated to ensure that newborn cells neither lack any necessary genetic information, nor suffer the consequences of containing excess DNA. The amount of DNA replication that occurs is primarily controlled at the level of replication initiation. The steps of replication initiation appear to be similar in Gram-negative and Gram-positive bacteria, although some of the proteins involved in these steps differ between the two groups. Prokaryotes possess multiple mechanisms for control over initiation, but Gram-negative and Gram-positive bacteria seem to possess different proteins that regulate this process. This thesis focuses on the roles of two essential proteins found only in Gram-positive bacteria that function in both replication initiation and replication control in the model Gram-positive organism Bacillus subtilis.

The three phases of DNA replication. DNA replication consists of three phases: replication initiation, replication elongation, and replication termination. Replication is mainly controlled at the initiation phase. The events that occur during replication initiation are common to both prokaryotes and eukaryotes. In both groups, replication initiation begins with the binding of an initiator protein to a specific DNA sequence called an origin of replication. In bacteria, this initiator protein is DnaA; in eukaryotes, the initiator complex consists of multiple ORC (origin recognition complex) proteins (Figure 1). After DnaA or ORC has bound to origins, double-stranded DNA in the origin region is unwound to create a bubble of single-stranded DNA (Figure 1). In both cases, the region of DNA at the origin that is unwound is A+T rich. This DNA unwinding is achieved by active DnaA protein in bacteria and by an unknown mechanism in eukaryotes (Baker and Bell, 1998; Bell and Dutta, 2002; Davey and O'Donnell, 2000;
binding of the initiator protein to the origin

(initiator is DnaA in prokaryotes, ORC in eukaryotes)

DNA unwinding

(unwinding by DnaA in prokaryotes)

DNA unwinding

(loading by helicase loader in prokaryotes)

entry of DNA primase and DNA polymerase

Figure 1.
Figure 1. A comparison of prokaryotic and eukaryotic replication initiation. The steps of replication initiation are conserved in prokaryotes and eukaryotes. In both groups, replication initiation begins with the binding of the initiator protein (DnaA in bacteria, ORC in eukaryotes) to the origin of replication. Following initiation binding, double-stranded DNA is unwound to single-stranded DNA by the action of DnaA in prokaryotes, and by an unknown mechanism in eukaryotes. Helicase (or the MCM complex in eukaryotes) is loaded onto DNA at the origin by a protein known as helicase loader in prokaryotes, and by the Cdc6 and Cdt1 proteins in eukaryotes. Helicase is loaded following DNA unwinding in prokaryotes, and most likely prior to DNA unwinding in eukaryotes. Finally, DNA primase and DNA polymerase load at the unwound DNA, and the process of replication elongation begins.
Kornberg and Baker, 1992; Leatherwood, 1998; Lemon et al., 2002; Messer and Weigel, 1996; Messer et al., 2001; Messer, 2002; Moriya et al., 1999; Yoshikawa and Wake, 1993).

During replication initiation, many replication proteins are recruited to the origin of replication, including the replicative helicase, DNA primase, and DNA polymerase (Figure 1). The replicative helicase (which most likely consists of the MCM proteins in eukaryotes) is loaded onto DNA at the origin by a protein called helicase loader in bacteria, or by the Cdc6 and Cdt1 proteins in eukaryotes. In prokaryotes, the replicative helicase is loaded onto the single-stranded DNA that has been created at the origin by the unwinding activity of DnaA (Baker and Bell, 1998; Bell and Dutta, 2002; Davey and O'Donnell, 2000; Kornberg and Baker, 1992; Leatherwood, 1998; Lemon et al., 2002; Messer and Weigel, 1996; Messer et al., 2001; Messer, 2002; Moriya et al., 1999; Yoshikawa and Wake, 1993). In contrast, in eukaryotes, the MCM proteins are most likely loaded onto double-stranded DNA at the origin, before any DNA unwinding occurs (Forsburg, 2004; Pape et al., 2003; Shin et al., 2003). In either case, upon activation, helicase begins to unwind extensive regions of DNA near the origin. Finally, DNA primase loads at the single-stranded region of the origin and synthesizes short RNA primers that will prime the leading and lagging strands. Replication initiation ends with the recruitment of DNA polymerases to the sites of these primers, at which point, replication elongation begins (Figure 1) (Baker and Bell, 1998; Bell and Dutta, 2002; Davey and O'Donnell, 2000; Kornberg and Baker, 1992; Leatherwood, 1998; Lemon et al., 2002; Messer and Weigel, 1996; Messer et al., 2001; Messer, 2002; Moriya et al., 1999; Yoshikawa and Wake, 1993). All of the events listed above have been most well characterized in the Gram-negative bacterium E. coli. The events of replication initiation in E. coli are diagrammed in greater detail in Figure 2.
Figure 2.

DnaA-induced unwinding

helicase loading and unwinding

replication elongation

primase loading and primer synthesis

polymerase loading

Figure 2.
**Figure 2. The steps of replication initiation.** The steps of replication initiation have been most well characterized in the Gram-negative bacterium *E. coli*. In *E. coli*, the binding of DnaA to *oriC* (the chromosomal origin) leads to nucleoprotein complex formation. Active DnaA protein induces the unwinding of DNA within the origin region, creating single-stranded DNA. Helicase is loaded onto this single-stranded DNA by the helicase loader protein. Primase is then recruited to the long stretches of single-stranded DNA created by helicase, where primase generates RNA primers. The replicative DNA polymerase is then recruited to sites of primed single-stranded DNA to begin the process of replication elongation. All of the steps listed here most likely occur in a similar fashion in the Gram-positive bacterium *B. subtilis*. 
Many organisms, such as eukaryotes and the bacterium *B. subtilis*, utilize multiple, distinct versions of DNA polymerase during the elongation phase of DNA replication (Dervyn et al., 2001; Hubscher et al., 2002; McHenry, 2003). In these organisms, one version appears to be dedicated to the leading strand, and another version may be dedicated to lagging strand replication. It is thought that some organisms have multiple replicative polymerases to serve the different needs for the leading and lagging strands. For instance, the lagging strand polymerase is recycled after the completion of each Okazaki fragment, while the leading strand polymerase replicates its strand in a much more processive manner (McHenry, 2003). However some organisms, such as *E. coli*, possess a single type of DNA polymerase that appears to be responsible for the elongation of both the leading and lagging strands. In *E. coli*, a single type of replicative polymerase may fulfill these different needs by possessing differential properties due to asymmetric association with other replisome subunits (Glover and McHenry, 2001; McHenry, 2003).

Replication elongation in both prokaryotes and eukaryotes proceeds bidirectionally outwards from the origin of replication to the site of termination. Bacteria such as *Escherichia coli* and *Bacillus subtilis* each possess a single circular chromosome with one origin of replication (Figure 3). In these organisms, replication termination occurs in a chromosomal region approximately half-way around the circular chromosome from the origin. Thus, in *E. coli* as in *B. subtilis*, a single circular chromosome exists that contains a single origin of replication (*oriC*) at the 0° position on the circle, and a predominant site of replication termination located near the 180° position on the circle (Figure 3). The Replication termination protein Rtp (or Tus in *E. coli*) binds specifically to the *ter* (termination) sites in the region of termination and inhibits helicase, thereby terminating replication at these sites (Kornberg and Baker, 1992; Lemon et al., 2002;
0°: the site of DNA replication initiation (oriC)

172°: the main ter site in the region of replication termination

~ 4.2 Mb
~ 4000 genes

Figure 3.
Figure 3. The single, circular *B. subtilis* chromosome. *B. subtilis* contains a single circular chromosome that contains about 4,000 genes in 4 megabases of DNA. One chromosomal origin of replication, oriC, is found on this circular chromosome. Approximately half-way around the circular chromosome exists the major site of replication termination, located in a large region of termination that contains several ter sites. All of the structural features of the *B. subtilis* chromosome described here are shared with the single circular chromosome of *E. coli.*
Messer and Weigel, 1996; Messer et al., 2001; Messer, 2002; Moriya et al., 1999; Yoshikawa and Wake, 1993).

In organisms whose genomes possess multiple origins of replication, replication elongation proceeds until the two sides of the replication bubble meet up with its neighboring replication forks to the right and left. Thus, replication termination in these organisms typically occurs in a sequence-non-specific manner where replication forks converge (Vengrova et al., 2002).

**The coordination of DNA replication and cell division.** During the prokaryotic and eukaryotic mitotic cell cycles, DNA replication initiation and cell division alternate. These processes are coordinated with each other so that each newborn cell inherits one full copy of the genome. In eukaryotic cells, DNA replication and cell division are temporally separated. Thus, a cell going through the mitotic cell cycle duplicates its genome, waits, and then goes through the process of cell division (Figure 4). Under slow growth conditions, the bacterial cell cycle mimics that of eukaryotes. Thus, slow-growing bacteria can similarly be thought as having separate G1, S, G2, and M phases, just as eukaryotes do (Figure 4) (Kornberg and Baker, 1992; Nordstrom and Austin, 1993; Zyskind and Smith, 1992).

During fast growth, however, bacteria do not temporally separate DNA replication and cell division. This is because replication of the ~4 Mb genome possessed by bacteria such as *E. coli* and *B. subtilis* cannot occur in less than about 40 minutes, but bacterial generation times can be as fast as 20 minutes. When growing very quickly, bacterial cells re-initiate replication before the previous round of replication has completed. This replication re-initiation results in dichotomous, or multi-fork, replication, during which multiple elongating replication forks exist on a single chromosome (Figure 5) (Kornberg and Baker, 1992; Nordstrom and Austin, 1993; Zyskind and Smith, 1992).
Figure 4.
Figure 4. The cell cycle in slow-growing prokaryotes. In eukaryotes and slow-growing prokaryotes, DNA replication and cell division are temporally separated, occurring in distinct phases of the cell cycle. Shown here is the cell cycle, containing the G1 (gap 1), S (synthesis), G2 (gap 2), and M (mitosis) phases. Next to each phase is a simplified diagram of a prokaryotic cell with a single circular chromosome in each phase of the cell cycle.
Figure 5. The cell cycle in fast-growing prokaryotes. Bacteria growing under fast growth conditions do not have separate phases of the cell cycle for DNA replication and cell division. Instead, cell division occurs while replication elongation is ongoing. The bacterial chromosomes of *B. subtilis* and *E. coli* cannot be replicated in less than approximately 40 minutes, although these bacteria can grow with doubling times of 20 minutes. Thus, these cells respond by initiating additional rounds of replication on actively replicating chromosomes before cell division occurs. This creates cells with 4 or even 8 chromosomal origins, leading to the creation of newborn cells with 2 or 4 origins. Thus, newborn bacterial cells grown under fast growth conditions are born with chromosomes that are already undergoing replication elongation.
cell division and replication elongation occur simultaneously

newborn cells are already undergoing elongation

the first round of replication completes

re-initiation occurs on an actively replicating chromosome

Figure 5.
Excess initiation of DNA replication. *E. coli* and *B. subtilis* undergo multi-fork replication in order to grow with doubling times of 20 minutes, and thus these bacteria are equipped to tolerate a certain amount of replication re-initiation (Kornberg and Baker, 1992; Nordstrom and Austin, 1993; Zyskind and Smith, 1992). This is in contrast to eukaryotic cells, many of which cannot tolerate any amount of excess DNA replication. In budding yeast, for example, a single round of over-replication leads to cell cycle arrest and lethality (Nguyen et al., 2001; Wilmes et al., 2004), whereas *E. coli* cells can tolerate having 4 or 8 origins per cell under fast growth conditions (Kornberg and Baker, 1992; Nordstrom and Austin, 1993; Zyskind and Smith, 1992). However, too much replication initiation is intolerable to bacterial cells (Boye et al., 2000; Braun et al., 1987; Donachie and Blakely, 2003; Katayama, 2001; Simmons and Kaguni, 2003; Simmons et al., 2004).

It is important to regulate the amount and timing of replication initiation in a cell, because either too much or too little initiation causes cellular defects (Figure 6) (Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001). If the frequency and timing of DNA replication initiation are affected and too few initiation events occur per cell division event, cell division will result in the formation of anucleate cells (cells lacking genetic information). If too many initiation events occur per bacterial cell division, a collection of consequences can result, including replication fork stalling during the elongation phase and aberrant timing or positioning of cell division events (Figure 6) (Bach and Skarstad, 2004; Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001; Simmons et al., 2004; Weitao et al., 1999). Over-initiation of replication is often also accompanied by increased replication asynchrony. Replication asynchrony refers to the firing of one origin of replication in a cell without the simultaneous
Figure 6. The importance of replication timing. Either too few or too many replication initiation events per cell division event cause disastrous consequences to the cell. Too few replication events per cell division event can lead to the formation of anucleate cells (cells lacking all genetic information). Too many replication initiation events per cell division event can lead to replication asynchrony, replication fork stalling, and aberrant cell division.
too much initiation

stalled forks

replication asynchrony

Figure 6.

too little initiation

cell division defects

anucleate cells
firing of its sister origin in the same cell (Figure 7). Synchronously replicating cells contain 1, 2, 4, or 8 origins, whereas asynchronously replicating cells can contain 3, 5, 6, or 7 origins. Such asynchrony leads to a single cell possessing multiple chromosomes that are at different stages in the replication cycle (Figure 7) (Katayama, 2001; Nordstrom and Austin, 1993; Skarstad et al., 1986).

**Mechanisms of replication control.** To avoid the problems associated with over-initiation, DNA replication is highly regulated. Regulation of replication occurs mainly at the stage of initiation (Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001). In bacteria, chromosomal replication and cell division each require a characteristic amount of time to occur, regardless of growth rate. Thus, the length of the cell cycle is mainly adjusted for different growth rates based on how long a newborn cell waits to initiate DNA replication. In minimal medium at low temperatures, bacterial cells grow very slowly, and many newborn cells are born with a single unreplicated circular chromosome. These cells wait for a period of time before undergoing replication initiation (Figure 4). In bacterial cells growing in rich medium at higher, more optimal temperatures, new cells are born with chromosomes that have initiated replication prior to the birth of the new cell (Figure 5). Thus, the cell is born with two or even four copies of the origin of replication (Kornberg and Baker, 1992; Nordstrom and Austin, 1993; Zyskind and Smith, 1992).

Although most known mechanisms of replication control operate at the stage of initiation, the bacterium *B. subtilis* possesses a mechanism of post-initiation control. When *B. subtilis* undergoes a starvation adaptation process known as the stringent response, new rounds of replication initiate at *oriC* but then stall reversibly a couple of hundred kilobases away, at sites
Figure 7. Contrasting synchronously and asynchronously replicating cells. Synchronously replicating cells contain chromosomes with origins that always initiate replication if their sister origins have initiated replication. Synchronously replicating cells can contain 2 or 4 (or 8) origins. Asynchronously replicating cells contain sister origins that have not fired in unison, and can therefore contain 3 origins, for example. Replication elongation in asynchronously replicating cells leads to cells possessing chromosomes that are at different stages in the replication cycle. Asynchronously replicating cells can also contain 5, 6, or 7 origins.
synchronously replicating cells

2 origins

4 origins

asynchronously replicating cells

3 origins

5 origins

5 origins

6 origins

7 origins

7 origins

Figure 7.
on the chromosome called the left and right STer (stringent termination or arrest) sites (Autret et al., 1999; Levine et al., 1991; Levine et al., 1995). These replication arrest sites may also operate in *B. subtilis* to manage excess replication initiation events under conditions when the stringent response is not activated. There is a temperature sensitive mutation that exists in the *B. subtilis* replication initiation machinery (*dnaB134ts*) that allows two rounds of replication to initiate shortly after cells containing this mutation are shifted from the non-permissive temperature to the permissive temperature. Evidence suggests that forks derived from one of these two rounds of replication are stalled at the stringent arrest sites, and are only allowed to proceed after the first round of replication terminates (Henckes et al., 1989).

The existence of stringent arrest sites on either side of *oriC* on the *B. subtilis* chromosome is an example of a mechanism through which bacteria may control the progression of excess replication initiation events. However, most bacterial replication control mechanisms prevent excess initiation from occurring in the first place (Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001). In the following sections, the major mechanisms of control over initiation in *E. coli* and *B. subtilis* are discussed. Mechanisms of bacterial replication control have been most extensively studied in *E. coli*. Three major means of regulation of replication initiation exist in *E. coli*: DnaA activation and inactivation, origin sequestration, and DnaA sequestration.

The activation and inactivation of DnaA in *E. coli*. Replication initiation is regulated in part at the level of DnaA activity (Messer and Weigel, 1996; Messer et al., 2001; Messer, 2002; Mizushima et al., 1997; Nishida et al., 2002; Sekimizu et al., 1987). DnaA is a nucleotide binding protein and a member of the AAA+ ATPase family (Koonin, 1993; Neuwald et al.,
DnaA-ATP is active for replication, whereas DnaA-ADP is not (Messer and Weigel, 1996; Messer et al., 2001; Messer, 2002; Mizushima et al., 1997; Nishida et al., 2002; Sekimizu et al., 1987). In *E. coli*, most DnaA is in the ATP bound form before replication initiation occurs. After initiation occurs, the pool of DnaA quickly changes so that DnaA-ADP becomes the predominant form (Kurokawa et al., 1999). This is thought to be a way in which further initiation events are prevented for a portion of the cell cycle immediately following origin firing (Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001).

The quick shift in predominance from DnaA-ATP to DnaA-ADP that occurs after replication initiation is due to the inactivation of DnaA by the *E. coli* replication elongation machinery (Katayama et al., 1998; Katayama and Sekimizu, 1999). Two proteins work together to inactivate DnaA during replication elongation by stimulating the intrinsic ATPase activity of DnaA. These two proteins are DnaN (Boye et al., 2000; Bruck and O'Donnell, 2001; Donachie and Blakely, 2003; Katayama, 2001; Kuriyan and O'Donnell, 1993) and Hda (Kato and Katayama, 2001). DnaN is a component of the replication machinery called the sliding clamp, or β clamp, which is analogous to Proliferating Cell Nuclear Antigen (PCNA) in eukaryotes. The *E. coli* sliding clamp is a protein ring that binds to DNA polymerase and also encircles replicating DNA. The link that the sliding clamp forms between DNA polymerase and the DNA accounts for the high processivity of *E. coli* DNA polymerase, which replicates DNA at a rate of 500-1000 nucleotides per second (Bruck and O'Donnell, 2001; Kuriyan and O'Donnell, 1993).

Hda is an *E. coli* protein that resembles DnaA and is also a AAA+ ATPase family member (Kato and Katayama, 2001). Hda acts as a physical bridge between DnaA and sliding clamp (Kurz et al., 2004; Su'etsugu et al., 2004). The function of Hda is necessary for the *in vitro* reconstitution of the inactivation of DnaA by the replication elongation machinery (Kato and Katayama, 2001).
and for controlled, synchronous replication initiation (Camara et al., 2003; Kato and Katayama, 2001).

The importance of the regulation of DnaA activity is demonstrated by studies of mutant forms of DnaA that have increased levels of activity. One such mutant, DnaAcos, is a form of DnaA that cannot hydrolyze ATP at its non-permissive temperature (Katayama, 1994; Katayama and Kornberg, 1994; Katayama and Crooke, 1995; Katayama et al., 1995). Expressing DnaAcos in *E. coli* cells results in replication asynchrony at its permissive temperature, and severe over-initiation at its non-permissive temperature (Braun et al., 1987; Kellenberger-Gujer et al., 1978; Simmons and Kaguni, 2003; Simmons et al., 2004). Elevating levels of DnaA or DnaAcos in the cell appears to swamp *E. coli* cells with so many replication forks that elongation arrests due to replication fork stalling and collapse (Simmons et al., 2004).

Controlling DNA replication through regulating DnaA activity most likely occurs in other bacteria in addition to *E. coli*. Elevating levels of DnaA in *B. subtilis* by five-fold leads to cellular toxicity and the induction of the SOS response, indicating the presence of single-stranded DNA that may have resulted from stalled or collapsed replication forks (Ogura et al., 2001). DnaA activity is not regulated through Hda in most bacteria, however, because bacteria outside of the β- and γ- proteobacteria (such as *E. coli* and its close relatives) do not contain homologs of *hda*. It is hypothesized that *B. subtilis* may contain a functional analog of Hda, called YabA, which is described below (Noirot-Gros et al., 2002). However, more studies need to be performed on YabA before any firm conclusions are drawn regarding its function.

**Origin sequestration in *E. coli***. *E. coli* also regulates the initiation of DNA replication by sequestering chromosomal origins at the membrane immediately after replication initiation occurs (Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001; Landoulsi et al., 1990;
Ogden et al., 1988). This appears to prevent re-initiation at the origins for about one-third of the cell cycle (Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001; Landoulsi et al., 1990).

Sequestration of origins in the membrane fraction of cells occurs due to a system involving the action of two proteins, the sequestration factor SeqA, and the Dam methylase (Figure 8). The Dam protein methylates the adenine nucleotide in the GATC sites on the *E. coli* chromosome, and SeqA opposes the function of Dam (Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001). After a round of semiconservative replication, a fully methylated chromosome yields two hemi-methylated chromosomes. Subsequently, the Dam methylase converts the hemi-methylated DNA into the fully methylated form (Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001). Before Dam can act upon the hemi-methylated GATC sites near *oriC*, however, the membrane-associated SeqA protein binds to these sites due to a binding preference for hemi-methylated GATC sites found enriched in the origin region of the chromosome (Brendler et al., 1995; d'Alencon et al., 1999; Kang et al., 1999; Shakibai et al., 1998; Skarstad et al., 2000; Slater et al., 1995; Taghbalout et al., 2000). This sequesters the origin region of the chromosome at the membrane (d'Alencon et al., 1999; Ogden et al., 1988; Shakibai et al., 1998; Slater et al., 1995), presumably rendering it inaccessible to methylation and to re-initiation (Figure 8) (Campbell and Kleckner, 1990; Kang et al., 1999; Russell and Zinder, 1987; Taghbalout et al., 2000; Torheim and Skarstad, 1999; Wold et al., 1998).

Cells lacking SeqA function display over-initiation (Bach and Skarstad, 2004; Boye et al., 1996; Lu et al., 1994; von Freiesleben et al., 1994; Weitao et al., 1999), as these cells cannot sequester origins in the membrane fraction of cells (Shakibai et al., 1998; Slater et al., 1995). *E. coli* cells that lack *seqA* also display phenotypes that often accompany over-initiation, such as
Dam methylase cannot act at replicated origins in seqA+ cells

Dam methylase acts at replicated origins in ΔseqA cells

Legend
- * fully methylated ds DNA
- * hemi-methylated ds DNA
- SeqA

Figure 8.
Figure 8. **SeqA-mediated origin sequestration in *E. coli***. *E. coli* DNA is methylated at the adenines in GATC motifs that are enriched in the origin-proximal region of the chromosome. Upon replication of the chromosome, these sites become hemi-methylated (methylated only on the parental strand). The Dam methylase functions to methylate the daughter strand of DNA. The SeqA protein binds to the hemi-methylated GATC sites near to *oriC*, however, and sequesters that region of the chromosome for about one third of the cell cycle, thereby preventing full methylation and replication re-initiation at *oriC*. 
increased replication asynchrony and asymmetric cell division (Bach and Skarstad, 2004; Boye et al., 1996; Lu et al., 1994; von Freiesleben et al., 1994; Weitao et al., 1999). Similar abnormalities are displayed by cells in which the GATC sites near to oriC have been mutated so that they are unrecognizable by SeqA (Bach and Skarstad, 2004). Because SeqA prevents the function of Dam methylase at origins, a deletion of seqA results results in a similar phenotype as over-producing Dam. Accordingly, cells over-producing the Dam methylase exhibit over-initiation (Boye and Lobner-Olesen, 1990).

The SeqA-mediated sequestration mechanism of replication control only appears to exist in E. coli and its close relatives (Hiraga, 2000). Genomic analysis reveals that no sequenced Gram-positive bacterial species contain homologs of SeqA, and many Gram-negative bacteria also lack homologs of SeqA. Only γ-proteobacteria, the group containing E. coli and its closest relatives, contain SeqA homologs.

**DnaA sequestration in E. coli.** Another characterized mechanism of E. coli replication control involves the datA locus. datA is a locus on the E. coli chromosome that has a high affinity for the DnaA protein (Kitagawa et al., 1996; Roth and Messer, 1998). datA contains five DnaA binding sites, several of which have been shown to be critical for datA function (Kitagawa et al., 1996; Kitagawa et al., 1998; Ogawa et al., 2002). The datA locus is thought to bind and sequester excess DnaA protein in the cell, preventing it from functioning at the origin of replication to stimulate re-initiation (Kitagawa et al., 1996). datA has been shown in vivo to have the ability to sequester 40-60% of the DnaA in the cell, thereby affecting both replication initiation and transcription from DnaA-regulated promoters (Kitagawa et al., 1996). The sequestration of DnaA by datA causes the addition of a few extra copies of datA to result in
delayed initiation, and the addition of many copies of data to be lethal (Morigen et al., 2003). This lethality can be suppressed by the over-production of DnaA (Morigen et al., 2003).

Cells in which the data locus has been deleted exhibit increased replication asynchrony and over-initiation (Kitagawa et al., 1998), demonstrating that data does play an important role in E. coli replication control. No site analogous to the E. coli data locus has been found in Bacillus subtilis, although extensive searches for such a region have not been reported.

**Known mechanisms of control over B. subtilis replication initiation.** The roles of four B. subtilis proteins in control of initiation have been previously characterized. These proteins are DnaA, YabA, Spo0J, and Soj. The roles of these four proteins in B. subtilis replication control are discussed below.

The amount of DnaA in the cell influences replication control in B. subtilis, as it does in E. coli (Moriya et al., 1990; Ogura et al., 2001). DnaA-ATP most likely stimulates replication initiation at oriC in B. subtilis, and over-production of DnaA probably leads to increased cellular DnaA-ATP pools. Notably, the situation is not as simple as an overexpression of DnaA leading to increased replication. This is because DnaA negatively regulates the expression of DnaN, an essential component of the replication elongation machinery. Thus, overexpression of DnaA alone actually leads to cellular toxicity, but co-overexpression of DnaA and DnaN does lead to over-initiation (Ogura et al., 2001).

Three known B. subtilis genes exist that, when deleted, cause over-initiation. These three known genes are yabA, spo0J, and soj. YabA, as aforementioned, has been proposed to regulate DnaA activity by performing a similar function to E. coli Hda, which is required for the replication elongation-dependent inactivation of DnaA. However, neither Hda nor any other E. coli protein is homologous to YabA. A deletion of the yabA gene causes over-initiation. In
addition, YabA acts as a bridge between *B. subtilis* DnaA and DnaN (the sliding clamp) in yeast three-hybrid experiments (Noirot-Gros et al., 2002). More experiments must be done, however, to establish a conclusive role for YabA in replication control.

SpoOJ and Soj both appear to negatively regulate replication initiation (Lee et al., 2003; Lee and Grossman, 2004). SpoOJ is a member of the conserved ParB family of chromosome partitioning proteins that binds to several binding sites located in the origin-proximal region of the *B. subtilis* chromosome (Bignell and Thomas, 2001; Hiraga, 2000; Lin and Grossman, 1998; Surtees and Funnell, 2003). Soj is the corresponding ParA homolog in *B. subtilis*, and is a transcription factor and a putative ATPase (Bignell and Thomas, 2001; Hiraga, 2000; Marston and Errington, 1999; Quisel et al., 1999; Surtees and Funnell, 2003).

*spoOJ* mutant cells exhibit over-initiation, increased DNA content, and increased asynchrony (Lee et al., 2003; Lee and Grossman, 2004; Ogura et al., 2003). It is not clear yet by what mechanism SpoOJ inhibits replication. It has been proposed that the binding of SpoOJ to the origin creates a nucleoprotein complex that may prevent access of essential replication proteins to the origin, thereby negatively regulating replication initiation at *oriC* (Lee et al., 2003; Lee and Grossman, 2004; Ogura et al., 2003).

The effect of Soj on replication initiation is less clear. In one report, a deletion of *soj* led to over-initiation (Lee and Grossman, 2004), whereas another report concluded that the overexpression of Soj leads to over-initiation (Ogura et al., 2003). It has been proposed that opposite effects of Soj-ADP and Soj-ATP on replication control could explain this discrepancy (Lee and Grossman, 2004). Soj may regulate replication by influencing the assembly of proteins at *oriC*, or Soj may affect replication indirectly through its activity as a transcription factor (Lee...
et al., 2003; Lee and Grossman, 2004; Ogura et al., 2003). Notably, there are no chromosomally encoded homologs of Spo0J or Soj in *E. coli*.

**Replication initiation proteins specific to low G+C content Gram-positive bacteria.** The lack of conservation of genes and loci involved in control of replication initiation between the Gram-negative bacteria and Gram-positive bacteria may reflect differences in the protein players involved in the process of replication initiation in these bacteria. All characterized bacteria have homologs of the *E. coli* replication proteins DnaA, helicase, primase, and DNA polymerase. The genomes of low G+C Gram-positive bacteria also contain three genes that encode replication initiation proteins that have no *E. coli* homologs. These three genes, *dnaB*, *dnaD*, and *dnaI*, are essential for replication initiation in *B. subtilis* (Bruand and Ehrlich, 1995; Bruand et al., 1995b; Hoshino et al., 1987; Ogasawara et al., 1986; Sueoka, 1998). Genes encoding DnaB, DnaD, and DnaI can also be found in the genomes of other low G+C Gram-positive bacteria including *Staphylococcus, Enterococcus, Lactobacillus, Listeria, Lactococcus*, and *Streptococcus* species.

Temperature sensitive mutations in the *dnaB*, *dnaD*, and *dnaI* genes were used to demonstrate that these genes are essential for replication initiation in *B. subtilis*. In *dnaBts*, *dnaDts*, or *dnalts* cells incubated at the non-permissive temperature, DNA replication ceases gradually. This is because ongoing rounds of replication elongation are able to finish, but no new rounds of replication are initiated. Thus, these mutations are in genes that are essential for the process of replication initiation in *B. subtilis* (Burnett and Wake, 1977; Gross et al., 1968; Karamata and Gross, 1970; White and Sueoka, 1973). Subsequently, a *dnaDts* mutation has been isolated in *Staphylococcus aureus*. Studies of this mutation demonstrate that DnaD is also essential for replication initiation in *S. aureus* (Li et al., 2004).
DnaB, DnaD, and DnaI contribute to the loading of helicase onto origins. DnaI is most likely the functional analog of *E. coli* helicase loader. Both DnaI and *E. coli* helicase loader are ATPases belonging to the AAA+ family (Davey et al., 2002; Koonin, 1993; Lee and Bell, 2000; Neuwald et al., 1999), and both proteins bind to their respective replicative helicases (Imai et al., 2000; Kobori and Kornberg, 1982; Lanka and Schuster, 1983; Noirot-Gros et al., 2002; Soultanas, 2002; Velten et al., 2003; Wickner and Hurwitz, 1975). In addition, both proteins expose the ATPase and unwinding activities of helicase, and yet at high concentrations inhibit these activities of helicase (Davey et al., 2002; Velten et al., 2003; Wahle et al., 1989a; Wahle et al., 1989b). For *E. coli*, it has been proposed that helicase loader might use its two opposing effects on helicase activity to act as a switch protein that holds and inhibits helicase when it is not loaded on DNA or is in the process of being loaded. Then, once helicase is properly loaded, helicase loader hydrolyzes ATP, which releases active replicative helicase for DNA duplex unwinding (Davey et al., 2002; Lee and Bell, 2000).

DnaB and DnaD are also thought to act at the step in initiation of loading the replicative helicase onto origins. It was originally proposed that DnaB and DnaD might be involved in helicase loading at oriC because both proteins are necessary to load helicase onto some *B. subtilis* plasmids (Bruand et al., 1995a; Bruand et al., 2001b). Subsequently, it was shown that DnaB physically interacts with DnaI-helicase complexes *in vitro*, and that DnaB stimulates the ATPase and unwinding activities of helicase (when complexed with DnaI) three-fold (Velten et al., 2003). In addition, *dnaB* is found in an operon with *dnaI* (Bruand and Ehrlich, 1995), which is significant because proteins that function together are often co-transcribed in bacteria.

In Chapter 2, we show that DnaB and DnaD function is necessary prior to the loading of helicase during initiation at oriC *in vivo*. We monitored helicase loading at origins in live cells,
and found that *dnaBts* and *dnaDts* mutations block replication initiation at the step before helicase loading (Rokop et al., 2004). DnaD may assist in helicase loading by acting as a physical link between the DnaB-DnaI-helicase complex and DnaA bound to *oriC*, as DnaD has been shown to interact with both DnaA (Ishigo-oka et al., 2001) and DnaB (Rokop et al., 2004). The physical interaction between DnaD and DnaB is examined and discussed in Chapter 2 and Appendix A.

**DnaB, DnaD, and DnaI are involved in the process of replication restart.** DnaB, DnaD, and DnaI are not only involved in helicase loading during replication initiation at *oriC*. These proteins are also involved in loading helicase at replication forks that have stalled along the chromosome (Bruand et al., 1995a; Bruand et al., 2001b; Marsin et al., 2001). Fork stalling can result from many different factors, such as protein roadblocks, DNA damage, and DNA structural changes such as supercoiling status. The process of reloading helicase at stalled replication forks is called replication fork restart, and this process is organized by PriA (Cox et al., 2000; Cox, 2001; Marians, 2000; Sandler and Marians, 2000). There are numerous recombination pathways that can convert a stalled or damaged replication fork into a fork that is ready to undergo PriA-mediated replication restart. The pathway that is utilized depends on the cause of the stalled fork (McGlynn and Lloyd, 2002; Michel, 2000; Michel et al., 2001).

Replication restart is a very important cellular process, as *priA* mutant cells are barely viable. A deletion of *priA* results in poor growth, inviability in rich medium, SOS induction, and sensitivity to DNA damaging agents in both *E. coli* and *B. subtilis* (Polard et al., 2002; Sandler et al., 1996). The inviability of *priA* mutant cells grown in rich medium has been used to support the hypothesis that most cells encounter blockage to a replication fork in each generation. Conservatively low estimates of the frequency of replication fork stalling are that 15-20% of
cells experience fork blockage and replication restart in every generation. The need for replication restart increases with growth rate, because the number of replication forks per chromosome and the number of replication forks per cell increase with growth rate (Cox et al., 2000; Cox, 2001; Marians, 2000; Sandler and Marians, 2000). PriA recognizes stalled forks by binding to a specific structure called the D loop. Binding of PriA to a D loop initiates the process of loading helicase at the stalled replication fork. By directing helicase loading at sites of replication fork stalling, PriA acts as the “replication initiator” for all sites on the chromosome except oriC (Cox et al., 2000; Cox, 2001; Marians, 2000; Sandler and Marians, 2000).

Both biochemical and genetic evidence supports the involvement of DnaB and DnaD in replication restart along with PriA. DnaD and DnaB are both multimeric DNA binding proteins that bind preferentially to forked DNA substrates that resemble replication fork intermediates (Marsin et al., 2001). The binding affinity of DnaD for forked DNAs is stimulated by PriA, and the binding affinity of DnaB for forked DNAs is stimulated by DnaD (Marsin et al., 2001). Higher order complexes can be detected forming on forked DNAs only if these proteins are added in the order: PriA, DnaD, DnaB (Marsin et al., 2001). In addition, a missense mutation in the dnaB gene suppresses all characterized defects of priA null mutant cells (Bruand et al., 2001b).

Contrasting E. coli and B. subtilis replication restart. Replication restart differs between E. coli and B. subtilis in several ways. One difference is that E. coli has a set of proteins that are dedicated to replication restart, whereas B. subtilis uses the same proteins during replication initiation and replication restart. In B. subtilis, the proteins known to be involved in replication restart are PriA, DnaD, DnaB, DnaI, helicase, and DNA primase. Thus, all proteins involved in B. subtilis replication initiation are involved in replication restart except for DnaA, for which
PriA is substituted (Bruand et al., 1995a; Bruand et al., 2001b; Marsin et al., 2001). In contrast, *E. coli* uses several proteins during replication restart that do not play any known role in replication initiation. These proteins are the PriB, PriC, and DnaT proteins. Thus, the *E. coli* replication restart pathway converges with the *E. coli* replication initiation pathway after the action of PriA, PriB, PriC, and DnaT, and upon entry of helicase loader, helicase, and DNA primase (Table 1) (Cox et al., 2000; Cox, 2001; Marians, 2000; Sandler and Marians, 2000).

Another difference between replication restart in *E. coli* and in *B. subtilis* lies in the different mutations that eliminate the need for PriA in each of these bacterial species. A missense mutation in the gene encoding helicase loader suppresses the growth defects of *priA* null mutant *E. coli* cells (Sandler et al., 1996). In contrast, a missense mutation in *dnaB* suppresses the growth defects of *priA* null mutant *B. subtilis* cells (Bruand et al., 2001b). This is despite the fact that *B. subtilis* DnaI is functionally analogous to *E. coli* helicase loader in many ways (such as being a AAA+ ATPase that both exposes and inhibits the ATPase and unwinding activities of helicase) (Koonin, 1993; Neuwald et al., 1999; Velten et al., 2003). In contrast, DnaB is not a AAA+ ATPase, and DnaB cannot expose or inhibit the activities of helicase (Velten et al., 2003). Yet a mutation in *dnaB*, and not in *dnaI*, suppresses the defects of *B. subtilis priA* null mutant cells. One explanation for this result is that *E. coli* helicase loader is responsible for both loading helicase and regulating the frequency and timing of helicase loading. These two functions may be separated in *B. subtilis*, so that DnaI is helicase loader, and DnaB regulates when helicase loader can perform its function. In this model, mutations that suppress the defects of *priA* mutant cells would be found in the protein that regulates the timing and frequency of helicase loading events, thereby allowing helicase loading to take place in a more unregulated fashion.
A mutant form of E. coli helicase loader that eliminates the need for PriA has been shown in vitro to be capable of loading helicase onto DNA in the absence of PriA. This mutant form of helicase loader is thus considered to be a promiscuous form of helicase loader. Typically, helicase loader requires PriA to displace single-stranded-DNA-binding-protein (SSB) from unwound DNA so that it can load helicase onto the naked single-stranded DNA. However the mutant helicase loader can either load helicase onto SSB-coated DNA, or is capable of stripping SSB off of DNA itself without help from PriA (Liu et al., 1999; Xu and Marians, 2000).

Interestingly, E. coli cells utilizing the ΔpriA-suppressing form of E. coli helicase loader have no obvious growth phenotype, even though one would think that promiscuous helicase loading could be detrimental due to an unregulated production of single-stranded DNA in the cell (Sandler et al., 1996). Similarly, B. subtilis cells containing dnaBS371P (the PriA-suppressing form of dnaB) do not grow with a rate that is obviously different from that of wild-type cells. Upon closer examination, however, we have found that dnaBS371P actually does cause cellular defects, as dnaBS371P cells exhibit aberrant replication control and cell division defects. In addition, cells over-producing DnaBS371P form filamentous and anucleate cells when grown in rich medium, most likely due to replication fork stalling and collapse. These phenotypes of dnaBS371P cells and cells over-producing DnaBS371P are examined and discussed in Chapters 2 and 3.

DnaB and DnaD affect recombinational repair. The process of replication restart is intimately connected to the processes of recombinational repair, as many stalled replication forks must be repaired or processed by recombination proteins before they are ready for replication restart (McGlynn and Lloyd, 2002; Michel, 2000; Michel et al., 2001). There is some evidence that DnaB and DnaD may also affect recombinational repair in B. subtilis cells. First, dnaDts
and dnaBts mutations stimulate recombination in B. subtilis cells at the non-permissive temperature. This stimulation of recombination could result because these mutations may render the cells unable to restart replication, which would lead to the prolonged presence of replication fork intermediates (Bruand et al., 2001a). Second, in Staphylococcus aureus, a temperature sensitive mutation in dnaD leads to stalled replication forks, DNA degradation, and sensitivity to both the DNA damaging agent mitomycin C and to UV irradiation at the non-permissive temperature (Li et al., 2004). Third, B. subtilis cells over-producing a mutant form of DnaB (DnaBS371P) display severe effects on replication and cell division that appear to result from a reduced ability to repair stalled replication forks. As discussed in Chapter 3, DnaBS371P-over-producing cells are hypersensitive to DNA damaging agents, which is an indication of damaged DNA that may exist at stalled forks that have not undergone proper repair. Finally, it is possible that DnaD also has a role in more general aspects of DNA repair, as dnaD is found in an operon with nth, which encodes a homolog of endonuclease III (a known DNA repair enzyme in E. coli) (Bruand et al., 1995b).

**DnaB is a proposed anchor of chromosomal origins in the membrane.** In addition to contributing to aspects of DNA metabolism that include replication initiation and replication restart, DnaB is necessary for the enrichment of plasmid and chromosomal origins in the cell membrane. Origin regions of the chromosome and of plasmids are enriched in the membrane fraction of cells (Funnell, 1996; Sueoka and Hammers, 1974; Sueoka, 1998; Yamaguchi and Yoshikawa, 1977). In dnaBts cells incubated at the non-permissive temperature, chromosomal origins are no longer enriched in the membrane fraction of cells (Funnell, 1996; Sato et al., 1991; Sueoka, 1998; Winston and Sueoka, 1980). Some temperature sensitive alleles of dnaB also affect the association of plasmids with the cell membrane, as well as affecting their ability to
replicate (Alonso et al., 1988; Funnell, 1996; Langer and Alonso, 1994; Sueoka, 1998; Watabe and Forough, 1987; Winston and Sueoka, 1980). Thus, it has been hypothesized that DnaB anchors origins in the membrane of B. subtilis cells. Accordingly, we show in Chapter 2 that DnaB is found in the membrane fraction of B. subtilis cells (Rokop et al., 2004). DnaB is not an integral membrane protein, however, as it can be solubilized from the membrane fraction of cells by high salt (Appendix B). Therefore, DnaB may require another factor to be membrane-associated.

Notably, the subcellular localization of DnaB as visualized by fluorescence microscopy does not lend support to the notion that DnaB is an anchor for chromosomal origins. The subcellular location of DnaB has been examined both by immuno-fluorescence (Imai et al., 2000) and by live cell microscopy using a DnaB-GFP fusion protein (Healy, Lemon, and Grossman, unpublished results). In both cases, DnaB did not appear to localize to mid-cell and cell-quarter positions in the cell, as origins of replication do. In addition, studies in which the localization of origin markers and DnaB were examined simultaneously in the same cells showed that DnaB does not colocalize with chromosomal origins unless replication initiation is inactivated using a dnaA4ts allele (Imai et al., 2000) (Healy, Lemon, and Grossman, unpublished results). Notably, in E. coli, the origin anchor protein SeqA does not colocalize with chromosomal origins when they are visualized by fluorescence microscopy either (Hiraga et al., 1998), even though SeqA clearly binds to E. coli oriC (Kang et al., 1999; Shakibai et al., 1998; Skarstad et al., 2000; Slater et al., 1995; Taghbalout et al., 2000; Torheim and Skarstad, 1999; Wold et al., 1998). Thus, the localization pattern of DnaB observed with fluorescence microscopy does not rule out the possibility that DnaB is an anchor for origins. A fraction of the DnaB in the cell may be acting
to anchor origins even though the majority of the protein can be visualized elsewhere, perhaps performing a different function such as restarting replication.

**DnaB and DnaD may be involved in a mechanism of *B. subtilis* replication control.** This thesis focuses on a newly proposed role that DnaB and DnaD have in controlling the frequency and timing of replication initiation. The existence of this role for DnaB and DnaD has been proposed based on the observation that DnaB and DnaD are found in separate subcellular fractions of wild-type cells, even though these two proteins are thought to work together to load helicase during replication initiation. DnaB is found in the membrane fraction of cells, whereas DnaD is found in the cytoplasmic fraction of cells (Rokop et al., 2004). Chapter 2 focuses on establishing that these two *B. subtilis* replication initiation proteins are spatially separated in the cell, and that the subcellular location of DnaD may be regulated. I hypothesize that DnaB and DnaD are kept separate from each other in the cell during the majority of the cell cycle, thereby preventing helicase loading. My model for replication initiation is that DnaB and DnaD physically interact with each other transiently, during active replication initiation. This physical interaction would bring DnaD to the membrane fraction of cells, where it could work together with DnaB on the membrane-associated chromosomal origins (Figure 9).

This model was formed based on studies of *B. subtilis* cells containing a missense mutation in *dnaB, dnaBS371P*. As described in Chapter 2 and Appendix A, we isolated this mutation in a screen for suppressors of the temperature sensitivity of *dnaDts* cells, and in a screen for suppressors of the temperature sensitivity of *dnaBts* cells. In *dnaBS371P* cells, a significant fraction of DnaD is brought to the membrane fraction of cells, most likely due to a direct physical interaction that occurs between DnaBS371P and DnaD (Rokop et al., 2004). We hypothesize that wild-type DnaB and DnaD also interact, but in a highly regulated and transient
Figure 9. A model for the spatial control over replication initiation in *B. subtilis*. In my model, the two essential replication proteins DnaB and DnaD are located in different subcellular locations for the majority of the cell cycle. DnaB is found in the membrane fraction of cells, while DnaD is found in the cytoplasmic fraction of cells. However, these proteins are thought to work together to load helicase at chromosomal origins. Thus, I hypothesize that DnaB and DnaD are found together at the membrane during replication initiation, when these two proteins need to function together at the membrane-associated origins. In my model, the subcellular location of DnaD is regulated by a physical interaction between DnaB and DnaD that occurs transiently, during active initiation. This model stems from studies of *dnaBS371P* mutant cells, in which a significant fraction of DnaD is found in the membrane fraction of cells. This recruitment of DnaD to the membrane appears to be a result of a direct physical interaction that can be detected between DnaB and DnaD by utilizing the DnaBS371P mutant form of this protein.
wild-type cells

Initiation not occurring
(majority of cell cycle)

\[
\text{origin}
\]

\[\text{Cytoplasm}\]

\[\text{dnaBS371P mutant cells}
\]

(majority of cell cycle)

\[
\text{OR}
\]

Active initiation
in wild-type cells

Figure 9.
manner, during active initiation. The \textit{dnaBS371P} mutation may induce a conformational change in DnaB that leads to a constitutive or misregulated interaction between DnaB and DnaD (Figure 9).

If the spatial separation of DnaD and DnaB in the cell is important for replication control, then \textit{dnaBS371P} cells should exhibit aberrant replication control. Indeed, we have found that \textit{dnaBS371P} cells grown in minimal medium over-initiate DNA replication, and exhibit the increased replication asynchrony and cell division defects that are often associated with over-initiation. Chapter 3 focuses on the effects of \textit{dnaBS371P} on replication and cell division.

A comparison of newly and previously identified mechanisms of bacterial replication control. The \textit{dnaBS371P} mutation that we have isolated differs from the previously identified mutations in \textit{hda}, \textit{seqA}, and \textit{datA} (in \textit{E. coli}) and \textit{yabA}, \textit{spo0J}, and \textit{soj} (in \textit{B. subtilis}) that result in aberrant replication control (Bach and Skarstad, 2004; Boye et al., 1996; Camara et al., 2003; Kato and Katayama, 2001; Kitagawa et al., 1998; Lee et al., 2003; Lee and Grossman, 2004; Lu et al., 1994; Noirot-Gros et al., 2002; Ogura et al., 2003; von Freiesleben et al., 1994). One difference is that none of these six previously identified genes are essential for replication initiation to occur, whereas \textit{dnaB} is essential for replication initiation. Another difference is that null mutations in any of these six previously identified genes leads to over-initiation, whereas the \textit{dnaBS371P} mutation that results in over-initiation in slow growing \textit{B. subtilis} cells is not a null mutation. Missense mutations in \textit{E. coli} DnaA that cause hyperactivity (such as \textit{dnaAcos} (Braun et al., 1987; Kellenberger-Gujer et al., 1978; Simmons and Kaguni, 2003; Simmons et al., 2004)) and a temperature sensitive mutation in \textit{B. subtilis} \textit{dnaB} (\textit{dnaB134ts} (Henckes et al., 1989)) are
the other examples of characterized bacterial mutations that cause over-initiation, are found in
genes that are essential for replication initiation, and are not null mutations.

dnaBS371P also appears to differ from other known mutations that cause aberrant replication
control because, although it causes over-initiation in cells grown in minimal medium, it does not
cause over-initiation in cells grown in rich medium. In fact, dnaBS371P cells grown in rich
medium contain slightly decreased DNA content per cell mass compared to wild-type cells
(Chapter 3). Thus, we believe that dnaBS371P is different from other known replication control
mutants because it renders DnaB either directly or indirectly insensitive to changes in growth
rate and the necessary corresponding changes in initiation frequency, rather than causing DnaB
to promote over-initiation under all conditions. The data supporting this hypothesis are
presented in Chapter 3.

In our model for B. subtilis replication initiation, DnaB interacts with DnaD and recruits it to
the membrane in a regulated way, such that these two proteins only interact and work together
during active initiation. This physical interaction may be regulated by a conformational change
that allows DnaB to interact with DnaD (Figure 9). I propose that, during fast growth, when
replication initiation occurs more frequently than during slow growth, DnaB interacts with DnaD
more frequently. dnaBS371P may cause the DnaB protein to interact with DnaD at a set
frequency or affinity that cannot be influenced by growth rate. Our data are consistent with
dnaBS371P causing DnaB and DnaD to interact at a set amount or frequency that is about equal
to what is necessary during fast growth conditions, but is excessive for cells growing under slow
growth conditions (Chapter 3).

A common link between replication initiation and cell division. In addition to exhibiting
aberrant replication control, dnaBS371P cells contain cell division rings placed asymmetrically,
or off-center in the cell (Chapter 3). *E. coli* mutant cells displaying over-initiation and replication asynchrony also undergo asymmetric cell division events. Such mutants include cells lacking *seqA*, and cells in which the origin-proximal GATC sites to which SeqA binds are mutated (Bach and Skarstad, 2004; Boye et al., 1996; Lu et al., 1994; von Freiesleben et al., 1994; Weitao et al., 1999).

The fact that cells lacking replication control, such as *dnaBS371P B. subtilis* cells and *seqA* *E. coli* cells, also display cell division defects demonstrates that a link exists between DNA replication and cell division in bacteria. In wild-type cells, cell division occurs in the center of the dividing cell, and creates two newborn cells of the same size that each contain the same amount of DNA (Figures 4, 5) (Addinall and Holland, 2002; Errington et al., 2003; Harry, 2001; Margolin, 2000; Margolin, 2001). One factor that plays a role in ensuring medial cell division is nucleoid occlusion. Nucleoid occlusion describes the tendency of cell division rings to form in DNA-free regions of the cell (Addinall and Holland, 2002; Errington et al., 2003; Harry, 2001; Margolin, 2000; Margolin, 2001; Wu and Errington, 2004). In wild-type exponentially growing cells, two nucleoids typically exist that contain the same amount of DNA because they are replicating synchronously. Because the two nucleoids contain the same amount of DNA, they take up the same amount of volume in the cell, each in a different half of the cell. This leaves only the center of the cell and the poles of the cell free of DNA and accessible to newly forming cell division rings. Division is inhibited at bacterial cell poles by the Min division inhibitor proteins, and thus cell division rings form almost exclusively in the center of the cell (Figure 10) (Addinall and Holland, 2002; Errington et al., 2003; Harry, 2001; Margolin, 2000; Margolin, 2001).
Figure 10. The positioning of the FtsZ cell division ring is influenced by nucleoid occlusion and the Min division inhibitors. The FtsZ cell division ring typically forms in the center of *E. coli* and *B. subtilis* cells due to two mechanisms, nucleoid occlusion and division inhibition by the Min proteins. Nucleoid occlusion describes the ability of the nucleoid (DNA and all associated proteins) to inhibit cell division. Cells preparing for division typically contain two nucleoids of similar size, as the chromosomes are replicating synchronously. Thus, the bulk of the cell is subject to nucleoid occlusion, except for the DNA-free zones of the cell: the cell poles, and mid-cell. Division is inhibited at the cell poles by division inhibitor proteins called the Min proteins. Thus, an FtsZ ring forms in the center of each cell, between its two replicated chromosomes.
Figure 10.
The action of nucleoid occlusion could be responsible for the asymmetric cell division events seen in cells that are over-initiating and replicating asynchronously. In a population of cells that are undergoing over-initiation, some cells have extra chromosomes and thus often have three nucleoids instead of two. Asynchronously replicating cells also often contain nucleoids that are of different sizes, because an origin in one nucleoid has re-initiated replication even though its sister origin in the other nucleoid has not (Figure 7) (Katayama, 2001; Nordstrom and Austin, 1993; Skarstad et al., 1986). As a result, cells are created that have two differentially sized nucleoids that take up different amounts of volume in the cell. This leads to the creation of a DNA-free zone in the cell that is asymmetrically placed along the length of the cell, which may subsequently lead to asymmetric division ring placement (Figure 11) (Bach and Skarstad, 2004). Such asymmetric cell divisions may allow the creation of newborn cells with more typical, even numbers of origins from asynchronously replicating cells. Asymmetric divisions could also help a population of cells that are over-initiating to constantly regenerate a sub-population of cells with a low number of origins that is manageable for the cell (Figure 11).

Asymmetric cell division rings have been observed in cells that are over-initiating replication by the visualization of FtsZ, the earliest acting component of the bacterial cell division machinery. FtsZ is the most conserved bacterial division protein, and is possessed by essentially all characterized bacteria. FtsZ is a tubulin-like GTPase that polymerizes into rings in the center of wild-type bacterial cells that are preparing to divide. The positioning of FtsZ rings in cells marks the future division sites accurately (Addinall and Holland, 2002; Errington et al., 2003; Harry, 2001; Margolin, 2000; Margolin, 2001). Thus, FtsZ can be used as a marker to visualize future division sites. FtsZ rings have been visualized in over-initiating dnaBS371P B. subtilis.
Asynchronous replication can lead to asymmetric cell division. Asynchronously replicating cells contain chromosomes possessing different amounts of replicated DNA. Thus, asynchronously replicating nucleoids take up different amounts of volume in the cell, unlike synchronously replicating nucleoids. Cells containing asynchronously replicating nucleoids therefore do not contain a DNA-free zone in the center of the cell. Instead, the DNA-free zone is offset from the center by the asymmetric distribution of the nucleoids. Nucleoid occlusion may therefore dictate that asynchronously replicating cells should divide asymmetrically, in the DNA-free, non-polar zone of the cell that is available for division ring formation.
Figure 11.
cells (Chapter 3) and in E. coli cells that have mutations in the SeqA binding sites near to oriC (Bach and Skarstad, 2004). In both cases, FtsZ rings that are asymmetrically placed can be visualized dividing a single cell into two cells of unequal sizes that contain different amounts of DNA (Bach and Skarstad, 2004) (Chapter 3).

A comparison of eukaryotic and prokaryotic mechanisms of replication control. As described above, in bacteria, over-replication of DNA can result in aberrant cell division, replication fork stalling, and replication asynchrony. Bacteria possess multiple mechanisms of replication control that prevent such cell cycle defects from occurring. In eukaryotes, over-replication of DNA results in lethality (Nguyen et al., 2001; Wilmes et al., 2004). Thus, it is critically important that eukaryotes possess multiple, redundant mechanisms that prevent over-replication.

In eukaryotes, three mechanisms that prevent re-initiation have been well-characterized, mainly through studies of the budding yeast S. cerevisiae. First, proteins in the initiator complex ORC are phosphorylated, which render the ORC complex inactive. Second, the helicase loader protein Cdc6 is phosphorylated, which leads to its degradation in some eukaryotes, and its export out of the nucleus in other eukaryotes. Third, the putative helicase proteins (the MCMs) are phosphorylated, which leads to their export out of the nucleus in budding yeast. Re-initiation in budding yeast is detectable only when the phosphorylations of all three of these components (ORC, Cdc6, and MCMs) are prevented (Bell and Dutta, 2002; Kearsey and Cotterill, 2003; Nguyen et al., 2001).

There are several major differences between the methods used to prevent re-initiation in the eukaryote S. cerevisiae and those that occur in the bacteria E. coli and B. subtilis. First, in
eukaryotes, multiple prevention mechanisms must be disrupted in order to allow for re-initiation (Bell and Dutta, 2002; Kearsey and Cotterill, 2003; Nguyen et al., 2001). In contrast, in *E. coli* and *B. subtilis*, mutations in single genes (such as *dnaA*, *hda*, *seqA*, and *datA* in *E. coli*, and *dnaA*, *dnaB*, *yabA*, *spo0J*, and *soj* in *B. subtilis*) can cause detectable amounts of re-initiation (Bach and Skarstad, 2004; Boye et al., 1996; Braun et al., 1987; Camara et al., 2003; Henckes et al., 1989; Kato and Katayama, 2001; Kitagawa et al., 1998; Kellenberger-Gujer et al., 1978; Lee et al., 2003; Lee and Grossman, 2004; Lu et al., 1994; Noirot-Gros et al., 2002; Ogura et al., 2003; Rokop et al., 2004; Simmons and Kaguni, 2003; Simmons et al., 2004; von Freiesleben et al., 1994)). Second, in budding yeast, re-initiation leads to cellular inviability (Nguyen et al., 2001; Wilmes et al., 2004), whereas many single mutations that cause over-initiation in *E. coli* and *B. subtilis* are tolerable to cells. Third, the initiator protein is inactivated through very different mechanisms in eukaryotes and prokaryotes. In eukaryotes, re-initiation is prevented by the phosphorylation of ORC, which occurs immediately after the first initiation event fires at each origin (Bell and Dutta, 2002; Kearsey and Cotterill, 2003; Nguyen et al., 2001). In contrast, in bacteria, re-initiation is prevented in part by the act of replication elongation, which inactivates the initiator protein DnaA because components of the replication elongation machinery stimulate the intrinsic ATPase activity of DnaA (Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001). A final difference is that regulated phosphorylation and degradation events are used to control replication in eukaryotes (Bell and Dutta, 2002; Kearsey and Cotterill, 2003; Nguyen et al., 2001), but these mechanisms are not known to control re-initiation in *B. subtilis* or *E. coli*. Notably, both phosphorylation and degradation are used to control the replication cycle in the Gram-negative bacterium *Caulobacter crescentus* (Ausmees and Jacobs-Wagner, 2003; Marczynski and Shapiro, 2002; Ryan and Shapiro, 2003).
Although there are many obvious differences between eukaryotic and prokaryotic initiation control mechanisms, both eukaryotes and prokaryotes use the general strategy of regulating replication initiation by rendering essential initiation factors unable to access the replication origin. While the prokaryotic and eukaryotic mechanisms differ in detail, in all cases, essential replication factors are excluded from accessing chromosomal origins. In eukaryotes, Cdc6 and Cdt1 (essential components of the helicase loading machinery) are either exported from the nucleus to the cytoplasm or degraded so that they cannot assist in helicase loading at origins in the nucleus. In budding yeast, MCMs, which are most likely the proteins that complex together to form helicase, are similarly exported to the cytoplasm so that they cannot access the chromosomal origins (Bell and Dutta, 2002; Kearsey and Cotterill, 2003; Nguyen et al., 2001).

In *E. coli* and *B. subtilis*, regulated proteolysis has not been found to regulate replication initiation, and export of initiation factors out of the nucleus is clearly not an option. Instead, *E. coli* and *B. subtilis* use two different strategies to prevent essential replication factors from accessing chromosomal origins. First, these bacteria utilize origin-binding proteins that can bind to chromosomal origins and prevent other essential replication proteins from binding at oriC. SeqA is the protein that plays this role in *E. coli* (Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001), and Spo0J may play a similar role in *B. subtilis* (Lee et al., 2003; Lee and Grossman, 2004; Ogura et al., 2003). Second, *E. coli* and *B. subtilis* prevent replication factors from accessing the origins by sequestering the essential initiation factors at a site distal to the origin. In *E. coli*, this is accomplished by the sequestration of DnaA at the *datA* chromosomal site (Kitagawa et al., 1996; Kitagawa et al., 1998). In *B. subtilis*, this is accomplished by maintaining DnaD in the cytoplasm, away from its partner (DnaB) and chromosomal origins (Chapters 2 and 3), both of which are membrane-associated (Funnell, 1996; Rokop et al., 2004;
Sueoka and Hammers, 1974; Sueoka, 1998; Yamaguchi and Yoshikawa, 1977). In conclusion, although the mechanisms of eukaryotic and prokaryotic replication control differ, both groups of organisms achieve a common goal of rendering chromosomal origins inaccessible to essential replication factors that could potentially function at origins to stimulate re-initiation.

**Conclusions.** It is important for cells to regulate the amount of DNA replication that precedes each cell division event. Most mechanisms that regulate replication operate at the step of replication initiation. Both prokaryotes and eukaryotes possess multiple mechanisms of control over the initiation of replication that are very important for the proper execution of the cell cycle. In both Gram-positive and Gram-negative bacteria, a loss of control over replication initiation causes problems with replication asynchrony, replication fork progression, and cell division.

In the Gram-positive bacterium *B. subtilis*, in which common proteins are used for the initiation, restart, and control of replication, a single mutation can lead to pleiotropic effects on DNA replication and cell division. An example of such a mutation is *dnaBS371P*, which is a missense mutation in a gene that encodes an essential replication initiation factor that also functions in replication restart. The following thesis focuses on the functions of wild-type DnaB, DnaBS371P, and DnaB’s interaction partner, DnaD. The work in this thesis explores the roles of DnaB and DnaD in replication initiation and replication fork restart. It also investigates the regulated physical interaction that occurs between DnaB and DnaD, which appears to be important for replication control in *B. subtilis*. Finally, this work places the roles of DnaB and DnaD and the regulation of replication initiation in *B. subtilis* in the context of the cell membrane, as it appears that the membrane plays an integral role in the spatial and temporal control of replication initiation in this bacterium.
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Table 1. The pathways of replication restart in *E. coli* and *B. subtilis*.

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REFERENCES


Chapter 2:
Control of DNA replication initiation by recruitment of an essential initiation protein to the membrane of *Bacillus subtilis*

Megan E. Rokop, Jennifer M. Auchtung, and Alan D. Grossman

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For this chapter, Jennifer Auchtung helped to set up the chromatin immunoprecipitation assay to study helicase loading in *B. subtilis* cells.
SUMMARY

The *B. subtilis* proteins DnaD and DnaB are essential for replication initiation and are conserved in low G+C content Gram-positive bacteria. Previous work indicated that DnaD and DnaB are involved in helicase loading during the process of restarting stalled replication forks. We have investigated the roles of DnaD and DnaB in replication initiation at *oriC* in vivo. Using chromatin immunoprecipitation (ChIP), we found that DnaD and DnaB functions are needed to load the replicative helicase at *oriC*. To further investigate the functions of DnaD and DnaB in replication initiation, we isolated and characterized suppressors of the temperature sensitivity of *dnaD* and *dnaB* mutant cells. In both cases, we isolated the identical missense mutation in *dnaB*, *dnaBS371P*. Using yeast two-hybrid analysis, we found that *dnaBS371P* uncovers a previously undetected physical interaction between DnaD and DnaB. We also found that DnaBS371P constitutively recruits DnaD to the membrane fraction of cells, where DnaB and *oriC* are enriched. Phenotypes of cells expressing DnaBS371P are consistent with aberrant replication control. We hypothesize that *B. subtilis* regulates replication initiation by regulating a physical interaction between two proteins essential for helicase loading at chromosomal origins.
INTRODUCTION

Cells need to properly replicate and segregate their DNA to produce viable progeny, and these processes are highly regulated. The events involved in the initiation of DNA replication are very similar in prokaryotes and eukaryotes (Baker and Bell, 1998; Leatherwood, 1998; Davey and O'Donnell, 2000). Origins of replication are recognized by the replication initiator DnaA in bacteria, and the origin recognition complex ORC in eukaryotes (Messer et al., 2001; Bell and Dutta, 2002; Messer, 2002). A region of DNA in the origin is unwound by DnaA to generate single-stranded DNA, and helicase (or the MCM complex in eukaryotes) is loaded at the replication fork. This loading occurs through the action of helicase loader proteins in bacteria, and through Cdc6 and Cdt1 in eukaryotes. Helicase rapidly unwinds and expands the bubble of single-stranded DNA to allow the entry of DNA primase. Once primase enters and synthesizes primers, the DNA polymerase machinery enters and replication elongation proceeds (Kornberg and Baker, 1992; Messer and Weigel, 1996; Baker and Bell, 1998; Bell and Dutta, 2002).

The Gram-positive bacterium Bacillus subtilis has obvious homologs of Escherichia coli DnaA, helicase, primase, and DNA polymerase; however, no homolog of helicase loader exists (Yoshikawa and Wake, 1993; Moriya et al., 1999; Lemon et al., 2002). The genome of B. subtilis contains essential genes encoding additional replication initiation factors, DnaD, DnaB, and DnaI (Ogasawara et al., 1986; Hoshino et al., 1987; Bruand and Ehrlich, 1995; Bruand et al., 1995b; Sueoka, 1998). DnaI is a AAA+ ATPase (Koonin, 1993; Neuwald et al., 1999), like E. coli helicase loader (Lee and Bell, 2000; Davey et al., 2002), and is analogous to E. coli helicase loader. B. subtilis DnaI interacts physically with B. subtilis helicase and unmasks the ATPase
and unwinding activities of helicase (Imai et al., 2000; Noirot-Gros et al., 2002; Soultanas, 2002; Velten et al., 2003).

The essential functions of *B. subtilis* DnaD and DnaB in replication initiation at oriC are not well defined. {Note that *B. subtilis* dnaB is not related to *E. coli* dnaB. The *E. coli* dnaB gene product is the replicative helicase. In *B. subtilis*, dnaC encodes the replicative helicase (Sakamoto et al., 1995)}. DnaD and DnaB are conserved among low G+C content Gram-positive bacteria including *Listeria, Staphylococcus, Enterococcus, Lactobacillus, Streptococcus*, and *Lactococcus* spp. In *B. subtilis* cells containing a temperature sensitive mutation in dnaB or dnaD, replication initiation is blocked at the non-permissive temperature (Gross et al., 1968; Karamata and Gross, 1970; White and Sueoka, 1973; Burnett and Wake, 1977).

*B. subtilis* DnaB and DnaD are also involved in the process of replication restart (Bruand et al., 2001; Marsin et al., 2001). In both *E. coli* and *B. subtilis*, chromosomal DNA replication initiates in a DnaA-dependent manner at the single origin on the circular chromosome, oriC. Elongation occurs as replication forks travel bidirectionally around the entire circular chromosome until the forks reach the region of replication termination (Messer and Weigel, 1996; Lemon et al., 2002). However, in a significant percentage of cell cycles, elongating forks stall at sites along the chromosome due to DNA lesions or protein roadblocks. In these situations, replication restarts at the stalled forks through the action of PriA, which functions to direct helicase loading independently of DnaA. Thus PriA is the “initiator” of replication for all sites on the chromosome except oriC (Cox et al., 2000; Marians, 2000; Sandler and Marians, 2000; Cox, 2001). A deletion of *priA* causes pleiotropic phenotypes, including poor growth (Sandler et al., 1996; Polard et al., 2002).
Biochemical and genetic evidence indicates that DnaD and DnaB are involved in helicase loading at stalled forks along with PriA. PriA, DnaD, and DnaB form higher order complexes on forked DNAs in vitro (Marsin et al., 2001). In *B. subtilis*, suppressors of the growth defects of priA null mutants are in dnaB (Bruand et al., 2001). In *E. coli*, suppressors of the growth defects of priA null mutants are in the gene encoding helicase loader (Sandler et al., 1996). This mutant form of *E. coli* helicase loader has gained the ability to load helicase onto DNA in vitro in the absence of PriA (Liu et al., 1999; Xu and Marians, 2000).

Studies using plasmid replication or purified components also provide evidence for the involvement of DnaD and DnaB in helicase loading. Helicase cannot load onto a DnaA-independent plasmid replicating in dnaDts cells at the permissive temperature and in dnaBts cells at the non-permissive temperature (Bruand et al., 1995a). In vitro, DnaB physically interacts with helicase and with the helicase/DnaI complex. DnaB also modulates the activities exhibited by helicase when complexed with DnaI. In the absence of DnaI, helicase does not exhibit translocase (single-stranded DNA-dependent ATPase) and unwinding activities. DnaI exposes these activities of helicase. DnaB further stimulates the translocase and unwinding activities of helicase in the presence of DnaI approximately three-fold (Velten et al., 2003).

We have investigated the roles of DnaD and DnaB in replication initiation at oriC in vivo. We found that both DnaD and DnaB are necessary to load helicase at oriC in vivo. We provide evidence indicating that DnaD and DnaB physically interact and we propose that this interaction is regulated. If this interaction is increased or occurs in a misregulated fashion, the subcellular location of DnaD is altered such that DnaD is brought to the cell membrane, where DnaB is. This misregulation results in phenotypes consistent with aberrant control of replication initiation.
We propose that *B. subtilis* uses the regulation of the DnaD-DnaB interaction to control the timing and location of helicase loading, and therefore of replication initiation.

**RESULTS and DISCUSSION**

*DnaD and DnaB function is necessary prior to the loading of helicase at oriC in vivo.* We monitored the loading of a Myc-tagged version of the replicative helicase at oriC in dnaB and dnaD mutant cells using a chromatin immunoprecipitation (ChIP) assay. The tagged helicase functions as the only helicase in the cell, and is expressed from its endogenous site on the chromosome under its endogenous promoter. We introduced the helicase-Myc (dnaC-myc) construct into cells containing the dnaD23ts mutation. We grew a culture of these dnaDts cells at permissive temperature to obtain asynchronously growing cells. The culture was then incubated at non-permissive temperature to inactivate DnaD and synchronize the culture. This treatment arrests replication at the step in initiation that requires DnaD function. Upon release back to the permissive temperature, most cells initiate replication synchronously (Gross et al., 1968). At each stage of the experiment, we monitored enrichment of helicase-Myc at oriC by ChIP.

We found that helicase was not enriched at oriC as compared to an origin distal site in the dnaDts mutant at non-permissive temperature (Figure 1A). Quantitation showed that, after 1 minute of release at the permissive temperature, helicase was 5-fold more enriched at oriC (as compared to an origin distal site) than it was at non-permissive temperature. We obtained similar results using a strain containing the dnaD134ts allele (Figure 1B). In these experiments, helicase became enriched at oriC 1 minute after release, and enrichment decreased after 5
Figure 1.
Figure 1. DnaD and DnaB function is necessary prior to the loading of helicase at oriC.

Chromatin Immunoprecipitation (ChIP) was used to detect the presence of helicase-Myc bound to the origin region (oriC) and to an origin distal region in dnaDts (MER555) cells (panel A) and in dnaBts (MER489) cells (panel B). Cells were grown at permissive temperature (30°C), shifted to non-permissive temperature (45°C) to synchronize the culture and inactivate DnaD or DnaB, and then released back into permissive temperature for 1 minute. Samples were prepared for immunoprecipitation and DNA in the immunoprecipitates was analyzed by PCR. Quantitation (not shown) indicated that helicase-Myc was enriched at least five-fold at oriC after release into permissive temperature. Enrichment was specific to the oriC region, and was dependent on the presence of antibody during the immunoprecipitation. Total DNA = total input DNA after crosslinking, lysis, and sonication; -Ab = mock immunoprecipitation with no antibody; +Ab = immunoprecipitation with anti-Myc antibody.
minutes of release as helicase proceeded away from the origin (data not shown). This timing is consistent with previous experiments showing that the entire chromosome is replicated within an hour after release of \textit{dnaBts} cells back into permissive temperature (Laurent and Vannier, 1973). We conclude that the function of DnaD and DnaB is necessary prior to the assembly of helicase at \textit{oriC}. In vitro, purified \textit{B. subtilis} DnaA is capable of binding to \textit{oriC} and inducing open complex formation (unwinding \textit{oriC}) (Krause et al., 1997). If this is also true in vivo, then our results indicate that DnaD and DnaB function is required after open complex formation and before the loading of helicase at \textit{oriC}.

**Isolation of the \textit{dnaBS371P} allele as a suppressor of the temperature sensitivity of \textit{dnaDts} and \textit{dnaBts} cells.** To further characterize the replication initiation defects of \textit{dnaDts} and \textit{dnaBts} cells, we isolated spontaneous suppressors of the temperature sensitivity of \textit{dnaDts} or \textit{dnaBts} mutant cells. We isolated 27 independent mutations that suppressed the temperature sensitive phenotype caused by the \textit{dnaD23ts} mutation. Five of the 27 suppressors were extragenic (not linked to \textit{dnaD}). Mapping and DNA sequence analysis demonstrated that all five extragenic suppressor mutations were identical and caused a serine to proline change at amino acid 371 of DnaB (DnaBS371P).

We also isolated suppressors of the temperature sensitivity of cells containing the \textit{dnaB134ts} allele. Of the 41 independent suppressor mutations we isolated, 5 were the identical \textit{dnaBS371P} allele that we isolated in the \textit{dnaDts} suppressor screen. Thus \textit{dnaBS371P} is an intragenic suppressor of the temperature sensitivity of \textit{dnaB134ts} cells.

Intriguingly, the \textit{dnaBS371P} mutation was isolated previously in a screen for bypass suppressors of the poor growth of \textit{priA} mutants (Bruand et al., 2001). PriA is the protein central
to the process of restarting replication forks that have stalled at sites along the chromosome (Cox et al., 2000; Marians, 2000; Sandler and Marians, 2000; Cox, 2001). Taken together, the results from these three genetic screens demonstrate that dnaBS371P suppresses a variety of defects in the processes of replication restart and replication initiation at oriC.

**DnaD- and DnaB-like domains are fused in putative phage initiation factors.** To gain more insight into the potential roles of DnaD and DnaB in replication, we used BLAST searches (Altschul et al., 1990) to identify proteins that share sequence similarity with DnaD and DnaB. We found that a region of DnaD has 53% sequence similarity (27% identity) with a 73 amino acid region of Gp49, a putative replication initiation protein from phage A118 of *Listeria monocytogenes* (Loessner et al., 2000). We also found that a region of DnaB has 52% sequence similarity (32% identity) with a 70 amino acid region of Gp49 that partially overlaps with the Gp49 region that is similar to DnaD (Figure 2). Thus, Gp49 appears to contain a fusion of a C-terminal region of DnaD to a C-terminal region of DnaB.

We found that a homolog of Gp49, the putative initiation protein Orf46 from *Staphylococcus aureus* phage ΦPVL (Kaneko et al., 1998), also contains neighboring DnaD- and DnaB-like domains. We also found a similar fusion domain in Protein 20 from *Lactobacillus plantarum* prophage Lp1 (Kleerebezem et al., 2003).

Oftentimes, if two proteins are fused into a single polypeptide in one organism, the two proteins physically interact in a second organism in which they are encoded separately (Marcotte et al., 1999). The fusion of DnaD and DnaB into single polypeptides involved in phage replication indicates that DnaD and DnaB may physically interact in *B. subtilis*, an organism in which the two proteins are encoded separately. However, previous studies using in vitro assays and yeast two-hybrid assays did not detect interaction between DnaD and DnaB (Ishigo-oka et
Figure 2. DnaD and DnaB both share sequence similarity with Gp49, a putative phage replication initiation protein. Gp49 is shown here as a representative member of a family of putative phage replication initiation proteins. Gp49 is from the *Listeria monocytogenes* phage A118. DnaD has 53% sequence similarity with a 73 amino acid region of Gp49 that is neighboring to and partially overlapping with a 70 amino acid long region of Gp49 that is 52% similar to DnaB. Similar fusion domains were found in Orf46 from *Staphylococcus aureus* phage ΦPVL and in Protein 20 from *Lactobacillus plantarum* prophage Lp1. The arrow indicates the position of the dnaBS371P missense mutation, which occurs just three amino acids upstream of the region of DnaB that is homologous to the phage initiation factors.
Figure 2.

DnaD

region of 53% similarity

Gp49

region of 52% similarity

DnaB

dnaBS371P
al., 2001; Marsin et al., 2001; Noirot-Gros et al., 2002). To reconcile this discrepancy, we postulated that DnaD-DnaB interactions might be transient and regulated, and that \textit{dnaBS371P} causes increased or inappropriately regulated interactions to occur between DnaB and DnaD. Interestingly, the serine to proline change caused by \textit{dnaBS371P} is three amino acids upstream of the region of DnaB that is similar to the phage initiation proteins described above (Figure 2). We suspect that this substitution causes a conformational change in DnaB.

**DnaD is recruited to the membrane fraction of cells by DnaBS371P.** We investigated the subcellular fractionation patterns of the DnaB and DnaD proteins to determine if these two putative interaction partners were in a common subcellular location. It was previously postulated that DnaB is a membrane protein (Hoshino et al., 1987). We performed subcellular fractionation experiments on \textit{dnaB+} cells and analyzed the fractions with Western blotting using an anti-DnaB antibody. We found that DnaB is located predominantly in the membrane fraction of cells (Figure 3A). DnaBS371P had the same fractionation pattern as DnaB, and this was not altered by the presence of the DnaD-Myc fusion used below (data not shown).

Our findings are consistent with previous studies showing that DnaB is functionally linked to the membrane. In both \textit{E. coli} and \textit{B. subtilis}, origin regions of the chromosome are enriched in the membrane fraction of cells (Sueoka and Hammers, 1974; Yamaguchi and Yoshikawa, 1977; Funnell, 1996; Sueoka, 1998). In \textit{B. subtilis}, DnaB function is necessary for the enrichment of chromosomal origins in the membrane (Winston and Sueoka, 1980; Sato et al., 1991; Funnell, 1996; Sueoka, 1998). Because both DnaB and chromosomal origins are found in the membrane fraction of cells, replication initiation is likely to occur at the membrane in \textit{B. subtilis}. 

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Figure 3. DnaD is recruited to the membrane fraction of cells by DnaBS371P.

Subcellular fractionations were probed for DnaB or DnaD-Myc by Western blotting. Tot = total extract, Cyt = cytoplasmic fraction, Mem = membrane fraction.

A) DnaB is enriched in the membrane fraction of cells. Extracts from dnaB+ (BDR524) cells were probed using anti-DnaB antibodies. The upper band in this Western blot is a non-specific cross-reacting band as its signal does not decrease after depletion of DnaB (data not shown).

B) DnaD. Extracts from dnaD+ (JH642), dnaD-myc (MER454), and dnaD-myc dnaBS371P (MER484) cells were probed using anti-Myc monoclonal antibodies. In this Western blot, 4.5-fold more material was loaded from the membrane fraction than from the total extract or cytoplasmic fraction. In quantitation (data not shown) less than 1-2% of DnaD-Myc was found in the membrane fraction of dnaB+ cells. In contrast, approximately 15% of the cellular content of DnaD-Myc was relocated to the membrane fraction of dnaBS371P mutants.
Figure 3.
We found that DnaBS371P recruits DnaD to the membrane fraction of cells. We performed subcellular fractionation experiments on cells expressing DnaD-Myc and analyzed the fractions with Western blotting using an anti-Myc antibody. The Myc-tagged DnaD functions as the only DnaD in the cell and is expressed at its endogenous site on the chromosome under the control of its endogenous promoter. We found that only approximately 1 - 2% of DnaD-Myc was in the membrane fraction of dnaB+ cells. In marked contrast, a significant proportion of DnaD-Myc (~15%) was found in the membrane fraction of dnaBS371P mutant cells (Figure 3B).

*dnaBS371P allows for the detection of the physical interaction between DnaD and DnaB.* The recruitment of DnaD by DnaBS371P to the membrane fraction of cells could be a result of direct interaction between the two proteins. However, previous work failed to detect direct interaction between DnaD and DnaB (Ishigo-oka et al., 2001; Marsin et al., 2001; Noirot-Gros et al., 2002). We chose to test for direct interaction using yeast two-hybrid analysis, one of the methods that previously failed to detect interactions between DnaD and DnaB (Ishigo-oka et al., 2001; Noirot-Gros et al., 2002).

Using yeast two-hybrid analysis, we found that DnaBS371P and DnaD directly interact. We fused DnaB, DnaD, or DnaBS371P to either the activation domain (AD) or the DNA binding domain (DBD) of the yeast transcription factor Gal4. A physical interaction between the Gal4 AD and DBD domains will drive expression of the ADE2 gene, thereby allowing for growth of the yeast on medium lacking adenine (James et al., 1996). We found that yeast expressing AD-DnaD and DBD-DnaBS371P (or AD-DnaBS371P and DBD-DnaD) grew on medium lacking adenine (Figure 4), indicating that DnaBS371P and DnaD physically interact.
Figure 4. DnaD interacts with DnaBS371P. Yeast two-hybrid analysis was used to examine physical interactions between DnaB and DnaD fused either to the activation domain (AD) or to the DNA binding domain (DBD) from the Gal4 transcription factor. A physical interaction activates the expression of ADE2, allowing for growth on medium lacking adenine. Plate sections 1 (AD-DnaD, DBD-DnaB) and 2 (AD-DnaB, DBD-DnaD) confirm previous results that DnaB and DnaD do not interact in the two-hybrid assay. Plate sections 3 (AD-DnaBS371P, DBD-DnaD) and 5 (AD-DnaD, DBD-DnaBS371P) show that DnaBS371P and DnaD do interact. Plate section 4 (AD-DnaB, DBD-DnaB) is a positive control that confirms the previous result that DnaB (a multimer) interacts with itself.
Figure 4.
In addition, we confirmed the previous findings that wild-type DnaB and DnaD do not
detectably interact by the two-hybrid assay. Yeast expressing AD-DnaD and DBD-DnaB (or
AD-DnaB and DBD-DnaD) did not grow on medium lacking adenine (Figure 4). As a positive
control and a test for the functionality of the DnaB fusion plasmids, we found that yeast
expressing AD-DnaB and DBD-DnaB grew on medium lacking adenine. DnaB has previously
been shown to interact with itself by two-hybrid analysis and to multimerize in vitro (Ishigo-oka
et al., 2001; Marsin et al., 2001; Noirot-Gros et al., 2002).

Based on our findings, we suggest that wild-type DnaB and DnaD directly interact, but that
this interaction is regulated, perhaps so that it only occurs when *B. subtilis* is actively initiating
replication. Thus, this interaction might be difficult to detect in the yeast two-hybrid assay, in
vitro using purified components, or in subcellular fractionation experiments on asynchronously
growing wild-type cells. The *dnaBS371P* mutation allows for increased or unregulated
interactions to occur between DnaB and DnaD. This makes the physical interaction detectable in
the subcellular fractionation assay and by the yeast two-hybrid assay.

**Phenotypes associated with dnaBS371P are consistent with aberrant replication control.**
We found that cells expressing DnaBS371P have two phenotypes indicative of inappropriate
regulation and timing of replication initiation. First, *dnaBS371P* mutant cells were more
sensitive to elevated levels of DnaA than were *dnaB*+ cells. We expressed DnaA from a LacI-
repressible-IPTG-inducible promoter at an ectopic locus in *dnaB*+ and *dnaBS371P* cells. We
found that *dnaBS371P* cells were not capable of colony formation in the presence of IPTG, but
*dnaB*+ cells were (Figure 5A). *dnaB*+ cells cannot tolerate extremely high overexpression of
Figure 5. Phenotypes associated with the dnaBS371P allele. A) dnaBS371P cells are hypersensitive to higher levels of DnaA than are wild-type cells. dnaB+ (MER616, right plate quadrants) and dnaBS371P (MER617, left plate quadrants) cells containing Pspank fused to dnaA were tested for growth on LB plates without (lower plate) or with (upper plate) IPTG to induce transcription from Pspank.

B) Cells overproducing DnaBS371P grow poorly and form filaments. Cells containing Pspank(hy) fused to dnaB (MER580, left panels) or dnaBS371P (MER582, right panels) were grown at 37°C in LB medium without (upper panels) or with (lower panels) IPTG to induce transcription from Pspank(hy). Cells were sampled during exponential growth, and cell nucleoids (that is, the DNA and all associated proteins) were stained with DAPI and visualized by fluorescence microscopy. Cell outlines were visualized using Nomarski microscopy, and the two images were merged to show the cell outlines in grey and the nucleoids in white.
A.

\[ P_{\text{IPTG}} - \text{dnaA} \]
\[ \text{dnaBS371P} \]
\[ P_{\text{IPTG}} - \text{dnaA} \]
\[ \text{dnaB} \]

+ IPTG

- IPTG

B.

\[ P_{\text{IPTG}} - \text{dnaB} \]
\[ P_{\text{IPTG}} - \text{dnaBS371P} \]

- IPTG

+ IPTG

Figure 5.
DnaA, but they can tolerate expression of DnaA to the levels provided by the construct we used (Ogura et al., 2001).

Second, we found that otherwise wild-type cells were sensitive to overexpression of dnaBS371P. Cells overproducing DnaBS371P grew poorly in rich media, whereas cells overproducing wild-type DnaB did not. Wild-type B. subtilis cells grow in chains that contain regularly spaced and sized nucleoids (DNA masses and all associated proteins). Using DAPI staining and live cell microscopy, we observed that cells overproducing DnaBS371P (but not those overproducing wild-type DnaB) form filaments and contain large, irregularly spaced cellular regions devoid of DNA (Figure 5B). This effect is dominant, as these cells are dnaB+ at the endogenous locus. These findings indicate that dnaBS371P causes altered control of replication initiation. This alteration is tolerable in otherwise wild-type cells, but is not tolerable in cells with elevated levels of DnaA or upon elevation of levels of DnaBS371P.

The events of chromosomal replication initiation in B. subtilis. Based on our results, we suggest that the initiation of replication in B. subtilis is controlled in part by regulating the interaction between DnaB and DnaD, and therefore by regulating the activity of a functional helicase loader. Our genetic and molecular studies of the dnaDts, dnaBts, and dnaBS371P alleles led to the following model for events that occur during replication initiation in B. subtilis. We hypothesize that, during active replication initiation, the membrane-associated initiation factor DnaB interacts with DnaD to recruit it to the membrane. Initiation can only occur when DnaB, DnaD, and chromosomal origins converge at the membrane. DnaB and DnaD promote initiation by facilitating the loading of helicase by DnaI onto origins unwind by DnaA. The DnaD-DnaB interaction could be triggered by DnaA-dependent open complex formation (the unwinding of oriC). The signal to interact could be sent through DnaD, as DnaD interacts
directly with DnaA as determined by yeast two-hybrid analysis (Ishigo-oka et al., 2001).

Alternatively, the signal could be sent through DnaB, a known DNA-binding protein that has higher affinities for DNA that contains structures similar to replication forks (Marsin et al., 2001).

DnaBS371P has several properties that are different from wild-type DnaB. In vitro, the affinity of DnaBS371P for DNA is higher than that of wild-type DnaB (Velten et al., 2003). Additionally, the unwinding activity of helicase (complexed with DnaI) is stimulated three-fold more by DnaBS371P than by wild-type DnaB (Velten et al., 2003). We found that DnaBS371P physically interacts with DnaD and causes it to associate with the membrane in a misregulated or increased manner. It is currently unclear which of these properties contribute to the ability of dnaBS371P to suppress the phenotypes of dnaBts, dnaDts, and ApriA mutants.

It has been proposed that together, DnaB and DnaI function as the helicase loader in B. subtilis (Velten et al., 2003). However, it is also possible that DnaI functions as the helicase loader and that together, DnaB and DnaD function to regulate where and when DnaI can perform this function. Having separate subunits that regulate the activity of helicase loader could allow B. subtilis to achieve stringent control over regulation of replication initiation. Since DnaD and DnaB facilitate the loading of helicase at oriC in vivo, and oriC and DnaB are enriched in the membrane fraction of cells (Sueoka and Hammers, 1974; Yamaguchi and Yoshikawa, 1977; Sueoka, 1998), then maintaining DnaD in the cytoplasm would prevent the occurrence of excess replication initiation events. B. subtilis could control the frequency and timing of replication initiation, in part, by regulating when the essential initiation factor DnaD is associated with the membrane. This spatial control over the timing of replication initiation appears to be disrupted
in *dnaBS371P* cells, in which the DnaB-DnaD interaction and the recruitment of DnaD to the membrane are misregulated.

*E. coli* regulates replication initiation, in part, through the sequestration of hemimethylated origin DNA by the SeqA protein (Lu et al., 1994; Slater et al., 1995; Shakibai et al., 1998). However, this system is not found in *B. subtilis* and other Gram-positive bacteria, nor is it found in many Gram-negative bacteria (Hiraga, 2000). We hypothesize that *B. subtilis* regulates initiation in part by regulating the interaction between DnaB and DnaD that occurs at the membrane. This thereby regulates the activity of a functional helicase loader. This spatial control of essential replication proteins provides another step at which the initiation of chromosome replication can be regulated.
EXPERIMENTAL PROCEDURES

Media and growth conditions. Rich medium was LB for routine growth and strain constructions for both *E. coli* and *B. subtilis*. *E. coli* and *B. subtilis* transformations were done by standard procedures (Harwood and Cutting, 1990; Sambrook and Russell, 2001). Defined minimal medium for *B. subtilis* was S7 medium, with MOPS buffer at 50 mM rather than 100 mM, supplemented with 1% glucose and 0.1% glutamate and required amino acids (tryptophan, 40 μg/ml; phenylalanine, 40 μg/ml) as needed (Vasantha and Freese, 1980; Jaacks et al., 1989). Antibiotics were used at the following concentrations: ampicillin at 100 μg/ml, spectinomycin at 100 μg/ml, chloramphenicol at 5 μg/ml, and erythromycin at 0.5 μg/ml with lincomycin at 12.5 μg/ml. Where indicated, isopropyl-beta-D-thiogalactopyranoside (IPTG) (Sigma) was used at 1 mM to induce expression from the LacI-repressible-IPTG-inducible promoters Pspank and Pspank(hy).

Strains, alleles, and plasmids. *E. coli* strains used for cloning included DH5α (*endA1 hsdR17 (rK- mK+) supE44 thi-1 recA1 gyrA (Nal') relA1 Δ(lacIZYA-argF)U169 deoR (ϕ80lac (lacZΔM15) and JM107 (F' traD36 lacF lacZΔM15 proA+ B'/e14- (mcrA-) Δ(lac-proAB) thi gyrA96 (Nal') endA1 hsdR17 (rK- mK+) relA1 supE44). *B. subtilis* strains are listed in Table 1, and are derivatives of JH642 (AG174) that contain the *trpC* and *pheA* mutations, unless otherwise indicated. Standard procedures were used for strain constructions (Harwood and Cutting, 1990).

The dnaDts allele used was dnaD23ts, which causes an alanine to threonine change at amino acid 166 of 232 (Bruand et al., 1995b). The dnaBts allele used was dnaB134ts, which causes a lysine to glutamic acid change at amino acid 85 of 472 (Sueoka, 1998). All strains containing temperature sensitive alleles were grown at 30°C.
To generate the DnaD-Myc and helicase-Myc fusion constructs, the 3' ends of \textit{dnaD} and \textit{dnaC} (helicase) were amplified by PCR and fused in frame to three tandem copies of the sequence encoding the Myc epitope inside the vector pUS19 (Benson and Haldenwang, 1993). The resulting fusion plasmids, pMR35 (\textit{dnaD-myc}) and pKL138 (\textit{dnaC-myc}), were integrated into the chromosome by single crossover to generate fusions of the entire \textit{dnaD} or \textit{dnaC} genes to \textit{myc} linked to spectinomycin resistance.

\textit{dnaB} and \textit{dnaBS371P} were overexpressed from fusions to the LacI-repressible-IPTG-inducible promoter Pspank(hy). \textit{dnaB} and \textit{dnaBS371P} were amplified by PCR and cloned into the vector pDR111 (a gift from D. Rudner) to generate the plasmids pMR64 and pMR65, which were used to introduce Pspank(hy)-\textit{dnaB} and Pspank(hy)-\textit{dnaBS371P} into the \textit{amyE} locus.

\textit{dnaA} was overexpressed using the LacI-repressible-IPTG-inducible promoter Pspank. \textit{dnaA} was amplified by PCR and cloned into the vector pDR110 (a gift from D. Rudner) to generate the plasmid pMR77, which was used to introduce Pspank-\textit{dnaA} into the \textit{amyE} locus.

The pDR110- and pDR111-derived constructs were linearized before transformation to ensure integration by double crossover. Transformants were selected for resistance to spectinomycin, and were screened for the disruption of \textit{amyE} by the loss of the ability to degrade starch.

**Chromatin Immunoprecipitation.** \textit{dnaDts} (MER555) and \textit{dnaBts} (MER489) mutants were grown in defined minimal medium with spectinomycin (to maintain selection for the \textit{dnaC-myc} fusion) at 30° to mid-exponential phase (OD\textsubscript{600} = 0.4). Cells were shifted to 45° for 45 minutes to prevent initiation of replication and to allow most ongoing rounds of replication to finish. Cells were then shifted back to 30° rapidly by adding an equal volume of medium that was at 15°C. Samples were taken at various times and fixed and crosslinked with 1% formaldehyde for
5 minutes at room temperature. Glycine was added (final concentration 120 mM) and samples were incubated for 5 minutes at room temperature to stop the crosslinking. Further processing, cell lysis, and immunoprecipitations were done essentially as described previously (Lin and Grossman, 1998; Quisel et al., 1999) with the following exceptions. The protease inhibitor 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF) (Sigma) was added during cell lysis and immunoprecipitations to a final concentration of 1 mM. Immunoprecipitations were performed overnight at 4°C using mouse anti-c-Myc antibody (Zymed) at 6 μg per immunoprecipitation (a dilution of 1:75 relative to the final volume of the immunoprecipitation) and then incubated with a 50% Protein A-Sepharose bead slurry (Pharmacia Biotech) for 1 hour at 4°C. Crosslinks were reversed overnight at 65°C, and DNA was purified essentially as described (Aparicio, 1999). DNA in the immunoprecipitates was analyzed by PCR using primers to the oriC region and to an origin distal site region (dnaD, which is located ~1,860 kbp from oriC).

PCR was done on two-fold serial dilutions of template DNA to obtain products in the linear range. Products were analyzed on 2% agarose gels stained with ethidium bromide using the ChemiImager gel documentation system and the accompanying software (Alpha Innotech). This allowed us to determine the relative amounts of DNA from the origin region compared to an origin distal site. This was done under permissive (30°C) and non-permissive (45°C) conditions, and the ratio of permissive to non-permissive is an indication of the relative amounts of helicase bound.

Suppressor Screens. Spontaneous suppressors were isolated by plating 10^7 or 10^8 dnaD23ts (KPL73) or dnaB134ts (KPL69) cells (grown in liquid LB at 30°C) onto LB plates incubated overnight at either 45°C or 48°C. Suppressors were isolated at frequencies of approximately 10^-6 to 10^-7. Colonies were purified three times and grouped by growth phenotypes on LB plates.
incubated at 30°, 37°, 42°, 45°, 48°, and 52°. Representatives from each group were tested for linkage to dnaA, dnaB, dnaC, and dnaD using markers linked to the wild-type version of each gene. In each case, the selectable marker linked to dnaA, dnaB, and dnaD was a Tn917 transposon insertion containing the chloramphenicol resistance gene (cat) from the plasmid pTV21Δ2 (Youngman et al., 1984; Vandeyar and Zahler, 1986). Tn917ΩHU163 is linked to dnaA in BB302, zhb-83::Tn917 is linked to dnaB in K11346, and Tn917ΩHU151 is linked to dnaD in KPL154 (Vandeyar and Zahler, 1986; Sandman et al., 1987). For linkage to dnaC (helicase), the spectinomycin resistance linked to the dnaC-myc fusion in KPL314 was used. For example, linkage tests for dnaA were done by transforming Tn917ΩHU163::pTV21Δ2-cat genomic DNA into the suppressor strain, selecting for chloramphenicol, and screening for conversion from temperature resistance back to the original phenotype of temperature sensitivity. Once linkage was identified, the genes were sequenced by the MIT Biopolymers Lab. DnaB was PCR amplified and either sequenced directly or cloned into pGEMcat (Youngman et al., 1989) for sequencing. In the dnaDts suppressor screen, five of the 27 suppressor mutations were dnaBS371P, and all others were linked to dnaD. In the dnaBts suppressor screen, five of the 41 suppressor mutations were dnaBS371P, twelve were linked to dnaB but were not dnaBS371P, and the remaining isolates have not been analyzed.

**Membrane fractionations.** Samples of JH642 (wild-type), MER454 (dnaD-myc), MER484 (dnaD-myc dnaBS371P), and BDR524 {dnaD+ dnaB+ amyE::(PxyLA-spoIVFB-gfpmut2)} were harvested in exponential growth (OD600 between 0.5 - 0.9) in LB medium at 37°C. MER454 and MER484 cells were grown in LB with spectinomycin to maintain selection for the dnaD-myc fusion. Protoplasting, lysis, and nuclease treatments were done essentially as described (Rudner and Losick, 2002) except that the protease inhibitor AEBSF was used at a concentration of 1
mM. Extracts were centrifuged for 1 hour at 4° in a Beckman Optima TL ultracentrifuge using the TLS-55 rotor at 39,000 rpm (100,000X.g). SDS loading buffer was added to the supernatant to yield the cytoplasmic fraction in a final concentration of 1X SDS loading buffer. The pellet was resuspended in 1X SDS loading buffer to yield the membrane fraction. The membrane protein SpoIVFB fused to GFP, expressed in strain BDR524 (Rudner and Losick, 2002), was used as a control in some experiments. Expression of the SpoIVFB-GFP fusion was controlled by a xylose-inducible promoter.

**Western blotting.** Protein amounts to be loaded from fractionation experiments were normalized with respect to the optical density of the culture. SDS-PAGE was performed using 7.5% Tris-HCl denaturing polyacrylamide gels (Biorad). Protein was electrotransferred to Immobilon-P membrane (Millipore) using the Trans-blot SD semi-dry transfer cell (Biorad). Membranes were blocked for 1 hour at room temperature in 1X TBS-0.05%Tween with 5% BSA. Membranes were probed with primary antibody diluted 1:500 in 1X TBS-0.05%Tween with 5% BSA for 1 hour at room temperature. The primary antibody to DnaB was affinity purified rabbit polyclonal antibody (Covance) made against purified DnaB-GST. The primary antibody to DnaD-Myc was mouse anti-c-myc antibody (Zymed). Horseradish peroxidase conjugated secondary antibody was diluted 1:3000 for goat anti-rabbit immunoglobulin-G (Biorad) and 1:10,000 for sheep anti-mouse immunoglobulin-G (Amersham) and incubated with membranes for 1 hour at room temperature. Signals were detected by incubation of membranes with Western Lightning chemiluminescence reagents (Perkin Elmer) for 1 minute and exposure to Kodak Biomax MR scientific film. A comparison of the signals from serial dilutions of extract from the cytoplasmic and membrane fractions allowed estimates of the amount of DnaD-Myc in the membrane fraction of cells to be made.
Two-hybrid analysis. *dnaB, dnaBS371P, and dnaD* were amplified by PCR from the appropriate chromosomal DNA and inserted into the pGAD-C1 or pGBDU-C3 vectors to create in frame fusions of DnaB or DnaD to the activation domain (AD) or DNA binding domain (DBD) of the Gal4 transcription factor (James et al., 1996). The resulting plasmids are pMR58 (encoding AD-DnaB), pMR59 (encoding AD-DnaBS371P), pMR60 (encoding AD-DnaD), pMR61 (encoding DBD-DnaB), pMR62 (encoding DBD-DnaBS371P), and pMR63 (encoding DBD-DnaD). Each combination of the AD and DBD fusions was transformed into the *Saccharomyces cerevisiae* strain DWY112 (ura3-52 leu2-3 his3 trp1 dph2Δ::HIS3 gal4Δ gal80Δ GAL-ADE2 lys2::GAL1-HIS3 met2::GAL-lacZ) (James et al., 1996). Transformations and colony purifications were done at 30°C on synthetic defined (SD) medium lacking uracil and leucine. Cells were then plated on SD medium lacking uracil, leucine, and adenine and scored for growth at 30°C.

Fluorescence microscopy. MER580 (*amyE::(Pspank(hy)-dnaB spc)*)) and MER582 (*amyE::Pspank(hy)-dnaBS371P spc)*) cells were grown in LB medium with or without IPTG at 37°C to mid-exponential phase (an OD600 between 0.3 and 0.7). The DNA stain 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) was added to 0.1 μg/mL and cells were placed on slides containing pads of 1% agarose in minimal salts with 1mM MgSO4. Fluorescence was viewed with a Nikon E800 microscope equipped with a 100X DIC objective using a UV filter. Images were obtained with a CCD camera (Hamamatsu, model C4742-95) using Improvision OpenLab software.
ACKNOWLEDGEMENTS

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Table 1. *B. subtilis* strains used.

<table>
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<th>Strain Name</th>
<th>Relevant genotype</th>
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<td>BB302</td>
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REFERENCES


Appendix A:

Isolation and characterization of suppressors of the temperature sensitivity of *dnaB* and *dnaD* *Bacillus subtilis* cells
SUMMARY

The *B. subtilis* genes *dnaD* and *dnaB* are essential for the initiation of DNA replication, and several temperature sensitive mutations in the *dnaB* and *dnaD* genes have been isolated. I performed three selections to isolate suppressors of the temperature sensitivity of *dnaD*23*ts*, *dnaB*134*ts*, and *dnaB*19*ts* mutant cells. I isolated a single extragenic mutation and several intragenic mutations that suppressed the temperature sensitive phenotype caused by *dnaD*23*ts*. We previously reported that the single extragenic suppressor *dnaBS*371*P*, a missense allele of *dnaB*, allows a direct physical interaction between DnaB and DnaD to be detected by yeast two-hybrid analysis. In this study, I found that one of the intragenic suppressors of the temperature sensitivity of *dnaD*23*ts* cells similarly allows for the detection of a physical interaction between DnaB and DnaD. I also isolated *dnaBS*371*P* as an intragenic suppressor of the temperature sensitivity of *dnaB*134*ts* cells, along with several other intragenic suppressors. I did not isolate *dnaBS*371*P* in my selection for *dnaB*19*ts* suppressors, but in this selection I did isolate several intragenic suppressors and several informational suppressors (compensatory mutations in tRNA genes that would restore the proper amino acid to the DnaB19*ts* protein). I conclude that *dnaBS*371*P* suppresses the defects caused by the *dnaD*ts* allele and by some but not all temperature sensitive mutations in *dnaB*. 
INTRODUCTION

The *B. subtilis* genes *dnaD* and *dnaB* are essential for the initiation of DNA replication (Bruand et al., 1995; Hoshino et al., 1987; Ogasawara et al., 1986; Sueoka, 1998). Because these two genes are essential, their function has traditionally been studied using temperature sensitive mutations that have been isolated in the *dnaB* and *dnaD* genes. Four temperature sensitive mutations have been isolated in *B. subtilis dnaB*: *dnaB134ts* (also called *dnaB37ts*), *dnaB1ts*, *dnaB27ts*, and *dnaB19ts* (Sueoka, 1998). One temperature sensitive mutation, *dnaD23ts*, has been isolated in the *B. subtilis dnaD* gene (Bruand et al., 1995). DnaD and DnaB are conserved among low G+C content Gram-positive bacteria, and two temperature sensitive alleles of *dnaD* have also been isolated in the Gram-positive bacterium *Staphylococcus aureus* (Li et al., 2004). Temperature sensitive mutations in *Staphylococcus aureus dnaB* have not yet been isolated.

I performed three selections to isolate suppressors of the temperature sensitivity of *dnaD23ts*, *dnaB134ts*, and *dnaB19ts* mutant *B. subtilis* cells. I isolated a missense allele of *dnaB*, called *dnaBS371P*, in two of these selections. *dnaBS371P* was isolated as an extragenic suppressor of the temperature sensitivity of *dnaD23ts* cells, and as an intragenic suppressor of the temperature sensitivity of *dnaB134ts* cells (Rokop et al., 2004). We previously reported that *dnaBS371P* allows for a direct physical interaction between DnaB and DnaD to be detected by yeast two-hybrid analysis (Rokop et al., 2004). DnaBS371P also recruits DnaD to the membrane fraction of cells, where DnaB is found (Rokop et al., 2004).

Here I describe the remaining suppressors that I isolated in my *dnaD23ts* and *dnaB134ts* selections, and I describe the suppressors I isolated in my *dnaB19ts* selection. I found that one of the intragenic suppressors of the temperature sensitivity of *dnaD23ts* cells allows for the
detection of a physical interaction between DnaB and DnaD, just like the extragenic suppressor
\textit{dnaBS371P} does (Rokop et al., 2004). This strengthens my hypothesis that the wild-type DnaB
and DnaD proteins are true interaction partners inside \textit{B. subtilis} cells. I did not isolate
\textit{dnaBS371P} in my selection for \textit{dnaB19ts} suppressors, but in this selection I did isolate several
intragenic suppressors and several informational suppressors (compensatory mutations in tRNA
genes that would mistakenly deliver the proper amino acid to the DnaB19ts protein). I conclude
that \textit{dnaBS371P} suppresses the defects caused by the \textit{dnaDts} allele and by some but not all
temperature sensitive mutations in \textit{dnaB}.

\textbf{RESULTS and DISCUSSION}

\textbf{Isolation of suppressors of the temperature sensitivity of \textit{dnaD23ts} cells.} I performed a
genetic selection to isolate spontaneous suppressors of the temperature sensitivity of \textit{dnaD23ts}
mutant cells (summarized in Table 2). The \textit{dnaD23ts} allele is \textit{dnaDA166T}, which causes an
alanine to threonine change at amino acid 166 of DnaD (Bruand et al., 1995). I isolated 25
independent mutations that suppressed the temperature sensitive phenotype caused by \textit{dnaD23ts}.
Five of the 25 suppressors were extragenic. All five extragenic suppressor mutations were the
allele \textit{dnaBS371P}, as described previously (Rokop et al., 2004).

The remaining 20 suppressor mutations fell into six classes of mutants by classification based
on growth phenotype at a series of temperatures. All nine members of one class grew as well as
wild-type through the full range of temperatures at which wild-type grows, and are thus
presumed to contain revertant mutations. However, no members of this class have been
sequenced.
The remaining classes contain intragenic suppressor mutations. One class consists of one isolate, which contains the suppressor mutation \textit{dnaDW188L}. Another class consists of three isolates, all of which contain the identical suppressor mutation, \textit{dnaDA138G}. Another class consists of one isolate, which contains the suppressor mutation \textit{dnaDT148M}.

Another class of intragenic suppressors consists of one isolate, which contains the suppressor mutation \textit{dnaDA166S}. This mutation causes an alanine to serine change at amino acid 166 of DnaD. The original \textit{dnaDts} mutation is an alanine to threonine change at amino acid 166 of DnaD (Bruand et al., 1995), and thus this suppressor mutation suppresses the temperature sensitivity caused by \textit{dnaDts} by changing the threonine at position 166 of the mutant DnaDts protein to a serine.

The final class of \textit{dnaD23ts} suppressors consists of five isolates, all of which contain the identical intragenic suppressor mutation that is a spontaneous deletion of six nucleotides from a region that contains a small nucleotide repeat. This six nucleotide deletion leads to the deletion of two amino acids in the DnaD protein, AA154 (asp) and AA155 (gln). I will refer to the allele of \textit{dnaD} that contains both this deletion and the original mutation that causes the temperature sensitivity as \textit{dnaD23A154-155}.

\textit{dnaD23A154-155} allows for the detection of the physical interaction between DnaD and DnaB. We previously reported that the only extragenic suppressor isolated in the \textit{dnaDts} selection, \textit{dnaBS371P}, allowed for a physical interaction to be detected between DnaB and DnaD (Rokop et al., 2004). Just as \textit{dnaBS371P} is located at the region of DnaB that is homologous to a family of phage replication proteins, the five intragenic suppressors of \textit{dnaDts} that I isolated were all located inside the phage homology region of DnaD (Rokop et al., 2004). I used yeast
two-hybrid analysis to test whether any of the dnaDts intragenic suppressors could allow for a physical interaction to be detected between mutant DnaD and wild-type DnaB.

Using yeast two-hybrid analysis, I found that DnaD23Δ154-155 and DnaB directly interact. I fused DnaB to the activation domain (AD) and DnaD23Δ154-155 to the DNA binding domain (DBD) of the yeast transcription factor Gal4. A physical interaction between the Gal4 AD and DBD domains will drive expression of the ADE2 gene, thereby allowing for growth of the yeast on medium lacking adenine (James et al., 1996). I found that yeast expressing AD-DnaB and DBD-DnaD23Δ154-155 grew on medium lacking adenine, indicating that DnaD23Δ154-155 and DnaB physically interact (Figure 1).

In contrast, I did not detect physical interactions between wild-type DnaB and the other mutant forms of DnaD that I isolated in my dnaDts selection. I found that wild-type DnaB does not detectably interact by the two-hybrid assay with either DnaD23T148M or DnaD23W188L (Figure 1). The intragenic suppressor dnaD23A138G did not yield reproducible results in this assay, and the intragenic suppressor dnaDA166S was not tested in this assay.

I hypothesize that the dnaD23Δ154-155 mutation allows for increased or unregulated interactions to occur between DnaB and DnaD, just as dnaBS371P does. The other suppressor mutations that do not promote an interaction between DnaB and DnaD most likely affect the structure or stability of DnaD at high temperatures. There is a report that the dnaD23ts mutation causes a reduction in the amount of DnaD in the cell (Bruand et al., 2001; Marsin et al., 2001), so it is quite feasible that these other intragenic mutations may return DnaD23ts to higher levels in the cell by increasing its stability. Antibodies against DnaD could be used to test the effects that these intragenic mutations have on levels of the DnaDts protein. In addition, over-expression
Figure 1.
Figure 1. DnaB interacts with DnaD23Δ154-155. Yeast two-hybrid analysis was used to examine physical interactions between DnaB fused to the activation domain (AD) and mutant forms of DnaD fused to the DNA binding domain (DBD) from the Gal4 transcription factor. A physical interaction activates the expression of ADE2, allowing for growth on medium lacking adenine. Plate section 1 (AD-DnaB, DBD-DnaD23Δ154-155) shows that DnaB and DnaD23Δ154-155 do interact. Plate section 2 (AD-DnaB, DBD-DnaD) confirms previous results that DnaB and DnaD do not interact in the two-hybrid assay. Plate sections 3 (AD-DnaB, DBD-DnaD23W188L) and 4 (AD-DnaB, DBD-DnaD23T148M) show that DnaB does not detectably interact with DnaD23W188L or DnaD23T148M in this assay.
studies could be used to test whether simply raising the levels of DnaDts in the cell leads to temperature resistance.

The fact that DnaD23Δ154-155 and DnaB physically interact in the two-hybrid assay further supports the hypothesis that wild-type DnaD and DnaB really do interact in B. subtilis cells. One could argue that the ability of DnaBS371P to directly physically interact with DnaD is actually a gain-of-function caused by the dnaBS371P mutation that is not indicative of what occurs between the wild-type proteins in the cell. However, the fact that I have isolated an analogous mutation in DnaD strengthens the argument that these two proteins are true interaction partners. In each putative interaction partner, a mutation exists that suppresses the temperature sensitivity of dnaDts cells, is found in the phage homology region of the protein, and allows for the detection of an interaction between these two proteins in the two-hybrid assay.

C-terminal Myc or GFP fusions to DnaD23Δ154-155 could not be constructed. We have previously shown that the mutant form of DnaB (DnaBS371P) that interacts by two-hybrid analysis with DnaD also recruits DnaD-Myc to the membrane fraction of cells (Rokop et al., 2004). I was therefore interested in determining whether DnaD23Δ154-155 was enriched in the membrane fraction of cells, but I was unable to create strains in which DnaD23Δ154-155 was fused to a C-terminal Myc or GFP tag. In contrast, I was able to create strains in which DnaD-Myc (Rokop et al., 2004) or DnaD-GFP (Rokop and Grossman, unpublished results) was the only version of DnaD in the cell. Thus, I hypothesize that Myc or GFP-tagged DnaD23Δ154-155 cannot function as the only DnaD in the cell.

Although I am interested in studying the dnaD23Δ154-155 mutation further, I feel that the next step in studies of this mutation would be to isolate the suppressor mutation alone (ie. create
cells containing *dnaDΔ154-155* that lack the original mutation that causes the temperature sensitivity. I have not yet separated the original mutation from the suppressor mutation, which is found only 11 amino acids away in the protein from the original missense mutation, A166T. Once the suppressor mutation is isolated alone, one can determine whether DnaDΔ154-155-Myc is enriched in the membrane fraction of cells. If DnaDΔ154-155 cannot be Myc-tagged, the subcellular fractionation pattern of this protein can be examined using antibodies raised against DnaD. It will also be interesting to examine whether *dnaDΔ154-155* suppresses the phenotypes caused by the other two mutations that *dnaBS371P* suppresses (namely, *dnaB134ts* (Rokop et al., 2004) and *AprIA* (Bruand et al., 2001)), and whether *dnaDΔ154-155* cells exhibit the same phenotype as *dnaBS371P* cells do (Chapter 3).

**Isolation of suppressors of the temperature sensitivity of *dnaB134ts* cells.**

*dnaBS371P* was isolated as a *dnaB134ts* suppressor. I also isolated suppressors of the temperature sensitivity of cells containing the *dnaB134ts* allele (summarized in Table 3), which causes a glutamic acid to lysine change at amino acid 85 of DnaB (Sueoka, 1998). Of the 41 independent suppressor mutations I isolated, 5 were the identical *dnaBS371P* allele that I isolated in the *dnaDts* selection. Thus, *dnaBS371P* is an intragenic suppressor of the temperature sensitivity of *dnaB134ts* cells (Rokop et al., 2004). A sixth suppressor isolated in this selection phenocopies these five, and thus most likely also contains this same mutation.

**Several *dnaB134ts* suppressors are presumed to be in tRNA or rRNA genes.** Two classes of the *dnaB134ts* suppressors were extragenic, as they were not linked to *dnaB*. One class contains a member whose suppressor mutation has been linked to the *trnO-rrnO* locus (a locus containing rRNA and tRNA genes), and the other class has not been linked to a specific locus. DNA sequence analysis showed that all four isolates in the class linked to the *trnO-rrnO* locus
contained wild-type \textit{trnO} genes, even though a plasmid containing the \textit{rrnO} and \textit{trnO} loci from one of these isolates confers temperature resistance to \textit{dnaB134ts} cells (data not shown). Therefore this isolate most likely contains a suppressor mutation in the \textit{rrnO} genes. It is possible that the suppressor mutation suppresses the temperature sensitive phenotype of \textit{dnaB134ts} cells by increasing the frequency of misreading events, thereby allowing for the translation of some wild-type DnaB protein. Mutations in ribosomal RNA genes that increase the frequency of misreading have been isolated (Allen and Noller, 1991). These "ram" (ribosomal ambiguity) mutations are hypothesized to cause decreased accuracy in translation because of defects in proofreading, like the "ram" mutations that have been isolated in ribosomal protein subunits do (Andersson and Kurland, 1983).

The second class of extragenic mutations is also presumed to contain informational suppressors in tRNA (or possibly rRNA) genes for the following reasons. Before doing this \textit{dnaB134ts} selection, I had performed a selection for \textit{dnaB19ts} suppressors (see below). In the \textit{dnaB19ts} selection, I isolated several suppressor mutations that were informational suppressors in tRNA genes. All such mutations caused allele-specific suppression and very slow growth, despite normal cellular appearance by live cell microscopy. The class of extragenic \textit{dnaB134ts} suppressors that I have not mapped also causes allele-specific suppression and very slow growth despite normal cellular morphology (data not shown). Thus, I hypothesize that the mutations in these strains are also informational suppressors in tRNA (or possibly rRNA) genes.

The remaining classes of \textit{dnaB134ts} suppressors contained intragenic mutations. The remaining \textit{dnaB134ts} suppressors fall into five classes of mutants by classification based on growth phenotype at a series of temperatures. All classes contain members that have been linked to the \textit{dnaB} locus. All five members of one class grow as well as wild-type through the full
range of temperatures at which wild-type grows, and are thus presumed to contain revertant mutations.

The remaining classes contain intragenic suppressor mutations that cause secondary site substitutions. One class contains 6 isolates, 2 of which have been sequenced and shown to contain the identical suppressor mutation, \textit{dnaBA164V}. Another class contains 5 isolates, 1 of which has been sequenced and shown to contain the suppressor mutation \textit{dnaBH65Y}. Another class contains 6 isolates, 3 of which have been sequenced and shown to contain the identical suppressor mutation, \textit{dnaBE288K}. The final class contains 6 isolates, one of which has been sequenced and shown to contain the suppressor mutation \textit{dnaBS151P}.

Isolation of suppressors of the temperature sensitivity of \textit{dnaB19ts} cells.

\textit{dnaBS371P} was not isolated as a \textit{dnaB19ts} suppressor. I isolated suppressors of the temperature sensitivity of cells containing the \textit{dnaB19ts} allele (summarized in Table 4), which causes an alanine to threonine change at amino acid 379 of DnaB (Sueoka, 1998). Of the 31 independent suppressor mutations I isolated, \textit{dnaBS371P} was not isolated. Thus, I believe that \textit{dnaBS371P} is mostly likely not able to intragenically suppress the temperature sensitivity of \textit{dnaB19ts} cells, as the mutation is obviously isolatable by the methods I used and yet was not isolated in this selection. I have not directly tested this hypothesis, however, as I have not constructed the double mutant \textit{dnaBS371P/A379T}.

Several \textit{dnaB19ts} suppressors were informational suppressors in tRNA genes. Three classes of \textit{dnaB19ts} suppressors were extragenic, as they were not linked to \textit{dnaB}. All extragenic suppressor mutations are presumed to be mutations in tRNA genes, as all classes contain members that contain anticodon mutations in alanine tRNA genes. One class consists of one isolate, which contains the exact missense mutation in the \textit{trnB-ala} gene that would allow the
alanine tRNA to read the mutant codon for threonine in the dnaB19ts mRNA and insert the proper alanine amino acid. Another class contains 4 isolates, two of which have been sequenced and shown to contain this same anticodon mutation in another gene encoding an alanine tRNA, called trnA-ala. The final class contains eight isolates, one of which has been sequenced and shown to contain the same anticodon mutation found in the above suppressors, but in the trnO-ala gene.

The isolation of informational suppressors of the temperature sensitivity of dnaB19ts cells has some interesting implications regarding DnaB function. The efficiency of suppressor tRNAs that suppress missense mutations is very low (Tsai and Curran, 1998), which implies that the majority of the DnaB present in the cell will contain the mutation caused by dnaB19ts. The fact that alanine tRNA mutations are able to suppress the temperature sensitivity of dnaB19ts cells implies that the amount of wild-type DnaB necessary for the cell is much lower than the amount of DnaB found in wild-type cells. It may be that there is excess DnaB in wild-type cells, and thus decreasing the concentration of functional DnaB is not harmful to cells. Alternatively, it may be that mixed multimers of wild-type and mutant DnaB are functional. DnaB has been shown to multimerize both in in vitro assays and by two-hybrid analysis (Ishigo-oka et al., 2001; Marsin et al., 2001; Noirot-Gros et al., 2002). If mixed multimers of DnaB and DnaB19ts are capable of functioning properly, then this could explain why the inefficient action of suppressor tRNAs can restore temperature resistance to dnaB19ts cells.

**The remaining classes of dnaB19ts suppressors contained intragenic mutations.** One class of intragenic suppressors contains 6 isolates, one of which has been sequenced and shown to contain a wild-type dnaB gene, indicating the presence of a reversion mutation back to the original sequence. Another class contains 5 isolates, one of which has been sequenced and
shown to contain the suppressor mutation \textit{dnaBT366N}. The final class contains 7 isolates, one of which has been sequenced and shown to contain the suppressor mutation \textit{dnaBT355I}.

\textbf{\textit{dnaBS371P} suppresses the temperature sensitivity caused by some but not all \textit{dnaBts} mutations.} A total of four temperature sensitive mutations in the \textit{B. subtilis} \textit{dnaB} gene have been isolated previously (Sueoka, 1998). Three of those mutations (\textit{dnaB134ts}, \textit{dnaB1ts}, and \textit{dnaB27ts}) cause missense mutations that are clustered in an N-terminal 40 amino acid region of this 472-amino-acid-long protein. The fourth temperature sensitive mutation, \textit{dnaB19ts}, causes a missense mutation over 250 amino acids away from this cluster, in the C-terminus of the DnaB protein (Sueoka, 1998). I have found that \textit{dnaBS371P} suppresses the temperature sensitivity caused by the N-terminal mutation \textit{dnaB134ts} (Rokop et al., 2004), but most likely does not suppress the temperature sensitivity caused by the C-terminal mutation \textit{dnaB19ts}.

I hypothesize that \textit{dnaBS371P} induces a conformational change in DnaB such that its C-terminus (which I believe to be its DnaD-interaction domain based on my phage homology studies) is exposed to interact with DnaD in a constitutive or misregulated fashion (Rokop et al., 2004). The \textit{dnaB19ts} mutation may cause a defect in the DnaD-interaction domain that affects the ability of DnaB to interact with DnaD. Thus, the defect caused by the \textit{dnaB19ts} mutation would not be suppressed by \textit{dnaBS371P}. \textit{DnaBS371P} would expose the C-terminus of DnaB19ts in a constitutive or misregulated fashion, but that C-terminus would still be unable to interact with DnaD. In contrast, \textit{dnaBS371P} does suppress the defect of \textit{dnaB134ts} cells, which occurs in the N-terminus of DnaB. I hypothesize that the N-terminus of DnaB is somehow involved in sensing a state of active replication initiation and responding by regulating when DnaD and DnaB interact. In this model, the \textit{dnaBS371P} mutation would bypass this regulation

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and allow the wild-type C-terminus of DnaB134ts to interact with DnaD in a constitutive or misregulated fashion.

To further test this hypothesis, it would be interesting to make double mutant forms of dnaB that contained dnaBS371P and each of the four dnaBts mutations. My model for the mechanism of the suppression of the temperature sensitivity of dnaB134ts cells by dnaBS371P would be supported by the ability of dnaBS371P to suppress the temperature sensitivity caused by dnaB1ts and dnaB27ts, but not by dnaB19ts.

It would also be informative to perform yeast two-hybrid analysis on fragments of DnaB and DnaD in order to determine which domains of DnaB and DnaD interact with each other. I have done preliminary analysis of this kind by testing for interactions between C-terminal fragments of wild-type DnaB and full-length wild-type DnaD. I have not detected interactions between DnaD and any of the fragments of DnaB that I have tested (data not shown). A diagram of the fragments of DnaB that I have tested is included in Figure 2. Possible extensions of this analysis include testing N-terminal fragments of DnaB, or testing fragments of DnaD. It may be more beneficial to first test fragments of DnaBS371P with wild-type DnaD, or to test fragments of DnaD23Δ154-155 with wild-type DnaB, as the full-length versions of these mutant proteins do exhibit interactions in this assay (Rokop et al., 2004). If minimal interaction domains of DnaBS371P and DnaD23Δ154-155 are identified this way, an interaction can then be tested using the wild-type versions of these fragments.
Figure 2.
Figure 2. Fragments of wild-type DnaB that have been tested for interaction with full-length wild-type DnaD. Yeast two-hybrid analysis was used to examine physical interactions between fragments of wild-type DnaB fused to the activation domain (AD) and full-length wild-type DnaD fused to the DNA binding domain (DBD) from the Gal4 transcription factor. None of these fragments of DnaB showed a detectable physical interaction with full-length wild-type DnaD in this assay.
EXPERIMENTAL PROCEDURES

Media and growth conditions. Media and growth conditions were as previously described (Rokop et al., 2004).

Strains, alleles, and plasmids. E. coli strains used for cloning and procedures used for strain constructions were as previously described (Rokop et al., 2004). B. subtilis strains are listed in Table 1, and are derivatives of JH642 (AG174) that contain the trpC and pheA mutations.

The dnaDts allele used was dnaD23ts, which causes an alanine to threonine change at amino acid 166 of 232 (Bruand et al., 1995). One dnaBts allele used was dnaB134ts, which causes a lysine to glutamic acid change at amino acid 85 of 472 (Sueoka, 1998). The other dnaBts allele used was dnaB19ts, which causes an alanine to threonine change at amino acid 379 of 472 (Sueoka, 1998). All strains containing temperature sensitive alleles were grown at 30°C.

Selections for suppressor mutations. Spontaneous suppressors were isolated by plating 10^7 or 10^8 dnaD23ts (KPL73), dnaB134ts (KPL69), or dnaB19ts (MER271) cells (grown in liquid LB at 30°) onto LB plates incubated overnight at either 45° or 48°. Suppressors were grouped and mapped as described previously (Rokop et al., 2004). Once linkage was identified, the genes were sequenced by the MIT Biopolymers Lab. dnaB, dnaD, and the alanine tRNA genes were PCR amplified and either sequenced directly or cloned into pGEMcat (Youngman et al., 1989) for sequencing.

EMS mutagenesis. In addition to isolating spontaneous suppressors in my dnaB19ts selection, I also isolated suppressors by EMS mutagenesis in this selection only. Strains MER273 - MER283 contain spontaneous suppressor mutations, and strains MER284 - MER304 contain suppressor mutations obtained through EMS mutagenesis. For the EMS mutagenesis,
*dnaB19ts* (MER271) cells were grown in liquid LB at 30°C to an OD₆₀₀ = 0.4. Cells were washed twice with LB and resuspended to an OD₆₀₀ = 1.0. Seven independent pools of cells were mutagenized in LB with 1.2% EMS (Sigma) for 30 minutes at 30°C and then washed twice with LB. Cells were grown for four generations at 30°C in LB without mutagen for recovery, and plated on LB at 45°C or 48°C for selection.

**Two-hybrid analysis.** *dnaD23Δ154-155, dnaD23W188L, dnaD23T148M,* and *dnaD23A138G* were amplified by PCR from the appropriate chromosomal DNA and inserted into the pGBDU-C3 vector to create in frame fusions of DnaD23Δ154-155, DnaD23W188L, DnaD23T148M, and DnaD23A138G to the DNA binding domain (DBD) of the Gal4 transcription factor (James et al., 1996). The resulting plasmids are pMR81 (encoding DBD-DnaD23Δ154-155), pMR82 (encoding DBD-DnaD23W188L), pMR83 (encoding DBD-DnaD23T148M), and pMR80 (encoding DBD-DnaD23A138G). The plasmid pMR58 (encoding AD-DnaB) was cotransformed with each of these DBD fusion plasmids into the yeast strain DWY112, and the yeast two-hybrid analysis was performed as described previously (Rokop et al., 2004).

Fragments of *dnaB* were amplified by PCR from wild-type chromosomal DNA and inserted into the pGAD-C1 vector to create in frame fusions of fragments of DnaB to the activation domain (AD) of the Gal4 transcription factor (James et al., 1996). The resulting plasmids are pMR99 (encoding AD-DnaB:AA299-472), pMR100 (encoding AD-DnaB:AA257-472), pMR101 (encoding AD-DnaB:AA201-472), pMR102 (encoding AD-DnaB:AA58-472), pMR66 (encoding AD-DnaB:AA364-472), and pMR68 (encoding AD-DnaB:372-472). The plasmid pMR63 (encoding DBD-DnaD) was cotransformed with each of these AD fusion plasmids into
the yeast strain DWY112, and the yeast two-hybrid analysis was performed as described previously (Rokop et al., 2004).

ACKNOWLEDGEMENTS

I thank Bill Burkholder, Jenny Auchtung, and Soni Lacefield Shimoda for reagents and very helpful advice.
Table 1. *B. subtilis* strains used.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Relevant genotype</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB302</td>
<td>Tn917ΩHU163::pTV21Δ2 (cat)</td>
<td>(this Tn917 insertion is linked to <em>dnaA</em>)</td>
</tr>
<tr>
<td>KI1346</td>
<td>zhb83::Tn917::pTV21Δ2 (cat)</td>
<td>(this Tn917 insertion is linked to <em>dnaB</em>)</td>
</tr>
<tr>
<td>KPL69</td>
<td>dnaB134ts-zhb83::Tn917 (mls)</td>
<td></td>
</tr>
<tr>
<td>KPL73</td>
<td>dnaD23ts-Tn917ΩHU151 (mls)</td>
<td></td>
</tr>
<tr>
<td>KPL154</td>
<td>Tn917ΩHU151::pTV21Δ2 (cat)</td>
<td>(this Tn917 insertion is linked to <em>dnaD</em>)</td>
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<tr>
<td>KPL314</td>
<td>dnaC-myc (spc)</td>
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<tr>
<td>MER271</td>
<td>dnaB19ts-zhb83::Tn917 (mls)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. A summary of suppressors of the temperature sensitivity of *dnaD23ts* cells.

<table>
<thead>
<tr>
<th>Mutation isolated</th>
<th>Names of strains containing <em>dnaD23ts and this mutation</em></th>
<th>Names of strains that have not been sequenced but phenocopy these strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>dnaBS371P</em></td>
<td>MER372, 378, 381, 385, 389</td>
<td>None</td>
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<tr>
<td>presumed revertants</td>
<td>None sequenced</td>
<td>MER371, 376, 377, 379, 380, 387, 390, 392, 393</td>
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<tr>
<td><em>dnaDA154,155</em></td>
<td>MER373, 374, 384, 386, 394</td>
<td>None</td>
</tr>
<tr>
<td><em>dnaDW188L</em></td>
<td>MER382</td>
<td>None</td>
</tr>
<tr>
<td><em>dnaDT148M</em></td>
<td>MER383</td>
<td>None</td>
</tr>
<tr>
<td><em>dnaDA138G</em></td>
<td>MER369, 388, 391</td>
<td>None</td>
</tr>
<tr>
<td><em>dnaDA166S</em></td>
<td>MER370</td>
<td>None</td>
</tr>
</tbody>
</table>

**The suppressor mutations in underlined strains in this column have been linked to *dnaD* even though they have not been sequenced.**

Note: MER369-394 were suppressor strains isolated in this selection; MER375 was discarded because it did not retain its temperature resistant phenotype.
Table 3. A summary of suppressors of the temperature sensitivity of *dnaB134ts* cells.

<table>
<thead>
<tr>
<th>Mutation isolated</th>
<th>Names of strains containing <em>dnaB134ts</em> and this mutation</th>
<th>Names of strains that have not been** sequenced but phenocopy these strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>dnaBS371P</em></td>
<td>MER505, 506, 507, 508, 509</td>
<td>MER511</td>
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<tr>
<td><em>dnaBA164V</em></td>
<td>MER510, 530</td>
<td>MER529, 531, 532, 533</td>
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<tr>
<td>presumed <em>rrnO</em></td>
<td>sequenced <em>trnO</em> loci but no mutations found in any of the four strains</td>
<td>MER498, 499, 500, 501</td>
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<tr>
<td>mutations</td>
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<td><em>dnaBH65Y</em></td>
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<td>MER513, 514, 515, 516</td>
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<td><em>dnaBE288K</em></td>
<td>MER517, 519, 521</td>
<td>MER518, 520, 522</td>
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<tr>
<td>presumed revertants</td>
<td>none sequenced</td>
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<tr>
<td><em>dnaBS151P</em></td>
<td>MER524</td>
<td>MER523, 525, 526, 527, 528</td>
</tr>
<tr>
<td>presumed tRNA or rRNA mutations</td>
<td>none sequenced</td>
<td>MER502, 503, 504</td>
</tr>
</tbody>
</table>

**The suppressor mutations in underlined strains have been linked to *dnaB* even though they have not been sequenced. The suppressor mutation in the strain in bold has been linked to the *rrnO* locus even though it has not been sequenced.

Note: MER493-535 were suppressor strains isolated in this selection; MER534 and 535 were discarded because they did not retain their temperature resistant phenotypes.
Table 4. A summary of suppressors of the temperature sensitivity of *dnaB19ts* cells.

<table>
<thead>
<tr>
<th>Mutation isolated</th>
<th>Names of strains containing <em>dnaB19ts</em> and this mutation</th>
<th>Names of strains that have not been** sequenced but phenocopy these strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Revertant</td>
<td>MER278</td>
<td>MER279, 289, 293, 296, 299</td>
</tr>
<tr>
<td><em>dnaBT366N</em></td>
<td>MER281</td>
<td>MER273, 275, 285, 295</td>
</tr>
<tr>
<td><em>dnaBT355I</em></td>
<td>MER284</td>
<td>MER287, 290, 292, 297, 300, 302</td>
</tr>
<tr>
<td><em>trnO-ala</em> anticodon</td>
<td>MER280</td>
<td>MER276, 282, 288, 291, 294, 298, 303</td>
</tr>
<tr>
<td><em>trnA-ala</em> anticodon</td>
<td>MER274, 301</td>
<td>MER286, 304</td>
</tr>
<tr>
<td><em>trnB-ala</em> anticodon</td>
<td>MER283</td>
<td>None</td>
</tr>
</tbody>
</table>

**The suppressor mutations in underlined strains have been linked to *dnaB* even though they have not been sequenced. The suppressor mutation in the strain in bold has been linked to the *trnO* locus even though it has not been sequenced.

Note: MER273-304 were suppressor strains isolated in this selection; MER277 was discarded because it did not retain its resistance to erythromycin and lincomycin.
REFERENCES


Appendix B:

The essential replication initiation factor DnaB from *Bacillus subtilis* is not an integral membrane protein.
**SUMMARY**

DnaB is an essential replication initiation protein in *B. subtilis*. It has long been hypothesized that DnaB is an integral membrane protein, as DnaB possesses a predicted transmembrane domain. We have shown previously that DnaB is found in the membrane fraction of *B. subtilis* cells. In addition, DnaB is functionally linked to the membrane, as its function is required for the enrichment of chromosomal origins in the membrane fraction of *B. subtilis* cells. Despite this, if DnaB were a bacterial transmembrane protein, it would be quite unusual. This is because DnaB does not possess a consensus signal sequence, but has a predicted extracellular N-terminus and intracellular C-terminus, which is rare among bacterial transmembrane proteins. In addition, DnaB has been previously purified without using detergent solubilization, which is commonly required for the purification of membrane proteins. I have found that DnaB is not an integral membrane protein, as it can be solubilized from the membrane fraction of *B. subtilis* cells using high salt (unlike integral membrane proteins). In addition, I have found that the N-terminus of DnaB is located intracellularly, as an N-terminal GFP fusion to DnaB fluoresces inside the cell and shows a localization pattern similar to that found by using a C-terminal GFP fusion to DnaB. Thus, I conclude that DnaB is most likely a peripheral membrane protein.
INTRODUCTION

DnaB is an essential replication initiation protein in B. subtilis (Hoshino et al., 1987; Ogasawara et al., 1986; Sueoka, 1998). DnaB has also been proposed to be an anchor for chromosomal origins in the membrane. In B. subtilis cells containing a temperature sensitive mutation in dnaB, replication initiation is blocked at the non-permissive temperature (Burnett and Wake, 1977; Gross et al., 1968; Karamata and Gross, 1970; White and Sueoka, 1973). Chromosomal origins are enriched in the membrane fraction of B. subtilis cells (Funnell, 1996; Sueoka and Hammers, 1974; Sueoka, 1998; Yamaguchi and Yoshikawa, 1977). However, in dnaBts cells incubated at the non-permissive temperature, origins are no longer enriched in the membrane fraction (Funnell, 1996; Sato et al., 1991; Sueoka, 1998; Winston and Sueoka, 1980). Consistent with this link between DnaB function and the cell membrane, wild-type DnaB is found in the membrane fraction of B. subtilis cells (Rokop et al., 2004). The fractionation patterns of the mutant versions of DnaB encoded by dnaBts mutations have not yet been examined.

Several labs have hypothesized that DnaB is an integral membrane protein, as DnaB possesses a predicted transmembrane domain (DAS (Cserzo et al., 1997), TMpred (Hofmann and Stoffel, 1993), TopPred (Claros and von Heijne, 1994)). B. subtilis DnaB is predicted to have a short extracellular N-terminus and a long intracellular C-terminus (TMpred (Hofmann and Stoffel, 1993)). DnaB is conserved among low G+C content Gram-positive bacteria, and this predicted transmembrane domain is well-conserved in the sequences of DnaB from these other organisms (Figure 1). The same region of the DnaB proteins from Bacillus halodurans, Streptococcus mutans, Enterococcus faecalis, Lactococcus lactis, and Staphylococcus aureus is also predicted to be a transmembrane domain (TMpred (Hofmann and Stoffel, 1993)).
Figure 1. The putative transmembrane domain of DnaB is well-conserved among low G+C content Gram-positive bacteria. Shown here is an alignment of the DnaB proteins from *Bacillus subtilis*, *Bacillus halodurans*, *Streptococcus mutans*, *Enterococcus faecalis*, *Lactococcus lactis*, and *Staphylococcus aureus*. Amino acid positions with high conservation are shown shaded in gray. The putative transmembrane domain, located at amino acid positions 34-53 of *B. subtilis* DnaB, is well-conserved among these species. Note that the two bolded GKT motifs in *B. subtilis* DnaB, which are putative ATP binding motifs, are not conserved.
Figure 1.
However, several pieces of evidence point to *B. subtilis* DnaB not being an integral membrane protein. First, DnaB does not possess a consensus signal sequence (PSORT (Gardy et al., 2003)), but is predicted to have an intracellular C-terminus (TMpred (Hofmann and Stoffel, 1993)). Such membrane proteins are relatively uncommon in bacteria, and they utilize an unusual mechanism of translocation that is independent of the Sec machinery. Two well-studied examples of such proteins are the bacteriophage coat protein Pf3 and ProW, a subunit of an *E. coli* ABC transporter (Dalbey et al., 1995). Second, the predicted topology of DnaB is not conserved among low G+C content Gram-positive bacteria. Although *B. subtilis* DnaB is predicted to have an intracellular C-terminus, the DnaB proteins from *Bacillus halodurans*, *Streptococcus mutans*, *Enterococcus faecalis*, *Lactococcus lactis*, and *Staphylococcus aureus* are predicted to have extracellular C-termini (TMpred (Hofmann and Stoffel, 1993)). Finally, DnaB has been purified previously, and the purification did not require detergent solubilization, as it typically does for transmembrane proteins (Marsin et al., 2001).

I have found that DnaB is not an integral membrane protein, as I have shown that DnaB can be solubilized from the membrane fraction of cells using high salt. Such a solubilization pattern is the hallmark of a peripheral membrane protein. This is in contrast to known integral membrane proteins, which cannot be solubilized from the membrane fraction of cells using high salt. In addition, I have shown that an N-terminal GFP fusion to DnaB does yield intracellular fluorescence. This indicates that the N-terminus of DnaB is inside the cell, which is in contrast to the computer prediction of an extracellular N-terminus. Given that DnaB is found in the membrane fraction of cells, is solubilized by high salt, and contains an intracellular N-terminus, I conclude that DnaB is most likely a peripheral membrane protein.
RESULTS AND DISCUSSION

_DnaB_ can be solubilized from the membrane fraction of cells with high salt. I investigated the subcellular fractionation pattern of DnaB to determine if this protein behaved as an integral membrane protein or a peripheral membrane protein. We have previously shown that DnaB is found in the membrane fraction of cells (Rokop et al., 2004). I repeated these subcellular fractionation experiments on _dnaB_+ cells and then used buffer or high salt buffer to attempt to solubilize DnaB from the membrane fraction of cells. I then analyzed the solubilized fractions with Western blotting using an anti-DnaB antibody. I found that DnaB can be solubilized from the membrane fraction of cells with high salt buffer, but not with buffer alone (Figure 2). Proteins found in the membrane fraction of cells that can be solubilized with high salt are typically peripheral membrane proteins.

I performed the same experiments on a control protein, the known integral membrane protein SpoIVFB fused to GFP (Rudner and Losick, 2002). The SpoIVFB-GFP fusion protein was expressed from an ectopic locus under the control of a xylose-inducible promoter. I performed subcellular fractionation and solubilization experiments on cells expressing SpoIVFB-GFP, and analyzed the solubilized fractions with Western blotting using an anti-GFP antibody. As expected, SpoIVFB was not solubilized by buffer or by high salt (Figure 2).

**The N-terminus of DnaB is found inside the cell.** If DnaB is a peripheral membrane protein, then both its N-terminus and C-terminus should be located inside the cell. This is in contrast to the computer prediction that DnaB has a single transmembrane domain that directs the N-terminus of the protein extracellularly. The intracellular location of a terminus of a protein can be tested using GFP fusion proteins, because GFP will not fluoresce if it is located
Figure 2.
Figure 2. DnaB can be solubilized from the membrane fraction of cells using high salt.

Subcellular fractionations were performed on dnaB+ amyE::PxyLA-spoIVFB-gfp (BDR524) cells grown in LB with xylose, and the extracts from these cells were probed using anti-DnaB and anti-GFP antibodies by Western blotting. The anti-GFP antibody detects SpoIVFB-GFP, a GFP-tagged version of a known integral membrane protein. Cyt = cytoplasmic fraction, Mem = membrane fraction. The membrane fractions (pellets) from these fractionation experiments were solubilized using either buffer or buffer with high salt, and centrifugation was performed again to separate the solubilized material from the insoluble pellet. Buffer = membrane fraction solubilized with buffer, Salt = membrane fraction solubilized with 1M NaCl. Soluble = soluble fraction, Pelleted = pelleted fraction (insoluble material).
extracellularly (Feilmeier et al., 2000). Previous results using a C-terminal fusion of GFP to 
DnaB showed that this construct does yield fluorescence inside live *B. subtilis* cells (Healy,
Lemon, and Grossman, unpublished results). DnaB-GFP forms foci in some, but not all, live 
cells, and these foci are not found at specific sub-cellular locations (Healy, Lemon, and 
Grossman, unpublished results). DnaB-GFP showed a localization pattern consistent with that 
observed for DnaB in fixed cells using immuno-fluorescence (Imai et al., 2000).

I constructed an N-terminal fusion of GFP to DnaB. The GFP-DnaB fusion protein is 
expressed from an ectopic locus under the control of a xylose-inducible promoter in cells that are 
dnaB+ at the endogenous locus. I used live-cell fluorescence microscopy to show that the GFP-
DnaB fusion protein does emit fluorescence inside *B. subtilis* cells (Figure 3). The fluorescence 
I observed showed a pattern consistent with the localization of DnaB observed using the C-
terminal DnaB-GFP. Namely, I observed foci of GFP-DnaB in some, but not all cells. Taken 
together, my work and previous studies indicate that both the N-terminus and C-terminus of the 
DnaB protein are located intracellularly, and that DnaB is found in the membrane fraction of 
cells. Thus, I conclude that DnaB is likely to be a peripheral membrane protein.
GFP-DnaB

Figure 3.
Figure 3. The N-terminus of DnaB is located intracellularly. Cells expressing GFP-DnaB (MER610) were grown at 30°C in LB supplemented with xylose to induce expression of the fusion protein. Cells were sampled during exponential growth, and were pelleted and resuspended in defined minimal medium to reduce background fluorescence. The cell membrane was stained with a vital membrane dye and visualized by fluorescence microscopy. Images of the vital membrane dye and of the GFP fluorescence were taken, and the two images were merged to show the cell membranes in red and the foci of GFP-DnaB in white.
EXPERIMENTAL PROCEDURES

Media and growth conditions. Media and growth conditions were as previously described (Rokop et al., 2004). Where indicated, xylose (Sigma) was used at 1% to induce expression from the xylose-inducible promoter PxyLA.

Strains, alleles, and plasmids. The E. coli strains used for cloning and the procedures used for strain constructions were as described previously (Rokop et al., 2004). B. subtilis strains are listed in Table 1, and are derivatives of JH642 (AG174) that contain the trpC and pheA mutations, unless otherwise indicated. The spoIVFB-gfp fusion construct contained in BDR524 was constructed as previously described (Rudner and Losick, 2002).

To generate the GFP-DnaB fusion construct, dnaB was amplified by PCR and fused in frame to the sequence encoding GFP inside the vector pEA18 (Quisel et al., 1999). The resulting fusion plasmid, pMR75, was integrated into the chromosome by double crossover into the amyE locus. The pEA18-derived construct was linearized before transformation to ensure integration by double crossover. Transformants were selected for resistance to chloramphenicol, and were screened for the disruption of amyE by the loss of the ability to degrade starch.

Membrane fractionations. Samples of BDR524 {amyE::(PxyLA-spoIVFB-gfpmut2)} were harvested in exponential growth (OD600 between 0.5 - 0.9) in LB medium at 37°C supplemented with 1% xylose. Protoplasting, lysis, and nuclease treatments were done as previously described (Rokop et al., 2004; Rudner and Losick, 2002). The membrane protein SpoIVFB fused to GFP, expressed in strain BDR524 (Rudner and Losick, 2002), was used as a known integral membrane
protein control. Expression of the SpoIVFB-GFP fusion was controlled by a xylose-inducible promoter.

**Membrane solubilizations.** The pellet (membrane fraction) from the fractionation experiments described above was resuspended in buffer H (20 mM HEPES at pH 7.6, 25 mM NaCl, 1 mM DTT, 1 mM of the protease inhibitor AEBSF) and split into thirds. To each third, a different buffer solution was added – buffer H alone, buffer H with NaCl that gave a final concentration of 1 M NaCl, or buffer H with Triton X-100 that gave a final concentration of 1% Triton X-100. The resuspensions were incubated on ice for 1 hour and then centrifuged for 1 hour at 4° in a Beckman Optima TL ultracentrifuge using the TLS-55 rotor at 39,000 rpm (100,000Xg). SDS loading buffer was added to the supernatant to yield the solubilized fraction in a final concentration of 1X SDS loading buffer. The pellet was resuspended in 1X SDS loading buffer to yield the insoluble fractions.

**Western blotting.** Western blotting was performed as described previously (Rokop et al., 2004). The primary antibody to DnaB was affinity purified rabbit polyclonal antibody (Covance) made against purified DnaB-GST. The primary antibody to GFP was a rabbit polyclonal antibody (a gift from David Rudner). The anti-DnaB antibody was used at a dilution of 1:500 and the anti-GFP antibody was used at a dilution of 1:10000. The secondary antibody used was horseradish peroxidase conjugated goat anti-rabbit immunoglobulin-G (Biorad) at a dilution of 1:3000.

**Fluorescence microscopy.** MER610 (amyE::gfp-dnaB cat) cells were grown in LB supplemented with 1% xylose at 30° to mid-exponential phase (an OD600 between 0.1 and 0.4). MER610 cells were grown in xylose to induce expression of the N-terminal GFP-DnaB fusion protein. Cells were pelleted at 14,000g for 1 minute and resuspended in defined minimal
medium to reduce background fluorescence. The DNA stain 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) was added to 0.1 μg/mL and the vital membrane dye FM4-64 (Molecular Probes) was added to 0.05 μg/mL. Cells were placed on slides containing pads of 1% agarose in minimal salts with 1mM MgSO₄. Fluorescence was viewed with a Nikon E800 microscope equipped with a 100X DIC objective using UV, TRITC, and FITC filters. Images were obtained with a CCD camera (Hamamatsu, model C4742-95) using Improvision OpenLab software.

ACKNOWLEDGEMENTS

I thank Esther Angert, David Rudner, Judy Healy, Jenny Auchtung, and Katherine Lemon for reagents and very helpful advice.
Table 1. *B. subtilis* strains used.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Relevant genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDR524</td>
<td><em>amyE::</em>(PxyI-A-spoIVFB-gfp cat) (in prototrophic PY79)</td>
</tr>
<tr>
<td>MER610</td>
<td><em>amyE::</em>(PxyI-A-gfp-dnaB cat)</td>
</tr>
</tbody>
</table>
REFERENCES


Appendix C:

The *dnaBS371P* allele causes a reduction in the ability of DnaB to multimerize in the yeast two-hybrid assay.
RESULTS AND DISCUSSION

dnaBS371P is an allele of DnaB that substitutes a serine for a proline at position 371 of DnaB (Rokop et al., 2004). The region of DnaB surrounding position 371 is predicted to be very rich in alpha helices by secondary structure prediction programs (Chapter 4). In addition, a region of the protein very near to position 371 (amino acids 383-406) is predicted to be a coiled-coil and a trimerization domain for DnaB (Chapter 4), a protein that behaves as a trimer in vitro based on its sedimentation coefficient and as approximately a heptamer based on its Stokes radius (Marsin et al., 2001). DnaB also multimerizes in the two-hybrid assay (Ishigo-oka et al., 2001; Noirot-Gros et al., 2002; Rokop et al., 2004). Based on the proximity of the substitution in dnaBS371P to several predicted alpha helices, a predicted coiled-coil domain, and a predicted trimerization domain, I tested whether dnaBS371P changed the ability of DnaB to interact with itself in the yeast two-hybrid assay. I found that DnaBS371P has a reduced ability to multimerize in this assay.

I fused DnaB or DnaBS371P to either the activation domain (AD) or the DNA binding domain (DBD) of the yeast transcription factor Gal4. A physical interaction between the Gal4 AD and DBD domains will drive expression of the ADE2 gene, thereby allowing for growth of the yeast on medium lacking adenine (James et al., 1996). I confirmed the previous result that yeast expressing AD-DnaB and DBD-DnaB grew on medium lacking adenine (Figure 1), indicating that DnaB interacts with itself in this assay (Ishigo-oka et al., 2001; Noirot-Gros et al., 2002; Rokop et al., 2004). I found that yeast expressing AD-DnaBS371P and DBD-DnaBS371P grew poorly on medium lacking adenine (Figure 1), indicating that DnaBS371P has lost the ability to strongly interact with itself in this assay.
Figure 1.
Figure 1. **DnaB interacts with itself more strongly than DnaBS371P.** Yeast two-hybrid analysis was used to examine the multimerization of DnaB and DnaBS371P. DnaB and DnaBS371P were fused to the activation domain (AD) and to the DNA binding domain (DBD) from the Gal4 transcription factor. A physical interaction activates the expression of ADE2, allowing for growth on medium lacking adenine. The left plate section (AD-DnaB, DBD-DnaB) confirms the previous result that DnaB interacts with itself in the two-hybrid assay. The right plate section (AD-DnaBS371P, DBD-DnaBS371P) shows that DnaBS371P possesses a reduced ability to interact with itself in this assay.
Although the ability of DnaBS371P to interact with itself is reduced, it is not eliminated. Therefore it is important to quantify the amount of signal that remains for the DnaBS371P-DnaBS371P interaction compared with wild-type using liquid β-galactosidase assays. It would also be interesting to examine the multimerization capabilities of DnaBS371P in vitro, as DnaB forms multimers in vitro (Marsin et al., 2001).

EXPERIMENTAL PROCEDURES

Two-hybrid analysis. dnaB and dnaBS371P were amplified by PCR from the appropriate chromosomal DNA and inserted into the pGAD-C1 or pGBDU-C3 vectors to create in frame fusions of DnaB or DnaBS371P to the activation domain (AD) or DNA binding domain (DBD) of the Gal4 transcription factor (James et al., 1996). The resulting plasmids are pMR58 (encoding AD-DnaB), pMR59 (encoding AD-DnaBS371P), pMR61 (encoding DBD-DnaB), and pMR62 (encoding DBD-DnaBS371P). The plasmids were transformed into the yeast strain DWY112, and the yeast two-hybrid analysis was performed as described previously (Rokop et al., 2004).
ACKNOWLEDGEMENTS

I thank Soni Lacefield Shimoda for reagents and very helpful advice.
REFERENCES


Chapter 3:

Effects of a mutation in dnaB on *Bacillus subtilis*

DNA replication and cell division

Megan E. Rokop, Alexi I. Goranov, Jade D. Wang, and Alan D. Grossman

(Manuscript in preparation)

For this chapter, Alexi Goranov performed many of the DNA/protein ratio experiments, and Jade Wang performed the genomic DNA microarray analysis.
SUMMARY

We previously showed that the essential replication proteins DnaB and DnaD are spatially separated from one another in *Bacillus subtilis* cells, even though these two proteins are thought to act together to load the replicative helicase. DnaB is found in the membrane fraction of cells, whereas DnaD is found in the cytoplasmic fraction. We previously isolated cells containing a missense mutation in *dnaB*, *dnaBS371P*, in which a significant fraction of DnaD is found in the membrane fraction of cells. In this work, we show that DnaBS371P causes an aberrant frequency of replication initiation events and an inability to properly undergo replication restart at stalled forks. When grown in rich medium, cells that are over-producing the DnaBS371P protein are filamentous and hypersensitive to DNA-damaging agents. These cells have decreased cellular DNA content, most likely due to a defect in restarting replication at stalled replication forks. When grown in minimal medium, *dnaBS371P* cells have increased cellular DNA content and abnormal distributions of cell lengths and chromosomal origin copy numbers. Many *dnaBS371P* cells also contain cell division rings that are asymmetrically positioned in the cell, a phenotype shared with *E. coli* cells that over-initiate replication. We conclude that *dnaBS371P* cells lack proper control over replication, which suggests that the spatial organization of DnaB and DnaD in the cell is an important mechanism of replication control.
INTRODUCTION

Coordinating DNA replication with cell division is an important part of cell cycle control. DNA replication is typically controlled at the stage of replication initiation. In a simplified bacterial cell cycle during slow growth, one cell division event follows each replication initiation event (Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001; Kornberg and Baker, 1992; Nordstrom and Austin, 1993; Zyskind and Smith, 1992). DNA replication initiates at the single origin on the chromosome, oriC. Replication elongation proceeds bidirectionally from oriC as the cell mass increases (Baker and Bell, 1998; Kornberg and Baker, 1992; Messer and Weigel, 1996; Messer et al., 2001; Messer, 2002). When the round of replication finishes, the cell divides in the center, generating two newborn cells of equal size that each contain one complete copy of the genome (Addinall and Holland, 2002; Errington et al., 2003; Harry, 2001; Kornberg and Baker, 1992; Margolin, 2000; Margolin, 2001).

The Gram-negative bacterium E. coli has a single replication initiator, the DnaA protein. DnaA binds to sequences in the replication origin oriC. There, DnaA induces unwinding of double-stranded DNA and helps helicase loader to bring the replicative helicase to the resulting single-stranded DNA. Once helicase is loaded at oriC, DNA primase and DNA polymerase III are recruited, and replication elongation begins (Baker and Bell, 1998; Kornberg and Baker, 1992; Messer and Weigel, 1996; Messer et al., 2001; Messer, 2002).

The Gram-positive bacterium Bacillus subtilis has homologs of E. coli DnaA, the replicative helicase, DNA primase, and DNA polymerase III (Lemon et al., 2002; Moriya et al., 1999; Yoshikawa and Wake, 1993), and a functional analog of E. coli helicase loader (Imai et al., 2000; Noirot-Gros et al., 2002; Soultanas, 2002; Velten et al., 2003). The two additional proteins DnaB and DnaD also function as essential components of the replication initiation
machinery in *B. subtilis* (Bruand et al., 1995b; Hoshino et al., 1987; Ogasawara et al., 1986; Sueoka, 1998). DnaB and DnaD are conserved among low G+C content Gram-positive bacteria, but do not have homologs in *E. coli* and other Gram-negative bacteria. In *B. subtilis* cells containing a temperature sensitive mutation in *dnaB* or *dnaD*, replication initiation is blocked at the non-permissive temperature (Burnett and Wake, 1977; Gross et al., 1968; Karamata and Gross, 1970; White and Sueoka, 1973). This block occurs *in vivo* at a step in replication initiation prior to the loading helicase onto chromosomal origins (Rokop et al., 2004). Thus, the function of DnaB and DnaD is required for helicase loading at *oriC* during initiation.

Loading helicase is not only necessary during the initiation of DNA replication at *oriC*. Helicase is also reloaded at elongating replication forks that have stalled along the chromosome due to protein roadblocks or DNA lesions (Cox et al., 2000; Cox, 2001; Marians, 2000; Sandler and Marians, 2000). DnaB and DnaD are also involved in loading helicase during the restart of replication at these stalled forks (Bruand et al., 1995a; Bruand et al., 2001; Marsin et al., 2001; Velten et al., 2003), a process that is initiated by the PriA protein (Cox et al., 2000; Cox, 2001; Marians, 2000; Sandler and Marians, 2000). A missense mutation in *dnaB*, *dnaBS371P*, suppresses the poor growth and sensitivity to rich medium exhibited by *priA* null mutants that cannot restart replication at stalled forks (Bruand et al., 2001). *dnaBS371P* also suppresses the temperature sensitivity of *dnaBts* cells and *dnaDts* cells that cannot initiate replication at the non-permissive temperature (Rokop et al., 2004). Although *dnaBS371P* suppresses the defects of mutant cells that cannot initiate or restart replication, we show in this study that expressing DnaBS371P in otherwise wild-type cells causes defects in both initiation and restart.

The multiple roles of DnaD in replication initiation and replication restart are reflected in the different phenotypes of the two mutations that have been isolated in *dnaD* from the Gram-
positive bacterium *Staphylococcus aureus* (Li et al., 2004). In *S. aureus*, one *dnaDts* mutation leads to replication fork stalling, DNA degradation, and sensitivity to DNA damaging agents at the non-permissive temperature. Presumably this phenotype results from a failure to properly execute replication fork restart, which would result in a prolonged presence of replication fork intermediates containing single-stranded DNA. The second *S. aureus dnaDts* mutation prevents replication initiation but allows elongation to proceed (Li et al., 2004), similar to the temperature sensitive allele of *dnaD* isolated in *B. subtilis* (Gross et al., 1968).

DnaB and DnaD are thought to work together to load helicase during replication initiation and replication fork restart (Bruand et al., 1995a; Bruand et al., 2001; Marsin et al., 2001; Rokop et al., 2004). Despite this, DnaB and DnaD are predominantly found in different subcellular fractions of wild-type cells. DnaB is found in the membrane fraction of cells, whereas DnaD is found in the cytoplasmic fraction (Rokop et al., 2004). Thus, two proteins that work together during replication initiation are separated from each other during the majority of the cell cycle. Regulating this separation of DnaB and DnaD could be a mechanism by which *B. subtilis* controls the frequency and timing of initiation events.

We previously isolated mutant cells in which DnaB and DnaD are no longer spatially separated (Rokop et al., 2004). We isolated the missense mutation *dnaBS371P* as an extragenic suppressor of the temperature sensitivity of *dnaDts* cells, and as an intragenic suppressor of the temperature sensitivity of *dnaBts* cells (Rokop et al., 2004). In *dnaBS371P* cells, the sub-cellular fractionation pattern of DnaD is altered such that a significant fraction of DnaD is found in the membrane fraction of these cells. This enrichment of DnaD in the membrane fraction of cells could occur as a result of the direct physical interaction that is observed between DnaBS371P and DnaD (Rokop et al., 2004). Wild-type DnaB and DnaD do not detectably physically interact.
in *in vitro* or two-hybrid assays (Ishigo-oka et al., 2001; Marsin et al., 2001; Noirot-Gros et al., 2002; Rokop et al., 2004), perhaps because the interaction between these two proteins is regulated. It is possible that *B. subtilis* controls the timing and location of replication initiation by regulating the DnaD-DnaB interaction. If this is true, then *dnaBS371P* cells may not be able to properly control DNA replication, as the DnaB-DnaD interaction and the recruitment of DnaD to the membrane fraction of cells occurs constitutively or in a misregulated fashion in these cells.

In this study, we have found that *dnaBS371P* cells have indeed lost proper control of replication. The *dnaBS371P* mutation results in increased cellular DNA content and increased replication asynchrony in cells grown slowly in minimal medium. *dnaBS371P* cells have abnormal distributions of cell lengths and origin copy numbers, and asymmetrically positioned cell division rings. Cells over-producing DnaBS371P growing quickly in rich medium contain decreased DNA content and exhibit hypersensitivity to DNA-damaging agents, most likely due to a defect in restarting replication at stalled forks. We conclude that regulating the interaction and spatial organization of essential replication proteins may be an important mechanism for controlling the frequency of replication initiation events and the progression of replication forks in *B. subtilis*.

**RESULTS and DISCUSSION**

*dnaBS371P* cells have increased DNA content when grown in minimal medium. We monitored the cellular DNA content of *dnaB*+ and *dnaBS371P* mutant cells by measuring the DNA/protein ratios of these cells when grown in defined minimal medium. We found that
\textit{dnaBS371P} cells contain about 40\% more DNA per protein mass than wild-type cells (Table 2). This 40\% increase in cellular DNA content is similar in range to the 10-50\% increase documented previously in characterized over-initiation mutants in both \textit{E. coli} (Bach and Skarstad, 2004; Kitagawa et al., 1998) and \textit{B. subtilis} (Lee et al., 2003; Lee and Grossman, 2004; Ogura et al., 2003). An increase in cellular DNA content was observed in \textit{dnaBS371P} cells grown in minimal medium at both 37°C and at 30°C.

The phenotype of increased cellular DNA content resulting from \textit{dnaBS371P} is dominant. We measured the DNA/protein ratios of cells producing wild-type DnaB or DnaBS371P from a LacI-repressible-IPTG-inducible promoter at an ectopic locus in \textit{dnaB+} cells. When grown in minimal medium, cells over-producing DnaBS371P have a normalized DNA/protein ratio of 1.35 when compared to the DNA/protein ratio of cells over-producing wild-type DnaB that is normalized to equal 1 (Table 2). The DNA/protein ratio of control cells expressing wild-type DnaB from two loci is statistically indistinguishable from that of wild-type \textit{dnaB+} haploid cells (data not shown). The increased DNA content of \textit{dnaB+} cells producing DnaBS371P from an ectopic locus was observed in minimal medium both at 30°C and at 37°C. In our subsequent experiments, cells were grown at 37°C, unless we were using a GFP fusion protein, in which case cells were grown at 30°C because the GFP fusions used display temperature sensitive phenotypes.

\textit{dnaBS371P} cells grown in minimal medium over-initiate and display increased replication asynchrony. DNA/protein ratios give an average measurement for all cells in a population. In order to measure the origin copy number of individual wild-type and \textit{dnaBS371P} cells, we used live-cell fluorescence microscopy. In these experiments, the origin regions of the chromosome were visualized using a well-characterized marker for origins, the fusion protein

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SpoOJ-GFP. SpoOJ is a protein that binds to DNA sequences found in the origin-proximal region of the chromosome (Lin and Grossman, 1998), and SpoOJ-GFP has been shown to display a very similar localization pattern to that of chromosomal origins (Lee et al., 2003; Lewis and Errington, 1997; Sharpe and Errington, 1998; Teleman et al., 1998). We compared the number of foci of SpoOJ-GFP per cell in dnaBS371P cells to that of wild-type cells. We found that there was a four-fold increase in the number of dnaBS371P cells containing more than four origins per cell, which is a high number of origins per cell for cells grown in minimal medium at 30°C (Table 3). This increase (and the corresponding increase in the average number of origins per cell) was observed despite the fact that dnaBS371P cells do not display an increased average in cell length (3.03 μm for dnaBS371P cells and 3.09 μm for dnaB+ cells). This is consistent with our result from the DNA/protein ratio measurements, and indicates that dnaBS371P cells are over-initiating.

We also found an increase in the number of dnaBS371P cells that contained 3, 5, 6, and 7 origins per cell. An increase in the number of cells with these numbers of origins indicates increased asynchrony of replication. Several previously characterized E. coli and B. subtilis mutants that over-initiate contain sister origins of replication that do not fire in unison (Bach and Skarstad, 2004; Boye et al., 1996; Camara et al., 2003; Kato and Katayama, 2001; Kitagawa et al., 1998; Lu et al., 1994; von Freiesleben et al., 1994), a condition known as replication asynchrony (Katayama, 2001; Nordstrom and Austin, 1993; Skarstad et al., 1986). The synchronous firing of origins would create a population of cells that each contains 1, 2, 4, or 8 origins. However, 28.8% of dnaBS371P cells contain numbers of origins that reflect asynchrony, as compared to 16.5% for dnaB+ cells. Taking our data using DNA/protein ratios
and live-cell microscopy together, we conclude that dnaBS371P cells display increased amounts of asynchrony and initiation of replication when grown in minimal medium.

Previously characterized over-initiation mutants in both E. coli (Lu et al., 1994) and B. subtilis (Lee et al., 2003; Lee and Grossman, 2004) exhibit skewed distributions of origin copy number, such that cells containing abnormally high numbers of origins and cells containing abnormally low numbers of origins are over-represented in the mutant populations. We found that dnaBS371P cells exhibit a similar skewed distribution of origin copy number. As described above, we found that there is a four-fold increase in the number of dnaBS371P cells that have greater than four origins per cell. We also found a four-fold increase in the number of dnaBS371P cells that have a single origin of replication (Table 3). Thus, the distribution of origin copy number in dnaBS371P cells is skewed towards both extremes, so that there is an increase in the proportion of cells with an unusually low number of origins, and an increase in the proportion of cells with an unusually high number of origins.

\textbf{dnaBS371P cells grown in minimal medium have an abnormal distribution of cell lengths.} A skewed distribution of cell lengths was also observed for dnaBS371P cells grown in minimal medium. We found that three times as many dnaBS371P cells are unusually long (greater than 5 microns in length) when compared to wild-type cells. We also found that three times as many dnaBS371P cells are unusually short (less than 2 microns in length) (Figure 1). This encompasses a thirteen-fold increase in the number of dnaBS371P cells that are under 1.8 microns long (0.5% in wild-type cells versus 6.3% in dnaBS371P cells). A tendency of dnaBS371P cells to undergo asymmetric cell division events would explain these data, as it would simultaneously create an increased number of short cells and an increased number of long cells in this mutant population. Asymmetric cell divisions in these mutant cells could also
Figure 1. *dnaBS371P* cells exhibit a skewed distribution of cell lengths. Cell lengths of *dnaB*+ (PAL657) cells (dark gray hatched bars, n = 873) and *dnaBS371P* (MER470) cells (light gray solid bars, n = 1554) were measured and plotted. Cells were grown in defined minimal medium at 30°C and were sampled during exponential growth. Cell membranes were stained with a vital membrane dye and visualized by fluorescence microscopy. Cell lengths (in microns) were measured and plotted along the X-axis against the percentage of cells in the population of each length, which was plotted along the Y-axis. The arrows indicate abnormally short and long cells, which are over-represented in *dnaBS371P* cells.
Figure 1.
explain the observation that the increase in the number of cells with many chromosomal origins is accompanied by an increase in the number of cells containing only one chromosomal origin. However, asymmetric division events cannot account for the overall increase in cellular DNA content seen for dnaBS371P cells, which is explained by the over-initiation that occurs in these cells.

\textit{dnaBS371P cells grown in minimal medium place their cell division rings asymmetrically.} To determine if dnaBS371P mutant cells contain cell division rings placed off-center in the cell, we compared the positioning of cell division rings in wild-type cells and in dnaBS371P cells grown in minimal medium. We found an eight-fold increase in the number of dnaBS371P cells that contain asymmetrically placed division rings as compared to wild-type cells. We used live-cell fluorescence microscopy to examine the placement of FtsZ-GFP in wild-type and dnaBS371P mutant cells (Figure 2A). FtsZ is the tubulin-like component of the bacterial cell division ring, and the formation of an FtsZ ring is the earliest known step in bacterial cell division (Addinall and Holland, 2002; Errington et al., 2003; Harry, 2001; Margolin, 2000; Margolin, 2001). The FtsZ-GFP fusion we used in this study functions as the only FtsZ in the cell, and is expressed from its endogenous site on the chromosome under its endogenous promoter.

In each cell that contained a single FtsZ-GFP ring, we measured the distance from that FtsZ-GFP ring to the nearest cell pole, and divided that distance by the total cell length. This calculation provides the fraction of the cell length at which the cell division ring was located. We found that 97.8\% of wild-type cells contain a division ring within the middle fifth of the cell (that is, 0.4-0.5 of cell length). Thus, only 2.2\% of wild-type cells (n = 361) contain a division
ring outside the middle fifth of cell length. In marked contrast, 17.2% of *dnaBS371P* mutant cells (n = 389) contain a division ring outside the middle fifth of cell length (Figure 2A). This proportion of cells containing asymmetric division rings is similar to that seen in characterized *E. coli* mutants that undergo over-initiation (Bach and Skarstad, 2004; Weitao et al., 1999).

**Asymmetric cell division rings form in nucleoid-free regions of *dnaBS371P* cells between unequally sized or asymmetrically distributed nucleoids.** We were interested in examining the nucleoid morphology in *dnaBS371P* cells containing asymmetric division rings, because one factor that influences the positioning of the cell division ring is nucleoid occlusion. Nucleoid occlusion describes the tendency of FtsZ cell division rings to form in cellular regions between the nucleoids (DNA masses and all associated proteins) (Addinall and Holland, 2002; Errington et al., 2003; Harry, 2001; Margolin, 2000; Margolin, 2001; Wu and Errington, 2004). Thus, we were interested in investigating whether the asymmetric division rings observed in *dnaBS371P* cells had formed over nucleoids, or in between unequally sized or asymmetrically distributed nucleoids.

We found that the majority (87%) of the asymmetric FtsZ rings observed in *dnaBS371P* cells had formed in DNA-free cellular regions located between unequally sized or asymmetrically distributed nucleoids (Figure 2B). Of the 46 *dnaBS371P* cells with an asymmetric FtsZ-GFP ring that we examined, 57% had an FtsZ-GFP ring placed in between two nucleoids of obviously different sizes. 24% of cells had an FtsZ-GFP ring placed in the cell such that it was separating a compartment harboring one nucleoid from another harboring two or three nucleoids. 7% of cells contained an FtsZ-GFP ring that was flanked by all of the DNA from the original cell on one side, and no DNA on the other side. The remaining 13% of cells contained an FtsZ-GFP ring that appeared to have formed over a nucleoid in the cell. Thus, it appears that nucleoid occlusion
A. FtsZ-GFP rings in \textit{dnaB}+ cells

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image1}
\caption{A.
FtsZ-GFP rings in \textit{dnaB}+ cells}
\end{figure}

B. FtsZ-GFP rings in \textit{dnaBS37IP} cells

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image2}
\caption{B.
\textit{dnaBS37IP} cells:
\begin{tabular}{ll}
FtsZ-GFP & DAPI-stained nucleoids \\
\end{tabular}}
\end{figure}
Figure 2. *dnaBS371P* cells contain asymmetric cell division rings that form between unequal amounts of DNA. Cells expressing *ftsZ-gfp* that were *dnaB+* (AG1752) or *dnaBS371P* (MER762) were grown at 30°C in defined minimal medium. Cells were sampled during exponential growth, and the cell membrane was stained with a vital membrane dye and visualized by fluorescence microscopy. Images of the vital membrane dye and of the GFP fluorescence were taken, and the two images were merged to show the cell membranes in red and the FtsZ-GFP division rings in white.

A) *dnaB+* (AG1752, upper panels) and *dnaBS371P* (MER762, lower panels) cells were examined for FtsZ-GFP cell division ring positioning. Almost all *dnaB+* cells with a single FtsZ-GFP ring contained the ring positioned in the middle fifth of the cell. In contrast, a significant portion of *dnaBS371P* cells contained an FtsZ-GFP ring outside of the middle fifth of the cell.

B) *dnaBS371P* (MER762) cells containing asymmetric cell division rings were examined for FtsZ-GFP cell division ring positioning and nucleoid morphology. Cell nucleoids (that is, the DNA and all associated proteins) were stained with DAPI and visualized by fluorescence microscopy. Images of the vital membrane dye and of the DAPI signal were taken, and the two images were merged to show the cell membranes in red and the nucleoids in white. The majority of *dnaBS371P* cells placed their asymmetric FtsZ-GFP rings in DNA-free zones of the cell, in between unequally sized or asymmetrically distributed nucleoids.
is largely intact in *dnaBS371P* cells, but that these cells often possess two nucleoid masses that are not uniform in size, or they possess three or more nucleoids. The asymmetric division rings observed in *dnaBS371P* cells form between two unevenly sized nucleoids, or between unequal numbers of nucleoids. Cell divisions at these sites would create two newborn cells of different sizes that contain unequal amounts of DNA from the single original cell.

Asymmetric division events have also been observed in mutant *E. coli* cells that over-initiate DNA replication (Bach and Skarstad, 2004; Weitao et al., 1999). Examples of mutant *E. coli* cells that over-initiate and divide asymmetrically are *seqA* null mutants and mutants in which the sequence of the SeqA binding sites near the chromosomal origin has been altered (Bach and Skarstad, 2004; Boye et al., 1996; Lu et al., 1994; von Freiesleben et al., 1994; Weitao et al., 1999). The SeqA protein negatively regulates *E. coli* replication initiation by binding to origins of replication and sequestering them in the cell membrane (Boye et al., 1996; d'Alencon et al., 1999; Lu et al., 1994; Shakibai et al., 1998; Slater et al., 1995; von Freiesleben et al., 1994), thereby rendering *oriC* inaccessible to replication proteins (Kang et al., 1999; Taghbalout et al., 2000; Torheim and Skarstad, 1999; Wold et al., 1998). The fact that cells lacking replication control, such as *dnaBS371P* *B. subtilis* cells and *seqA* *E. coli* cells, display cell division defects highlights the coordination of DNA replication and cell division that exists in these bacteria.

**dnaBS371P cells do not have increased DNA content when grown in rich medium.** We were interested in investigating whether *dnaBS371P* caused increased DNA content under conditions of faster growth in rich medium, as it does under conditions of slower growth in minimal medium. Thus, we measured the DNA/protein ratios of wild-type and *dnaBS371P* cells grown in rich medium (Table 4). We found that *dnaBS371P* cells do not contain increased DNA
content when grown under conditions of fast growth. Instead, \textit{dnaBS371P} cells contain similar but slightly lower DNA contents per mass than wild-type cells grown under these conditions.

This result indicates that \textit{dnaBS371P} is more appropriately described as a replication control mutant than an over-initiation mutant. We hypothesize that the \textit{dnaBS371P} mutation may cause replication initiation to occur at a constant frequency. This frequency would be higher than that appropriate for slow growth in defined minimal medium, and very close to, but slightly lower than, that appropriate for fast growth in rich medium. Thus, we propose a model in which \textit{dnaBS371P} renders DnaB insensitive to proper regulatory control. Wild-type DnaB may be able to (directly or indirectly) sense replication status, and promote initiation in a regulated fashion by interacting with DnaD and recruiting it to the membrane fraction of cells with appropriate timing. In our model, DnaB and DnaD can work together to load helicase at origins during initiation only when these two proteins converge at the membrane (where DnaB (Rokop et al., 2004) and \textit{oriC} (Funnell, 1996; Sueoka and Hammers, 1974; Sueoka, 1998; Yamaguchi and Yoshikawa, 1977) are enriched). Wild-type DnaB may respond to fast growth conditions by interacting with DnaD more frequently than it does during slow growth conditions, when initiation events are less frequent. In contrast, \textit{dnaBS371P} may render DnaB insensitive to such regulation, and instead, DnaBS371P may interact with DnaD and recruit it to the membrane at a constant frequency or affinity, regardless of growth rate. We hypothesize that DnaBS371P interacts with DnaD at a set amount or frequency that is close to that of wild-type cells grown quickly in rich medium, but excessive for cells growing slower in minimal medium.

\textbf{Cells over-producing DnaBS371P grown in rich medium have decreased cellular DNA content.} Although \textit{dnaBS371P} cells lack proper control of replication initiation, \textit{dnaBS371P} cells have no obvious growth defects. We previously found that cells over-producing
DnaBS371P grown in rich medium form long filaments that contain large cellular regions devoid of DNA (Rokop et al., 2004). We were interested in determining if the growth defects of these cells were due to a loss of control over replication initiation. Instead, our data support the hypothesis that the abnormalities of cells over-producing DnaBS371P result from a defect in completing replication elongation due to decreased processivity of elongating replication forks. We hypothesize that this decreased processivity results from a reduced ability of DnaBS371P over-producing cells to undergo replication restart at stalled replication forks.

We measured the DNA/protein ratios of dnaB+ cells over-producing DnaBS371P from an ectopic locus, and found that these cells have a significantly decreased cellular DNA content when grown in rich medium. Cells over-producing DnaBS371P have a DNA/protein ratio of 0.72 compared to the DNA/protein ratio of cells over-producing wild-type DnaB, which was normalized to equal 1 (Table 4). These data indicated that cells over-producing DnaBS371P might be under-replicating their DNA.

To confirm that cells over-producing DnaBS371P grown in rich medium are under-replicating, we used DNA microarrays to measure the relative copy numbers of all regions of the chromosome (Khodursky et al., 2000; Simmons et al., 2004) in wild-type cells (Figure 3A), dnaBS371P cells (Figure 3B), and cells over-producing DnaBS371P (Figure 3C) grown in rich medium. We found that cells over-producing DnaBS371P have a significantly lower ratio of origin/terminus copy number than wild-type cells, confirming the results from the DNA/protein ratio experiments. The origin/terminus ratio of wild-type exponentially growing cells was equal to 3.4; in contrast, the origin/terminus ratio of cells over-producing DnaBS371P was 1.7. To obtain this ratio, we plotted the log2 of the copy number of each open reading frame in the genome on the Y-axis against the position of each open reading frame along the chromosome on
the X-axis. The difference in height between the region of highest copy number (the origin of replication) and the region of lowest copy number (the terminus of replication) allows the origin/terminus ratio to be calculated. (The ratios reported above have been converted from their log2 values.) This difference is much larger for wild-type cells (Figure 3A) than it is for DnaBS371P over-producing cells (Figure 3C). In contrast, the pattern of relative copy numbers for all open reading frames in dnaBS371P cells (Figure 3B) is indistinguishable from that of wild-type cells (Figure 3A).

Cells over-producing DnaBS371P grown in rich medium contain stalled replication forks. The under-replication seen in cells over-producing DnaBS371P could be due to a defect in initiating replication or to a defect in completing replication. Further analysis of our array data supported the hypothesis that cells over-producing DnaBS371P have defects in completing replication. Previous studies have shown that using the genomic DNA array analysis described above on wild-type cells yields a plot in which the log2 of the copy number of each gene varies linearly with respect to the position of each gene on the chromosome (Simmons et al., 2004). Thus, a plot of the log2 of the copy number of each gene on the Y-axis against the position of each gene on the X-axis yields two straight lines that meet at the origin of the chromosome and end at the terminus region of the chromosome (Figure 3A). Previous studies have also shown that the genomic DNA isolated from mutant cells that do not complete many rounds of replication due to stalled replication forks yields a plot that is not linear (Simmons et al., 2004). Our analysis demonstrated that the plot we obtained for wild-type cells was significantly more linear than that obtained for DnaBS371 over-producing cells (r² of linear regression = 0.917 for wild-type cells and 0.630 for DnaBS371P over-producing cells). This indicates that many replication forks that initiate at the origin in DnaBS371P over-producing cells do not
Figure 3. Cells over-producing DnaBS371P grown in rich medium contain low origin/terminus copy number ratios and stalled forks. Wild-type cells (JH642, panel A), dnaBS371P cells (MER468, panel B), or cells over-producing DnaBS371P from an IPTG-inducible promoter (MER582, panel C) were grown at 37°C in LB medium with IPTG. Cells were sampled during exponential growth, and genomic DNA was isolated from the cells. This genomic DNA was digested, labeled with a fluorescent dye, and then hybridized to DNA microarrays along with differentially labeled genomic DNA from a reference culture of cells grown in stationary phase. The copy number of all regions of the stationary phase chromosomes is about 1, so the ratio of the signal from the experimental, exponential phase genomic DNA to that from the reference, stationary phase genomic DNA allows the copy number of each region of the experimental culture’s chromosomes to be calculated. In this graph, the position of each gene along the chromosome is plotted along the X-axis, with the origin in the center of the graph, and the terminus at either end of the axis. The log2 of the relative copy number of each gene is plotted along the Y-axis. In exponentially growing wild-type or dnaBS371P cells, such a plot gives two straight lines that start at the edges of the graph, at the terminus of replication. The two straight lines meet in the center of the graph, at the origin of replication. In contrast, the genomic DNA from cells over-producing DnaBS371P does not give this wild-type pattern. The difference in height between the region of highest copy number (the origin) and the region of lowest copy number (the terminus) is much smaller in cells over-producing DnaBS371P, indicating that the origin/terminus ratio is much lower in these mutants. In addition, the plot from cells over-producing DnaBS371P is significantly less linear than the plot from wild-type cells and dnaBS371P cells. This indicates that some initiation events that occur in cells over-producing DnaBS371P result in elongating replication forks that stall along the chromosome.
A. *dnaB*+ compared to reference

Position along the chromosome

B. *dnaBS371P* compared to reference

Position along the chromosome

C. *P*$_{IPTG}$-*dnaBS371P* compared to reference

Position along the chromosome

Figure 3.
proceed to the terminus with the processivity of replication forks from wild-type cells. This decreased processivity of replication forks in cells over-producing DnaBS371P could result from the prolonged stalling of elongating forks along the chromosome due to defects in repairing stalled replication forks.

Cells that have defects in repairing stalled replication forks are often sensitive to DNA-damaging agents (Li et al., 2004; Polard et al., 2002; Sandler et al., 1996). Consistent with the conclusions from our DNA array experiments, we found that cells over-producing DnaBS371P are hypersensitive to the DNA-damaging agent mitomycin-C when grown in rich medium. In these experiments, we plated cells on levels of mitomycin-C that are sub-lethal for wild-type cells and dnaBS371P cells, which do not exhibit hypersensitivity to mitomycin-C (data not shown). Cells over-producing DnaBS371P were not capable of colony formation in the presence of mitomycin-C, but cells over-producing wild-type DnaB were capable of growth under these conditions (Figure 4). The results of our mitomycin-C sensitivity assay and DNA array experiments support the model that cells over-producing DnaBS371P contain stalled replication forks along their chromosomes when grown in rich medium. The presence of these stalled forks disrupts the processivity of replication forks attempting to proceed from the origin to the terminus region of the chromosome in cells over-producing DnaBS371P, and renders these cells hypersensitive to DNA-damaging agents.

*dnABS371P* and over-produced wild-type DnaB suppress the defects of *ApriA* cells better than over-produced DnaBS371P. It seemed counterintuitive that over-producing DnaBS371P led to stalled replication forks that inhibit fork progression, given that *dnABS371P* suppresses the effects of the stalled forks in *ApriA* cells (Bruand et al., 2001). Therefore, we
Figure 4. Cells over-producing DnaBS371P are hypersensitive to mitomycin-C. dnaB+ cells over-producing wild-type DnaB (MER580, right plate quadrants) or DnaBS371P (MER582, left plate quadrants) were tested for growth on LB IPTG plates without (upper plate) or with (lower plate) a concentration of the DNA-damaging agent mitomycin-C (0.0625 μg/mL) that is sub-lethal to wild-type cells.
Figure 4.
investigated the differential abilities of dnaBS371P, over-produced wild-type DnaB, and over-produced DnaBS371P to suppress the growth defects of ApriA cells. Consistent with previous reports, we found that ApriA dnaB+ cells were inviable when grown in rich medium (Bruand et al., 2001; Polard et al., 2002), and that ApriA dnaBS371P cells were fully capable of growing in rich medium with a doubling time equal to that of wild-type cells (Bruand et al., 2001). The appearance of these cells as visualized by live-cell microscopy was also similar to wild-type (Figure 5).

We found that over-producing DnaB changes the abilities of DnaB and DnaBS371P to suppress the growth defects of ApriA cells. As expected, ApriA dnaB+ cells possessing an IPTG-inducible dnaB+ gene at an ectopic locus grew poorly in rich medium without inducer. However, when inducer was added, ApriA cells over-producing wild-type DnaB grew as well as wild-type cells, demonstrating that the over-production of wild-type DnaB fully suppresses the growth defects of ApriA cells (Figure 5). In contrast, ApriA dnaB+ cells possessing an IPTG-inducible dnaBS371P gene at an ectopic locus grew very well without inducer (due to basal expression of dnaBS371P from the promoter). Upon induction, however, ApriA cells over-producing DnaBS371P grew poorly in rich medium and formed long filaments with large cellular regions devoid of DNA (Figure 5). These findings indicate that over-producing DnaB changes the abilities of DnaB and DnaBS371P to suppress the defects of ApriA cells. This makes it more plausible that over-producing DnaBS371P leads to a defect in restarting stalled replication forks, even though dnaBS371P in a single copy fully suppresses the defects of ApriA cells that are caused by stalled forks (Bruand et al., 2001).
Figure 5. *dnaBS371P* and over-produced wild-type DnaB suppress the defects of *ApriA* cells better than over-produced *DnaBS371P*. *ApriA* cells containing *dnaBS371P* (MER776, upper panels), Pspank(hy) fused to *dnaB* (MER778, center panels), or Pspank(hy) fused to *dnaBS371P* (MER780, lower panels) were grown at 37°C in LB medium without (left panels) or with (right panels) IPTG to induce transcription from Pspank(hy). Cells were sampled during exponential growth, and cell nucleoids were stained with DAPI and visualized by fluorescence microscopy. Cell outlines were visualized using Nomarski microscopy, and the two images were merged to show the cell outlines in grey and the nucleoids in white.
Figure 5.
DnaBS371P causes defects in replication control and in replication restart. In *B. subtilis*, the DnaB protein functions in both replication initiation (Hoshino et al., 1987; Ogasawara et al., 1986; Sueoka, 1998) and restarting replication at stalled forks (Bruand et al., 1995a; Bruand et al., 2001; Marsin et al., 2001). A single mutation in *dnaB* (*dnaBS371P*) suppresses both the temperature sensitivity of *dnaBts* cells and *dnaDts* cells that cannot initiate replication (Rokop et al., 2004), and the growth defects of *ApriA* mutant cells that cannot restart replication (Bruand et al., 2001). In this study, we show that expressing DnaBS371P in otherwise wild-type cells results in defects in initiation and restart, and that these defects depend on the level of expression of the mutant protein and on the growth conditions. At endogenous levels of expression, DnaBS371P causes a loss of proper control over replication initiation. *dnaBS371P* cells grown under slow growth conditions in minimal medium over-initiate, whereas *dnaBS371P* cells grown under fast growth conditions in rich medium contain slightly decreased cellular DNA contents. Therefore we hypothesize that *dnaBS371P* cells lack the ability to adjust the frequency of replication initiation in response to growth rate, and instead initiate replication at a set frequency. This inability of *dnaBS371P* cells to properly control replication initiation supports the model that the spatial separation of DnaB and DnaD in the cell (which is disrupted in *dnaBS371P* cells (Rokop et al., 2004)) is an important mechanism of replication control in *B. subtilis*.

Cells over-producing DnaBS371P have also lost proper control of replication initiation, as evidenced by the increased DNA contents of cells over-producing DnaBS371P grown in minimal medium. A loss of initiation control most likely also occurs in cells over-producing DnaBS371P grown in rich medium. In these cells, however, the effects of a more severe defect in replication
restart become overwhelmingly apparent. Cells over-producing DnaBS371P grow poorly in rich medium, form filaments (Rokop et al., 2004), have low cellular DNA content, and exhibit sensitivity to DNA damaging agents. These abnormalities are shared by cells with defects in replication restart, such as $ApriA$ cells (Polard et al., 2002; Sandler et al., 1996). Based on this evidence and the results of our genomic DNA array analysis, we conclude that cells over-producing DnaBS371P most likely contain replication forks that are stalled for prolonged periods of time due to a defect in restarting replication. The defect in restarting replication of cells over-producing DnaBS371P is most likely apparent in rich medium because cells grown in rich medium contain many more replication forks due to the multi-fork replication that occurs in fast-growing bacterial cells (Kornberg and Baker, 1992; Nordstrom and Austin, 1993; Zyskind and Smith, 1992).

The defect in replication restart that exists in cells over-producing DnaBS371P may result from the increased ability of DnaBS371P to bind to its partner, DnaD (Rokop et al., 2004), or to DNA substrates that resemble replication fork intermediates (Velten et al., 2003). DnaB also interacts directly with the replicative helicase (Velten et al., 2003), and the $dnaBS371P$ mutation could affect this interaction. Upon over-production of the DnaBS371P protein, its increased ability to interact with DNA or its binding partners could lead to a sequestration of DnaD, helicase, or the sites of stalled forks away from other functional components of the replication restart machinery. Experiments are underway to test these models to explain the defect of cells over-producing DnaBS371P in performing replication restart.
EXPERIMENTAL PROCEDURES

Media and growth conditions. Rich medium was LB for routine growth and strain constructions for *B. subtilis*. *B. subtilis* transformations were done by standard procedures (Harwood and Cutting, 1990). Defined minimal medium for *B. subtilis* was S7 medium, with MOPS buffer at 50 mM rather than 100 mM, supplemented with 1% glucose and 0.1% glutamate and required amino acids (tryptophan, 40 μg/ml; phenylalanine, 40 μg/ml) as needed (Jaacks et al., 1989; Vasantha and Freese, 1980). Antibiotics were used at the following concentrations: spectinomycin at 100 μg/ml, chloramphenicol at 5 μg/ml, and erythromycin at 0.5 μg/ml with lincomycin at 12.5 μg/ml. Where indicated, isopropyl-beta-D-thiogalactopyranoside (IPTG) (Sigma) was used at 1 mM to induce expression from the LacI-repressible-IPTG-inducible promoter Pspank(hy).

Strains, alleles, and plasmids. *B. subtilis* strains are listed in Table 1, and are derivatives of JH642 (AG174) that contain the trpC and pheA mutations. Standard procedures were used for strain constructions (Harwood and Cutting, 1990).

The dnaBS371P mutation present in MER468, MER470, MER762, and MER776 was isolated as previously described (Rokop et al., 2004). The construction of the amyE::(Pspank(hy)-dnaB spc) construct contained in MER580 and MER778 and the amyE::(Pspank(hy)-dnaBS371P spc) construct contained in MER582 and MER780 were described previously (Rokop et al., 2004).

To generate the SpoOJ-GFP and FtsZ-GFP fusion constructs, the 3' ends of spo0J and ftsZ were amplified by PCR and fused in frame to the sequence encoding GFP inside the vector pUS19 (Benson and Haldenwang, 1993) for Spo0J and pGEMcat (Youngman et al., 1989) for FtsZ. The resulting fusion plasmids, pPL52 (spo0J-gfp) and pPL51 (ftsZ-gfp), were integrated
into the chromosome by single crossover to generate fusions of the entire spo0J or ftsZ genes to gfp linked to spectinomycin resistance for Spo0J or to chloramphenicol resistance for FtsZ.

To generate the ΔpriA::cat construct, an internal fragment of priA was amplified by PCR and inserted into the vector pGEMcat (Youngman et al., 1989). The resulting plasmid, pMR36, was integrated into the chromosome by single crossover to generate a disruption of priA by cat, the gene encoding chloramphenicol resistance.

**Measuring DNA/protein ratios.** Samples of JH642 (wild-type), MER468 (Tn917ΩHU151 (mls) dnaBS371P), MER580 (amyE::(Pspank(hy)-dnaB spc), and MER582 (amyE::(Pspank(hy)-dnaBS371P spc) were harvested in exponential growth (OD_{600} between 0.4 - 0.6) in LB medium or in defined minimal medium at 37°C or at 30°C. MER580 and MER582 cells were grown in LB or in defined minimal medium with IPTG to induce transcription from Pspank(hy). Nucleic acid and protein fractionations were performed and DNA/protein ratios were calculated as described previously (Kadoya et al., 2002; Lee et al., 2003; Lee and Grossman, 2004).

**DNA microarrays.** Samples of JH642 (wild-type), MER468 (Tn917ΩHU151 (mls) dnaBS371P), and MER582 (amyE::(Pspank(hy)-dnaBS371P spc) were harvested in exponential growth (OD_{600} between 0.4 - 0.6) in LB medium at 37°C. MER582 cells were grown in LB with IPTG to induce transcription from Pspank(hy). An equal volume of cold methanol was added, cells were pelleted by centrifugation, and decanted pellets were stored at -20°C. Genomic DNA was prepared using the Qiagen Genomic Tip 100 Protocol, as directed by the manufacturer. The amount of DNA was quantified using a spectrophotometer. The genomic DNA was digested with HaeIII (NEB) and purified using Qiaquick PCR purification spin columns as directed by the manufacturer.
For labeling, 0.5 μg of DNA was incubated with 7.5 μg of random hexamers at 95°C for 5 min, and then placed on ice. dNTPs (0.5mM dATP, 0.5mM dCTP, 0.5mM dGTP, 0.1mM dTTP (Invitrogen), 0.4mM aminoallyl-dUTP (Ambion)) and 20 units of Klenow exo- (NEB) were added and the reaction was incubated at 30°C overnight. DNA was purified using MinElute spin columns (Qiagen). 50mM NaHCO$_3$ (pH 9.0) and the fluorescent dye Cy5 (Amersham) were added, and the dye-coupling reaction was incubated for 1 hour at room temperature in the dark. The labeling was quenched with 1.2M hydroxylamine for 15 min at room temperature. The Cy5-labeled genomic DNA from the experimental culture was mixed with Cy3-labeled genomic DNA isolated from stationary phase cells (the reference DNA), in which the copy number of all chromosomal regions equals ~1. This mixture of experimental and reference DNA was purified using MinElute spin columns as directed by the manufacturer, except that 75% ethanol was substituted for the PE buffer used to wash the columns prior to elution.

Before hybridization of the DNA to the microarray, 10 μg salmon sperm DNA and 0.8 μg yeast tRNA were added to each sample. The samples were incubated at 100°C for 5 min, and mixed with an equal volume of 2X hybridization buffer (0.1% SDS, 10X SSC, 50% formamide). The DNA was hybridized to a microarray for at least 16 hours at 42°C. Arrays were washed with 1X SSC, 0.2% SDS for 5 min at 42°C, and then with 0.1X SSC, 0.2% SDS for 5 min at room temperature. Arrays were then washed in 0.1X SSC at room temperature for 5 min and dried with nitrogen gas. Construction of the DNA microarrays and scanning were performed as described previously (Britton et al., 2002). The comparison of the relative signals from each fluorescent dye allowed the copy number of all chromosomal regions from the experimental culture to be calculated (Khodursky et al., 2000; Simmons et al., 2004). Data analysis was performed using GenePix Pro, Spotfire, and Microsoft Excel software.
**Fluorescence microscopy.** AG1752 (ftsZ-gfp (cat)), MER470 (spo0J-gfp (spc)) Tn917ΩHU151 (mls dnaBS371P), MER762 (ftsZ-gfp (cat) Tn917ΩHU151 (mls) dnaBS371P), and PAL657 (spo0J-gfp (spc)) cells were grown in defined minimal medium at 30° to an OD₆₀₀ between 0.1 and 0.4. MER470 and PAL657 cells were grown in defined minimal medium with spectinomycin to maintain selection for the spo0J-gfp fusion. AG1752 and MER762 cells were grown in defined minimal medium with chloramphenicol to maintain selection for the ftsZ-gfp fusion. MER776 (ΔpriA::cat dnaBS371P), MER778 (ΔpriA::cat amyE::(Pspank(hy)-dnaB spc)), and MER780 (ΔpriA::cat amyE::(Pspank(hy)-dnaBS371P spc)) cells were grown in LB medium with or without IPTG at 37° to mid-exponential phase (an OD₆₀₀ between 0.1 and 0.5). MER776, MER778, and MER780 cells were grown in LB with chloramphenicol to maintain selection for the ΔpriA::cat disruption plasmid.

The DNA stain 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) was added to 0.1 μg/mL and the vital membrane dye FM4-64 (Molecular Probes) was added to 0.05 μg/mL. Cells were placed on slides containing pads of 1% agarose in minimal salts with 1mM MgSO₄. Fluorescence was viewed with a Nikon E800 microscope equipped with a 100X DIC objective using UV, TRITC, and FITC filters. Images were obtained with a CCD camera (Hamamatsu, model C4742-95) using Improvion OpenLab software. Cell lengths and the positioning of the FtsZ-GFP rings inside the cells were measured and tabulated using Improvion OpenLab software and Microsoft Excel.
ACKNOWLEDGEMENTS

We thank Petra Levin for reagents, and Lyle Simmons, Jenny Auchtung, Milan deVries, and C. Lee for very helpful comments. This work was supported in part by an HHMI Predoctoral Fellowship to M.E.R., by a Damon Runyon Postdoctoral Fellowship to J.D.W., and by NIH grant GM41934 to A.D.G.
Table 1. *B. subtilis* strains used.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Relevant genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG1752</td>
<td><em>ftsZ-gfp</em> (cat)</td>
</tr>
<tr>
<td>MER468</td>
<td>Tn917ΩHU151 (mls) <em>dnaBS371P</em></td>
</tr>
<tr>
<td>MER470</td>
<td><em>spo0J-gfp</em> (spc) Tn917ΩHU151 (mls) <em>dnaBS371P</em></td>
</tr>
<tr>
<td>MER580</td>
<td><em>amyE</em>::(<em>Pspank(hy)-dnaB</em> spc)</td>
</tr>
<tr>
<td>MER582</td>
<td><em>amyE</em>::(<em>Pspank(hy)-dnaBS371P</em> spc)</td>
</tr>
<tr>
<td>MER762</td>
<td><em>ftsZ-gfp</em> (cat) Tn917ΩHU151 (mls) <em>dnaBS371P</em></td>
</tr>
<tr>
<td>MER776</td>
<td>Δ<em>priA</em>::cat Tn917ΩHU151 (mls) <em>dnaBS371P</em></td>
</tr>
<tr>
<td>MER778</td>
<td>Δ<em>priA</em>::cat amyE::(<em>Pspank(hy)-dnaB</em> spc)</td>
</tr>
<tr>
<td>MER780</td>
<td>Δ<em>priA</em>::cat amyE::(*Pspank(hy)-dnaBS371P spc)</td>
</tr>
<tr>
<td>PAL657</td>
<td><em>spo0J-gfp</em> (spc)</td>
</tr>
</tbody>
</table>

Table 2. DNA content of *dnaBS371P* cells and cells over-producing DnaBS371P grown in defined minimal medium at 37°C, as measured by DNA/protein ratios.

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Normalized DNA/protein ratio +/- standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>dnaB</em>+</td>
<td>1.00 +/- 0.16</td>
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<tr>
<td><em>dnaBS371P</em></td>
<td>1.45 +/- 0.23</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Normalized DNA/protein ratio +/- standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pspank(hy)-dnaB</em></td>
<td>1.00 +/- 0.17</td>
</tr>
<tr>
<td><em>Pspank(hy)-dnaBS371P</em></td>
<td>1.35 +/- 0.19</td>
</tr>
</tbody>
</table>
Table 3. Percentage of cells containing each number of origins per cell, as measured by number of Spo0J-GFP foci.

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>1 origin</th>
<th>2 origins</th>
<th>3 origins</th>
<th>4 origins</th>
<th>5+ origins</th>
</tr>
</thead>
<tbody>
<tr>
<td>spo0J-gfp dnaB+</td>
<td>1.5%</td>
<td>61.7%</td>
<td>14.5%</td>
<td>20.2%</td>
<td>2.1%</td>
</tr>
<tr>
<td>n = 793</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>spo0J-gfp dnaBS371P</td>
<td>6.0%</td>
<td>43.9%</td>
<td>20.8%</td>
<td>21.2%</td>
<td>8.3%</td>
</tr>
<tr>
<td>n = 773</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. DNA content of dnaBS371P cells and cells over-producing DnaBS371P grown in rich medium at 37°C, as measured by DNA/protein ratios.

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Normalized DNA/protein ratio +/- standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>dnaB+</td>
<td>1.00 +/- 0.04</td>
</tr>
<tr>
<td>dnaBS371P</td>
<td>0.90 +/- 0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Normalized DNA/protein ratio +/- standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pspank(hy)-dnaB</td>
<td>1.00 +/- 0.09</td>
</tr>
<tr>
<td>Pspank(hy)-dnaBS371P</td>
<td>0.72 +/- 0.07</td>
</tr>
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</table>
REFERENCES


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Chapter 4:

Discussion
DISCUSSION

This thesis explores the roles of the DnaB and DnaD proteins in replication initiation, replication control, and replication fork restart in the Gram-positive bacterium *Bacillus subtilis*. DnaB and DnaD function to allow the replicative helicase to be loaded at chromosomal origins and at stalled replication forks (Bruand et al., 1995; Bruand et al., 2001; Marsin et al., 2001; Rokop et al., 2004; Velten et al., 2003). A missense mutation in *dnaB*, *dnaBS371P*, has been isolated in three different genetic screens as a suppressor of the temperature sensitivity of *dnaBts* cells, of the temperature sensitivity of *dnaDts* cells, and of the growth defects of *ApriA* mutant cells (Bruand et al., 2001; Rokop et al., 2004). My work focused on the effects of *dnaBS371P* on DnaB and DnaD, and on DNA replication and cell division. I also investigated the spatial separation of DnaB and DnaD in the cell, which has implications regarding when these two proteins can function together to allow for helicase loading to occur.

**A physical interaction between DnaB and DnaD.** I found that the two essential replication factors DnaB and DnaD are found in distinct subcellular fractions of asynchronously growing *B. subtilis* cells (Rokop et al., 2004). DnaB is found predominantly in the membrane fraction of cells, and DnaD is found predominantly in the cytoplasmic fraction of cells (Rokop et al., 2004). I hypothesize that these two proteins transiently interact with each other at the membrane, where chromosomal origins are enriched in *B. subtilis* (Funnell, 1996; Sueoka and Hammers, 1974; Sueoka, 1998; Yamaguchi and Yoshikawa, 1977), during active replication initiation events. Thus, these two proteins would function together to assist in loading helicase at chromosomal origins during periods of active replication initiation, but not during the majority of the cell cycle. This temporal and spatial regulation of helicase loading activity would be accomplished
through a regulated interaction between DnaB and DnaD. Although this is my model, a direct physical interaction between wild-type DnaB and wild-type DnaD has not been observed (Ishigo-oka et al., 2001; Marsin et al., 2001; Noirot-Gros et al., 2002; Rokop et al., 2004). I have also not observed wild-type DnaD coming to the membrane in dnaB+ cells (Rokop et al., 2004).

Despite the failure to detect an interaction between wild-type DnaB and DnaD, there are many pieces of evidence that suggest that DnaB and DnaD interact directly. First, a physical interaction can be detected by two-hybrid analysis between wild-type DnaD and DnaBS371P (Rokop et al., 2004). Second, in dnaBS371P cells, DnaD becomes enriched in the membrane fraction of cells, where DnaB is located (Rokop et al., 2004). Third, a physical interaction can be detected in the yeast two-hybrid assay using wild-type DnaB and a mutant form of DnaD, DnaD23tsΔ154-155. The dnaD23tsΔ154-155 allele was isolated as a suppressor of the temperature sensitivity of dnaD23ts cells, just as dnaBS371P was isolated (Appendix A). Fourth, wild-type DnaD stimulates the affinity of wild-type DnaB for single-stranded DNA (Marsin et al., 2001). Fifth, homologs of the C-termini of DnaB and DnaD are found fused together into single polypeptides predicted to function in replication initiation in phages that infect low G+C Gram-positive bacteria (Rokop et al., 2004). This is significant because oftentimes, if two proteins are fused into a single polypeptide in one organism, the two proteins physically interact in a second organism in which they are encoded separately (Marcotte et al., 1999).

Interestingly, the Clostridium acetobutylicum homologs of DnaB and DnaD are fused into a single polypeptide in this bacterium. I observed using BLAST searches (Altschul et al., 1990) that almost all Gram-positive bacterial genomes encoding a DnaB homolog also encoded a DnaD homolog. The exception to this rule was Clostridium acetobutylicum. The genome of C.
acetobutylicum contains a gene annotated as "C. acetobutylicum dnaD," but does not contain a
gene annotated as "C. acetobutylicum dnaB." Further sequence analysis of "C. acetobutylicum
DnaD" (Ca-DnaD) revealed that Ca-DnaD is actually a fusion of a DnaD homolog to a DnaB
homolog (Figure 1). B. subtilis DnaD shares 48% sequence similarity (25% identity) with a 105
amino acid region of Ca-DnaD. This region of Ca-DnaD partially overlaps with a region that
shares 46% sequence similarity (23% identity) with a 137 amino acid region of B. subtilis DnaB
(Figure 1). The regions of B. subtilis DnaB and DnaD that are homologous to Ca-DnaD
encompass the phage-homology domains of these proteins (Rokop et al., 2004). The fact that
homologs of DnaB and DnaD are fused into one polypeptide in C. acetobutylicum provides
further support for my model that these two proteins interact in B. subtilis.

Support for a conformational change in DnaB that alters its protein-protein
interactions. As discussed above, there are several pieces of evidence that support the existence
of a direct physical interaction between wild-type DnaB and DnaD. However, an interaction
between the wild-type versions of these proteins has not been detected in the yeast two-hybrid
assay or in in vitro assays (Ishigo-oka et al., 2001; Marsin et al., 2001; Noirot-Gros et al., 2002;
Rokop et al., 2004). I hypothesize that the wild-type proteins do not interact with each other in
yeast or in in vitro assays because, under these conditions, the proper signal is not present to
trigger a conformational change in DnaB that reveals a cryptic DnaD-interaction site, or
stabilizes the interaction between these two proteins. This model stems from studies of
DnaBS371P, a mutant form of DnaB that contains a substitution of a serine to a proline three
amino acids upstream of the phage homology region of DnaB. dnaBS371P allows DnaB to
detectably interact with DnaD (Rokop et al., 2004). A proline at this position could induce a
Figure 1.
Figure 1. A single protein from Clostridium acetobutylicum shares sequence similarity with both B. subtilis DnaD and DnaB. “DnaD” is the annotated name of this protein from the genome sequence of C. acetobutylicum. The genome of C. acetobutylicum has no separate homolog of DnaB. B. subtilis DnaD has 48% sequence similarity with a 105 amino acid region of Ca-DnaD that is partially overlapping with a 137 amino acid long region of Ca-DnaD that is 46% similar to B. subtilis DnaB. The regions of B. subtilis DnaD and DnaB that share homology with this protein encompass the regions of these B. subtilis proteins that are homologous to phage initiation factors. The phage homology regions of B. subtilis DnaD and DnaB are shown here shaded in gray.
conformational change in DnaB because prolines are capable of disrupting regions of secondary structure, especially those that are rich in alpha helices (Vanhoof et al., 1995).

I have used secondary structure prediction programs to show that the region of DnaB surrounding amino acid 371 is predicted to be very rich in alpha helices. In fact, the entire DnaB protein is predicted to be rich in alpha helices by the secondary structure prediction programs Jpred (Cuff et al., 1998) and PredictProtein (Rost et al., 2003). Alpha helices are especially abundant in the region of the protein around amino acid 371. All amino acids in the region of residues 353-400 are predicted to be alpha helical except for amino acids 366 and 384-388. The insertion of a proline at amino acid 371 could be disruptive to the secondary structure of this region.

Notably, amino acids 383-406 of DnaB are predicted to be the only multimerization domain in DnaB by the prediction program Multicoil (Wolf et al., 1997). Multicoil also predicts whether the multimerization domain is more likely to be a dimerization domain or a trimerization domain, and these amino acids in DnaB are predicted to be a trimerization domain. This computer prediction is consistent with the finding that DnaB is a multimer in vitro (Marsin et al., 2001). DnaB behaves as a trimer using its sedimentation coefficient and as approximately a heptamer using its Stokes radius (Marsin et al., 2001). The same amino acids (383-406) predicted to be the multimerization domain of DnaB are also predicted to be a coiled-coil domain by the prediction program COILS (Lupas et al., 1991).

Based on the secondary structure properties predicted for the region surrounding amino acid 371, I investigated whether dnaBS371P altered the ability of DnaB to multimerize using the yeast two-hybrid assay. DnaB has previously been shown to interact with itself by two-hybrid
analysis (Ishigo-oka et al., 2001; Noirot-Gros et al., 2002; Rokop et al., 2004). I found that DnaBS371P has lost the ability to strongly interact with itself in the yeast two-hybrid assay, as compared to wild-type DnaB (Appendix C).

Based on my results using yeast two-hybrid analysis and secondary structure prediction programs, I hypothesize that the dnaBS371P mutation causes a change in the secondary structure of the region of DnaB surrounding amino acid 371. In my model, this region of the protein is involved in the multimerization of DnaB, and the C-terminus of DnaB is the DnaD-interaction domain of the protein. I hypothesize that DnaB has two conformations. In one conformation, which is adopted by wild-type DnaB throughout the majority of the cell cycle, DnaB is a multimer that does not have its C-terminus exposed for interaction with DnaD. During active initiation, DnaB adopts a conformation in which the secondary structure near amino acid 371 is altered. This reduces the ability of DnaB to multimerize and allows DnaB to interact with DnaD. My data support the hypothesis that DnaBS371P is in the DnaD-interaction conformation for the majority of the cell cycle. This model is diagrammed in Figure 9 of Chapter 1 (the Introduction).

In this model, DnaBS371P is a mutant form of DnaB that adopts one of the two conformations of wild-type DnaB too often, or in a misregulated manner. Support for the model that DnaBS371P is performing a role of the wild-type protein with increased frequency, or in a misregulated manner, comes from the fact that over-producing wild-type DnaB suppresses the defects of priA null mutant cells as well as dnaBS371P does when expressed at endogenous levels (Chapter 3). This result supports my hypothesis that dnaBS371P is a hypermorphic allele that causes increased or misregulated activity of a wild-type function of DnaB. This result is not consistent with dnaBS371P being a neomorphic allele that causes DnaBS371P to perform a function that wild-type DnaB cannot perform. Based on the ability of over-produced DnaB to
suppress the defects of priA null mutant cells, it would be interesting to examine whether DnaD is brought to the membrane fraction of cells over-producing wild-type DnaB.

**B. subtilis and E. coli replication initiation in the context of the cell membrane.** I have shown that DnaB is located in the membrane fraction of cells (Rokop et al., 2004). DnaBS371P shows a fractionation pattern indistinguishable from that of DnaB. Thus, I hypothesize that both conformations of DnaB (the multimerization conformation and the DnaD-interaction conformation) exist at the cell membrane. Chromosomal origins are also enriched in the membrane fraction of *B. subtilis* cells (Funnell, 1996; Sueoka and Hammers, 1974; Sueoka, 1998; Yamaguchi and Yoshikawa, 1977). Thus, replication initiation may be restricted to occurring at the membrane in *B. subtilis*, because that is where oriC (the chromosomal site of initiation) and DnaB (an essential initiation factor) are located.

In support of this hypothesis, the ability of the cell membrane to bind chromosomal origins correlates with the ability of *B. subtilis* cells to initiate replication. In dnaBts cells incubated at the permissive temperature, replication initiation can occur, and chromosomal origins are enriched in the membrane fraction of cells. In dnaBts cells incubated at the non-permissive temperature, replication initiation is blocked, and chromosomal origins are no longer enriched in the membrane (Burnett and Wake, 1977; Funnell, 1996; Gross et al., 1968; Karamata and Gross, 1970; Sato et al., 1991; Sueoka, 1998; White and Sueoka, 1973; Winston and Sueoka, 1980).

In contrast to *B. subtilis*, *E. coli* replication initiation can occur when origins are not bound by the cell membrane. In fact, association with the cell membrane inhibits replication initiation in *E. coli* (Boye et al., 2000; Donachie and Blakely, 2003; Katayama, 2001; Landoulsi et al., 1990; Ogden et al., 1988). As in *B. subtilis*, *E. coli* chromosomal origins are enriched in the membrane fraction of wild-type cells (Funnell, 1996). However, the binding of *E. coli* origins by
the membrane is not correlated with the ability to initiate replication. In \( \Delta \text{seqA} \) mutant cells, origins are no longer bound by the cell membrane (Shakibai et al., 1998; Slater et al., 1995), and yet these cells are fully capable of initiating replication. In fact, these cells over-initiate, demonstrating that the membrane-associated SeqA protein is a negative regulator of replication initiation (Bach and Skarstad, 2004; Boye et al., 1996; Lu et al., 1994; von Freiesleben et al., 1994). Thus, the SeqA-mediated sequestration of origins in the membrane is not necessary for executing replication initiation in \( E. \text{coli} \), but is critical for proper negative control over initiation. In conclusion, although the cell membrane is utilized during DNA replication in both \( B. \text{subtilis} \) and \( E. \text{coli} \), the membrane may be the obligate site of initiation in one bacterium, and the site of inhibition of excess initiation in another bacterium. It will be interesting to examine the contribution of the cell membrane to replication initiation and replication control in other bacterial species.

The subcellular location of replication restart in \( B. \text{subtilis} \). DNA replication initiation may occur at the membrane in \( B. \text{subtilis} \) because the essential initiation factor DnaB is found in the membrane fraction of cells (Rokop et al., 2004). In addition to being involved in initiation, DnaB is also involved in replication restart at stalled forks (Bruand et al., 1995; Bruand et al., 2001; Marsin et al., 2001; Velten et al., 2003). Therefore, replication restart may also occur at the membrane in \( B. \text{subtilis} \). Several experiments could be performed to address this issue. It would be interesting to investigate whether other proteins involved in replication restart, such as PriA, DnaI, and helicase, are found in the membrane fraction of cells. It would also be interesting to examine whether PriA, DnaB, and DnaD co-localize in cells. GFP fusions to PriA, DnaB, and DnaD show that PriA-GFP (Simmons, Grossman, and Walker, unpublished results), DnaB-GFP (Healy, Lemon, and Grossman, unpublished results), and DnaD-GFP (Rokop and
Grossman, unpublished results) form foci in some but not all cells. However the simultaneous localization of these proteins in the same cells has not been performed. It is certainly feasible that the localization pattern of DnaB seen using fluorescence microscopy reflects the action of DnaB at stalled replication forks, as DnaB does not colocalize with chromosomal origins, its other main site of action (Imai et al., 2000).

**Conclusions.** The *B. subtilis* proteins DnaB and DnaD function in replication restart, replication initiation, and replication control. Correspondingly, mutations in *dnaB* and *dnaD* lead to a wide range of phenotypes that reflect the roles of these multifunctional proteins. Many Gram-positive bacteria contain homologs of *dnaB* and *dnaD*, but do not contain homologs of many of the proteins involved in replication restart or of the proteins involved in replication control in the Gram-negative bacterium *E. coli*. Therefore it may be that using proteins that function in the initiation, restart, and control of replication is a conserved strategy in low G+C content Gram-positive bacteria, whereas using proteins that are each dedicated to one of these processes is a conserved strategy of the Gram-negative γ-proteobacteria. It will be interesting to characterize the proteins involved in replication initiation and restart in other bacterial phyla to examine how these critical cell cycle processes are executed and controlled in distantly related bacteria.
ACKNOWLEDGEMENTS

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REFERENCES


