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Ordered association of helicase loader proteins with the *Bacillus subtilis* origin of replication in vivo

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Summary

The essential proteins DnaB, DnaD, and DnaI of *Bacillus subtilis* are required for initiation, but not elongation, of DNA replication, and for replication restart at stalled forks. The interactions and functions of these proteins have largely been determined in vitro based on their roles in replication restart. During replication initiation in vivo, it is not known if these proteins, and the replication initiator DnaA, associate with *oriC* independently of each other by virtue of their DNA binding activities, as a (sub)complex like other loader proteins, or in a particular dependent order. We used temperature sensitive mutants or a conditional degradation system to inactivate each protein and test for association of the other proteins with *oriC* in vivo. We found that there was a clear order of stable association with *oriC*; DnaA, DnaD, DnaB, and finally DnaI-mediated loading of helicase. The loading of helicase via stable intermediates resembles that of eukaryotes and the established hierarchy provides several potential regulatory points. The general approach described here can be used to analyze assembly of other complexes.

Keywords

*Bacillus subtilis*; DnaB; DnaD; helicase; replication initiation

Introduction

Faithful replication and its regulation are crucial for genome stability. In many types of bacteria, replication initiates from a single chromosomal origin of replication, *oriC*, and proceeds bidirectionally around a circular chromosome. Initiation of replication involves binding of the replication initiator and, in bacteria, local melting of the origin DNA, loading of the replicative helicase at the origin leading to further DNA unwinding, and assembly of the rest of the DNA synthesis machinery to form the replisome.

The replication initiator DnaA is found in virtually all bacteria and is functionally analogous to ORC in eukaryotes and archaea. DnaA is an AAA+ ATPase and also functions as a transcription factor (reviewed in Messer, 2002; Kaguni, 2006). DnaA binds to specific sequences in *oriC* and needs to be in the ATP-bound form to cause melting of an AT-rich sequence necessary for replication initiation (Bramhill & Kornberg, 1988; Kornberg & Baker, 1992; Messer *et al.*, 2001; Speck & Messer, 2001).

After the action of the initiator protein the replicative helicase is loaded onto *oriC*. Different organisms use different mechanisms for loading helicase. In yeast, loading of the MCM helicase requires two accessory proteins: CDC6 and CDT1 (Sivaprasad *et al.*, 2006). *E. coli*
uses a single protein, DnaC (reviewed in Kornberg & Baker, 1992; Davey & O'Donnell, 2003), to load helicase at oriC and at stalled replication forks (replication restart). In contrast, B. subtilis uses three proteins, DnaB, DnaD, and DnaI, to load helicase at oriC and at stalled forks during replication restart (Bruand et al., 1995; Bruand et al., 2001; Marsin et al., 2001; Polard et al., 2002; Velten et al., 2003; Rokop et al., 2004; Bruand et al., 2005; Ioannou et al., 2006). DnaB, DnaD, and DnaI are conserved in low G+C Gram-positive bacteria and are required for replication initiation in some of these organisms (Bruck & O'Donnell, 2000; Li et al., 2004; Li et al., 2007), indicating that the mechanism of helicase loading used by B. subtilis is likely conserved. It is not known if the B. subtilis helicase loading proteins DnaD, DnaB, and DnaI form a complex or sub-complexes before association with oriC and assembly of helicase, if they associate with oriC independently of each other, possibly through their DNA binding activity, or if there is ordered dependent association of the individual proteins with oriC.

There are several protein-protein and protein-DNA interactions involving DnaA, DnaB, DnaD, and DnaI in B. subtilis. Some, perhaps all, of these interactions are important for replication initiation. In addition to binding specifically to sequences in oriC, DnaA interacts with DnaD in a yeast two-hybrid assay (Ishigo-Oka et al., 2001). DnaB and DnaD bind to dsDNA and ssDNA, and although specific binding sites have not been identified (Marsin et al., 2001; Turner et al., 2004; Zhang et al., 2005; Zhang et al., 2006), it is possible that they associate with oriC independently of other replication initiation proteins. DnaB and DnaD interact weakly (Marsin et al., 2001; Rokop et al., 2004; Bruand et al., 2005). DnaB is membrane-associated (Hoshino et al., 1987; Sueoka, 1998; Rokop et al., 2004) and interacts with helicase and the DnaI-helicase complex and facilitates helicase loading onto DNA (Velten et al., 2003). In addition, it is generally thought that replication initiation occurs at the inner surface of the cell membrane in both E. coli and B. subtilis (Garner et al., 1998; Sueoka, 1998).

Using assays for the association of DnaA, DnaB, DnaD, and helicase with oriC during replication initiation in vivo, we determined which initiation proteins were required for the association of the others. DnaA associates with oriC independently of any of the other replication initiation proteins. However, we found that association of DnaD with oriC was dependent on DnaA, but not on DnaB or DnaI, and association of DnaB with oriC was dependent on DnaA and DnaD, but not on DnaI. Finally, assembly of the helicase was dependent on DnaA, DnaD, DnaB, and DnaI. These results demonstrate a hierarchical order of assembly of replication initiation proteins at oriC. This ordered assembly provides several potential points for regulation.

Results

Rationale and approaches

We determined the dependence and order of association of replication initiation proteins during the early stages of assembly of the replisome at oriC in vivo. Our approach was to inactivate a specific replication initiation protein, using temperature sensitive replication initiation mutants or conditional degradation (see below), allow ongoing rounds of replication to finish and replication to arrest at the execution point of the inactivated protein, and to use a chromatin immunoprecipitation (ChIP) assay to determine which initiation proteins were associated with oriC.

Temperature sensitive mutations—We used temperature sensitive replication initiation mutants and inactivated the mutant protein by shift to non-permissive temperature. Some of these mutants are able to re-initiate replication in a relatively synchronous manner after shift-down to permissive temperature, allowing for a stronger signal in

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immunoprecipitations in comparison to an asynchronously growing population of cells. In these mutants, we also measured association of the replication proteins with the oriC region after resumption of replication. The use of temperature sensitive mutants took advantage of previously characterized mutations and allowed for the rapid inactivation and re-activation of some of the mutant proteins. However, each of the proteins participates in multiple interactions and functions and it is generally not known if all or only some of these interactions and functions are defective in the temperature sensitive mutants.

**Conditional degradation alleles**—In contrast to temperature sensitive mutations, degradation of a protein should eliminate all its interactions and functions. We constructed conditional degradation alleles of dnaD, dnaB, dnaI, and dnaC (helicase) and used these to degrade each protein. We inserted a modified E. coli ssrA (ssrA*) at the 3’-end of the target gene. Degradation of the gene product is induced by expression of the E. coli adaptor protein SspB that is required for efficient ClpXP-mediated degradation of the ssrA*-tagged protein (Griffith & Grossman, 2008). In each case, we tested the resulting strains for degradation of the tagged protein, effects on replication, and association of the other proteins with oriC.

**Degradation of helicase or helicase-loader components causes an arrest in replication**

Strains in which ssrA* was at the 3’ end of genes for helicase (dnaC) and the helicase loader components (dnaD, dnaB, and dnaI) had a conditional lethal phenotype upon expression of sspB. In all cases, the ssrA*-tagged gene was the only copy in the cell, was expressed from the native promotor, and supported cell growth. Typically, by 30 min after SspB induction, the amount of the ssrA*-tagged protein was greatly reduced (Fig. 1A–D). In addition, expression of sspB in strains containing the ssrA*-tagged alleles caused a marked decrease in plating efficiency (<1×10^{-3}), consistent with degradation of an essential protein.

Degradation of helicase (DnaC-ssrA*) caused a rapid decrease in the rate of replication. Shortly after induction of SspB, the rate of DNA synthesis was strongly reduced (Fig. 1E), as measured by incorporation of ^3H-thymidine into DNA. This reduction was comparable to that caused by addition of HPUrA (Fig. 1E), an inhibitor of the replicative DNA polymerase PolC (Brown, 1970). This decrease is also comparable to that caused by shifting temperature sensitive helicase mutants (dnaCts) to non-permissive temperature (Karamata & Gross, 1970; Sakamoto *et al.*, 1995). These findings indicate that degradation of the replicative helicase causes DNA synthesis to stop quickly, i.e., a “fast-stop” phenotype. They also indicate that even when helicase-ssrA* is part of the replisome, it is sensitive to SspB-mediated ClpXP degradation.

In contrast to the fast stop in DNA synthesis caused by degradation of helicase-ssrA*, degradation of the initiation proteins DnaD-ssrA*, DnaB-ssrA*, and DnaI-ssrA*, caused a slower, more gradual decrease in replication (a “slow stop” phenotype). Incorporation of ^3H-thymidine slowly decreased after induction of SspB (Fig. 1E), similar to the effects of shifting temperature sensitive dnaD, dnaB, and dnaI mutants to the non-permissive temperatures (Karamata & Gross, 1970; Li *et al.*, 2004; Li *et al.*, 2007). Together, our results show that expression of SspB causes rapid degradation of the ssrA*-tagged replication proteins, a defect in growth, and corresponding defects in replication. Since each ssrA*-tagged protein is degraded, all interactions and functions of that protein should be compromised shortly after expression of SspB. We have not used a dnaA-ssrA* allele. Instead, we have used the temperature sensitive dnaA1ts (dnaA1ts) mutation. The DnaA1 mutant protein is rapidly degraded at non-permissive temperature causing a block in replication initiation (Moriya *et al.*, 1990). It is not known if any of the other replication initiation proteins associate with the oriC region after loss of DnaA.
Association of replication initiation proteins DnaD and DnaB and helicase with oriC depends on dnaA

We found that association of helicase and the replication initiation proteins DnaD and DnaB with oriC depends on DnaA. We shifted the dnaAts mutant to non-permissive temperature (52°C) for 60 min and measured association of DnaA, DnaD, DnaB, and helicase (DnaC) with oriC using ChIP. There was no significant association of any of these proteins with oriC under these conditions (Fig. 2). We were not able to reliably detect association of DnaI with oriC under our experimental conditions, likely because this association is transient (Ioannou et al., 2006).

We also tested association of the replication proteins with oriC after release of the replication block, that is, after return of the dnaAts mutant to permissive temperature. Since the DnaA1 mutant protein is unstable (Moriya et al., 1990), re-initiation of replication after shift-down to permissive temperature is asynchronous due to the differences in accumulation of DnaA and concomitant replication initiation in individual cells. This asynchrony makes it difficult to detect association of specific replication proteins at oriC. To restrict movement of the replisome away from the origin and trap the replication complex near oriC after return to the permissive temperature, we simultaneously added the replication inhibitor HPUra (Brown, 1970). We found that there was significant association of DnaD, DnaB, and helicase with oriC by 15 minutes after shift-down to permissive temperature in the presence of HPUra (Fig. 2). We also observed origin-association of the single stranded DNA binding protein Ssb, using an Ssb-myc fusion (Fig. 2), indicative of DNA unwinding at oriC.

Together, the results indicate that association of helicase and the replication initiation proteins DnaD and DnaB with oriC depends on dnaA. Association of DnaA with oriC in vivo is independent of each of the other replication initiation proteins (Goranov et al., 2005; Breier & Grossman, 2009).

Association of DnaD with oriC does not require dnaB or dnaI

DnaD binds dsDNA and ssDNA in vitro in the absence of other proteins (Marsin et al., 2001; Turner et al., 2004; Zhang et al., 2005; Zhang et al., 2006). We found that in vivo, specific association of DnaD with oriC was dependent on DnaA (Fig. 2), but not on DnaB or DnaI (Fig. 3A, B). DnaD was associated with oriC in the dnaB134ts (dnaBts) and dnaI2ts (dnaIts) mutants after 60 min at the non-permissive temperature (Fig. 3A). We also monitored association of DnaD with oriC in the dnaB-ssrA* and dnaI-ssrA* degradation mutants. Before induction of SspB, there was some association of DnaD with oriC (Fig. 3B). This level is indicative of the amount of DnaD at oriC in a population of asynchronously growing cells. Degradation of DnaB-ssrA* or DnaI-ssrA* prevented initiation of replication while ongoing replication finished (Fig. 1B), essentially causing all cells to arrest at the start of replication. Under these conditions, when most cells are poised to initiate a new round of replication, association of DnaD with oriC increased relative to that in the asynchronous population (Fig. 3B), indicating that association of DnaD with oriC was independent of DnaB and DnaI. The enrichment of oriC in the ChIP signal was not due to a cross-reacting protein since there was no significant enrichment after degradation of DnaD-ssrA* (Fig. 3B) or in the dnaD23ts (dnaDts) mutant at non-permissive temperature (Fig. 3A).

The lack of association of DnaD with oriC in the dnaDts mutant was rapidly reversible. Two minutes after shift-down of the dnaDts mutant cells to permissive temperature, there was association of DnaD with oriC (Fig. 3A). Two minutes after shift-down of the dnaBts and dnaIts mutants to permissive temperature, DnaD remained associated with oriC, at a level similar to that after shift-down in the dnaDts mutant. Together, these results indicate that association of DnaD with oriC requires DnaA but not DnaB or DnaI.

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Association of DnaB with oriC requires dnaD, but not dnaI

DnaB is part of the helicase loader and interacts with DnaD (Velten et al., 2003; Rokop et al., 2004; Bruand et al., 2005). DnaB also binds DNA in the absence of the other initiation proteins (Marsin et al., 2001; Zhang et al., 2005; Zhang et al., 2006), and is required for the enrichment of the origin region in membrane fractions (Hoshino et al., 1987; Sueoka, 1998). We found that association of DnaB with oriC was dependent on DnaA (Fig. 2) and DnaD, but not DnaI (Fig. 3C, D). There was little or no association of DnaB with oriC after 60 min at non-permissive temperature in the dnaDts mutant (Fig. 3C). In contrast, there was significant association of DnaB with the oriC region in the dnaIts mutant (Fig. 3C). Similar results were obtained with the dnaD-ssrA* and dnaI-ssrA* mutants. During exponential growth in the asynchronous population, before induction of SspB, there was low but detectable association of DnaB with oriC (Fig. 3D). Sixty minutes after induction of SspB to induce degradation of the DnaD-ssrA*, there was little or no association of DnaB with oriC (Fig. 3D). In contrast, DnaB was associated with oriC following degradation of DnaI-ssrA* (Fig. 3D). The enrichment of oriC in the ChIP signal was most likely due to immunoprecipitation of DnaB and not a cross-reacting protein since the ChIP signal was greatly reduced after degradation of DnaB-ssrA* (Fig. 3D) and in the dnaBts mutant at non-permissive temperature (Fig. 3C).

The lack of association of DnaB with oriC at the non-permissive temperature in the dnaDts and dnaBts mutants was rapidly reversible. Two minutes after shift-down of the dnaDts or dnaBts mutant to permissive temperature, there was significant association of DnaB with oriC (Fig. 3C). Two minutes after shift-down of the dnaIts to permissive temperature, DnaB remained associated with oriC (Fig. 3C). Together, these results indicate that association of DnaB with oriC requires DnaA and DnaD, but not DnaI.

Requirements for association of helicase with oriC

We determined which proteins were required for the association of helicase with oriC. We found that helicase was not detectably associated with oriC in the dnaAts (Fig. 2), dnaBts, dnaDts or dnaIts mutants after incubation at the non-permissive temperature (Fig. 3E), consistent with previous studies of assembly of helicase onto DNA (Bruand et al., 1995; Imai et al., 2000; Bruand et al., 2001; Velten et al., 2003; Rokop et al., 2004; Bruand et al., 2005; Ioannou et al., 2006). During exponential growth in an asynchronous population, we observed little if any association of helicase with oriC (Fig. 3F), as expected for a population of cells at different stages of the replication cycle. Sixty minutes after induction of SspB to induce degradation of the DnaD-ssrA*, DnaB-ssrA*, or DnaI-ssrA*, and to synchronize replication at the initiation stage, there was little or no detectable association of helicase with oriC (Fig. 3F). Together, these results indicate that DnaA, DnaD, DnaB, and DnaI are needed for loading of the replicative helicase at oriC and that the DnaIts and DnaI-ssrA* proteins are inactivated under non-permissive conditions.

In contrast to the lack of helicase association with oriC at non-permissive temperature, there was significant association after release of the replication initiation block. Two minutes after shift-down of the dnaDts and dnaBts mutants to permissive temperature, there was >20-fold enrichment of the oriC region in the immunoprecipitates of helicase (Fig. 3E). Enrichment was lower in the dnaIts mutant after shift-down, probably because of the longer time for replication to initiate and lack of synchrony in this mutant.
Discussion

Ordered assembly of a helicase-loading complex at oriC

*B. subtilis* has four essential gene products that are needed for replication initiation but not replication elongation: DnaA, DnaB, DnaD and DnaI. Other low-GC Gram-positive organisms appear to use the same four essential products (Bruck & O'Donnell, 2000; Li et al., 2004; Li et al., 2007), and it is therefore believed that the mechanism of replication initiation is conserved. Our work describes a general approach to determine ordered dependence of assembly of a complex in vivo. Applying this approach, we found that there is an ordered hierarchy in the assembly of the replication initiation proteins. DnaA binds to oriC independently of the other initiation proteins (Goranov et al., 2005; Breier & Grossman, 2009). DnaD association with oriC depends on DnaA, but does not require DnaB or DnaI. DnaB association with oriC requires DnaA and DnaD, but not DnaI. Finally, assembly of the helicase depends on all four replication initiation proteins DnaA, DnaD, DnaB, and DnaI. Thus, the order of assembly is DnaA, DnaD, DnaB, then DnaI-helicase.

In addition to their roles in replication initiation at oriC, DnaD, DnaB, and DnaI are needed for replication restart at stalled forks. Much of what is known about DnaD, DnaB, and DnaI is from elegant analyses of replication restart in vitro (Bruand et al., 2001; Marsin et al., 2001; Polard et al., 2002). However, in contrast to replication initiation, replication restart requires PriA and not DnaA, and can occur wherever a replication fork stalls instead of just at oriC. Despite these differences, our findings show that the hierarchal assembly in vivo at oriC is largely similar to the in vitro findings for replication restart.

Conditional degradation system

We used a conditional degradation system that relies on the highly conserved protease ClpXP and its ability to interact with the adaptor protein SspB that facilitates degradation of certain substrates (McGinness et al., 2006; Griffith & Grossman, 2008). The degradation tag that is useful in *B. subtilis*, ssrA*, was designed to be relatively stable and to require the adaptor protein SspB from *E. coli* to stimulate rapid degradation (Griffith & Grossman, 2008). Most proteins we have tagged with ssrA* have been functional and are efficiently degraded (e.g., Fig. 1 and Griffith & Grossman, 2008). We anticipate that this system will be broadly applicable for the analysis of many different biological processes and especially for dissecting assembly pathways involving essential proteins. ClpXP orthologs are found in most bacteria, mitochondria and chloroplasts and because of this conservation, the system has the potential to be extended to other organisms.

Model for sequential assembly of a helicase-loading complex

Based on the data presented here, in combination with previous work, we propose the following model for the assembly of the helicase-loading complex in vivo at oriC in *B. subtilis*, and other Gram-positives (Fig. 4). First, DnaA binds to oriC. Based on in vitro work, this binding event induces local melting of an AT-rich sequence in oriC and melting depends on the presence of DnaA-ATP (Bramhill & Kornberg, 1988; Kornberg & Baker, 1992). Next, DnaD associates with oriC. This association could be by virtue of direct interaction with DnaA, via interaction with the ssDNA or other non-B-type DNA generated by DnaA, or both. Next, DnaB associates with oriC, probably through a direct interaction with DnaD. Because DnaB is associated with the membrane and is required for enrichment of oriC in membrane fractions of cells (Hoshino et al., 1987; Sueoka, 1998), this interaction probably brings the origin region and its associated proteins to the inner surface of the cell membrane. Once this complex is assembled, we propose that a complex of DnaI-helicase interacts with DnaB and the ssDNA in the melted AT-rich region and helicase is assembled into a hexamer encircling the ssDNA.
Loading of helicase via hierarchical and stable association of proteins is also observed in yeast (Sivaprasad et al., 2006). Loading of the MCM/helicase complex requires two accessory proteins, Cdc6 and Cdt1, in addition to the origin recognition complex ORC. Association of Cdc6 with the origin depends on ORC, but does not require Cdt1 (Bowers et al., 2004; Speck et al., 2005; Randell et al., 2006). Though Cdt1 interacts with ORC directly, it requires Cdc6 for its recruitment to the origin in vivo (Chen et al., 2007). Thus, stable hierarchical assembly during helicase loading seems to be conserved in prokaryotes and eukaryotes.

Temporal and spatial regulation of replication initiation

Replication initiation is highly regulated. In contrast to B. subtilis with four essential replication initiation proteins, E. coli has only two, the initiator DnaA (Marszalek & Kaguni, 1994; Mott et al., 2008) and the E. coli helicase loader DnaC (Baker et al., 1986; Baker et al., 1987; Kornberg & Baker, 1992). Most of the known mechanisms for controlling replication initiation in E. coli affect DnaA and its interactions with oriC. For example, there are mechanisms for sequestration of the oriC region for a period of time after replication initiates (Lu et al., 1994; von Freiesleben et al., 1994), mechanisms for modulating interaction of DnaA with the oriC (Ishida et al., 2004; Keyamura et al., 2007), and mechanisms that couple nucleotide hydrolysis with ongoing replication elongation (Kato & Katayama, 2001). However, the proteins known to modulate replication initiation in E. coli are not widely conserved and are not found in Gram-positive bacteria.

In B. subtilis, DnaA and its interactions with oriC are also likely targets for controlling replication initiation. The level of DnaA is regulated by transcriptional autoregulation (Ogura et al., 2001), like in E. coli (Atlung et al., 1985; Braun et al., 1985). In addition, two different non-essential proteins, Soj and YabA, modulate replication initiation and interact with DnaA (Noirot-Gros et al., 2002; Noirot-Gros et al., 2006; Murray & Errington, 2008), although the mechanisms by which these regulators function are not known.

The existence of three additional replication initiation proteins that assemble in a hierarchical manner at oriC provides additional potential regulatory points. Although the mechanisms of action of regulators of replication initiation in B. subtilis and other Gram-positives are not known, we anticipate that one or more will affect specific steps in the hierarchical assembly of the initiation complex. Consistent with this hypothesis, we have found that a mutation in dnaB (dnaBS371P, also known as dnaB75) affects the frequency of replication initiation in vivo (Rokop et al., 2004). This mutation was isolated as a suppressor of a dnaDts mutation (Rokop et al., 2004; Bruand et al., 2005), and also suppresses the need for priA in replication restart (Bruand et al., 2001). The mutant DnaB protein has increased interaction with DnaD and DnaD is enriched in membrane fractions of cells (Rokop et al., 2004). Moreover, interaction between DnaB and the DnaI-helicase complex stimulates translocase and helicase activities (Velten et al., 2003), whereas DnaD destabilizes the complex of DnaI and helicase in vitro (Turner et al., 2004). Although not yet known, we suspect that there are regulators that normally modulate the interaction between DnaD and DnaB affecting the helicase loading process.

One important consequence of the ordered association of helicase loading proteins described here is to ensure spatial regulation of replication initiation. Replication initiation is thought to take place at the inner face of the membrane. In E. coli, DnaA interacts with the membrane directly (Garner et al., 1998). In addition, E. coli DnaA recruits helicase directly to the origin (Marszalek & Kaguni, 1994; Mott et al., 2008), where it is loaded by a single loader protein. In B. subtilis no evidence exists for a direct interaction between DnaA and helicase or DnaA and the membrane. Yet, like in E. coli, replication initiation is thought to occur at the inner face of the membrane (Winston & Sueoka, 1980; Watabe & Forough,
Enrichment of \textit{B. subtilis} oriC in membrane fractions depends on DnaB (Hoshino et al., 1987; Sueoka, 1998). DnaD bridges the oriC-DnaA complex and the DnaB-membrane complex, which in turn is responsible for bringing in the DnaI–helicase complex. The use of multiple proteins for helicase loading in vivo, and potentially the regulation of their interactions, allows for both temporal and spatial control and provides a mechanism by which oriC, the membrane, and the replicative helicase are coordinately brought together for replication initiation.

**Experimental Procedures**

**Media and growth conditions**

Cells were grown in LB, or defined minimal medium with 0.1% glutamate, supplemented with required amino acids (typically trp and phe), 1% glucose as a carbon source, and 1mM IPTG as inducer as necessary. For strains carrying a xylose-inducible construct (Pxy), glucose was replaced with arabinose. Xylose was added to 1% to induce expression from Pxy, as indicated. Single crossover constructs were maintained under antibiotic selection throughout the experiments.

**Strains**

\textit{B. subtilis} strains used are listed in Table 1. All are isogenic and contain the \textit{trpC2} and \textit{pheA1} alleles. \textit{dnaA1}, \textit{dnaB134}, \textit{dnaD23} and \textit{dnaI2} (Karamata & Gross, 1970; Moriya et al., 1990; Bruand et al., 2001; Bruand et al., 2005) are temperature sensitive alleles that prevent replication initiation at the non-permissive temperature. The transposon insertions Tn\text{917}\Omega\text{HU163}, Tn\text{917}\Omega\text{HU151}, and \textit{zhr83::Tn917} are linked to \textit{dnaA}, \textit{dnaD}, and the \textit{dnaB-dnaI} operon, respectively.

An Ssb-myc strain was constructed by cloning a PCR product carrying the operon promoter (P\textit{rpsF}), the first gene in the operon, \textit{rpsF}, and \textit{ssb} from an \textit{ssb-gfp} plasmid (Berkmen & Grossman, 2006) and fused in-frame to a linker and a 3xmyc tag and cloned into pSac-Kan (Middleton & Hofmeister, 2004). This construct was integrated into the \textit{B. subtilis} chromosome at \textit{sacA} by a double crossover.

\textit{ssrA}* is a modified \textit{Escherichia coli} \textit{ssrA}-tag that allows for user-controlled degradation of the tagged protein by ClpXP when a heterologous adapter protein (SspB) is expressed (Griffith & Grossman, 2008). PCR products carrying a C-terminal fragment of \textit{dnaC}, \textit{dnaD} and \textit{dnaI} were cloned into pKG1268 (Griffith & Grossman, 2008) to give plasmids pGCS-dnaC, pGCS-dnaD and pGCS-dnaI. For \textit{dnaB}, a similar product was cloned into p1292, a derivative of pMutin2 (Vagner et al., 1998) deleted for lacZ and carrying an \textit{ssrA}* tag, resulting in p1292-dnaB. The plasmids were integrated by single crossover after natural transformation into wild type \textit{B. subtilis} (AG174). Chromosomal DNA from these strains was used to introduce the constructs into strains carrying loci for the controlled expression of SspB, and transformants were screened for a growth defect on LB plates with 1mM IPTG (\textit{DnaC}, \textit{DnaD}, \textit{DnaI}) or 1% xylose (\textit{DnaB}). Although growth rates of tagged and untagged strains were within 10–15% of each other, we found that cultures of \textit{dnaD-ssrA}* and to lesser extent \textit{dnaI-ssrA}* mutants accumulated suppressors that were resistant to IPTG. For that reason, experiments were carried out using fresh transformants. Strains containing ts alleles were routinely checked for temperature sensitivity. Expression of SspB in cells without tags caused no detectable phenotypes (Griffith & Grossman, 2008).

**Antibodies and chromatin immunoprecipitation (ChIP)**

The presence of various proteins in cell lysates was assayed by Western blotting as described (Griffith & Grossman, 2008). Chromatin immunoprecipitation of DNA bound to
the various proteins was done essentially as described (Goranov et al., 2005), except that DNA was precipitated in the presence of glycogen (20 µg) as a carrier. For DnaB, DnaC, and DnaD, polyclonal antibodies from rabbit were used (Covance). For Ssb-myc monoclonal anti-cMyc antibodies (Zymed) were used.

We chose to use polyclonal antibodies as we found that epitope-tagged replication proteins, while functional, were not completely wild type and sometimes had synthetic phenotypes with other replication mutations. In addition, the use of polyclonal antibodies allowed us to immunoprecipitate multiple proteins from the same cell extracts, helping to minimize experimental variation. We verified that the antibodies recognized the protein of interest on Western blots comparing the mobilities of untagged and tagged protein and comparing signals before and after degradation of ssrA*-tagged proteins. We also verified that following formaldehyde-mediated crosslinking, each antiserum was able to deplete the protein from a cell extract under ChIP conditions. Furthermore, and most importantly, for all of the replication initiation proteins, association of the protein of interest with oriC was severely decreased or eliminated when the protein was inactivated or degraded (see above).

**Quantitative real time PCR (qRT-PCR)**

qRT-PCR was performed on a Roche LightCycler 480 II. 2 µl samples of immunoprecipitated DNA were analyzed in triplicate in a 20 µl reaction volume that contained Sybr green, using primers designed for oriC (5’-GGAGGACGTTATCATAGA-3’ and 5’-TAGGGCTGTGATTTGTG-3’) or the yabM locus (5’-TAGGGCTTAAACCGCATTTG-3’ and 5’-GACAGCATGACCGCAAATAC-3’) for which no binding was expected (Breier & Grossman, 2009). Signals were analyzed using the LightCycler 480 SW 1.5 software (Roche), according to the manufacturer (Advanced RelQuant; 2nd derivative of Max, using Median Cp for calculation). Signals were normalized against standard curves of a dilution series of total chromosomal DNA obtained from dnaB134ts (KPL69) arrested cells.

**Measurement of DNA replication**

Replication rates were determined by pulse-labeling exponentially growing cells with $^3$H-thymidine (70–90Ci/mmol; 1.0 mCi/ml; Perkin-Elmer; 7 µl with 200 µl of culture) for 1 min at 37°C essentially as described (Wang et al., 2007). Trichloroacetic acid-precipitable counts were determined and background was subtracted.

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**References**


Figure 1. Degradation of replication initiation proteins leads to an arrest in replication
Cells containing the indicated ssrA* fusion were grown in defined minimal medium and expression of the adaptor SspB was induced with either IPTG (from Pspank-sspB) or xylose (from Pxyl-sspB) at time=0. Samples were taken at the indicated times for determination of levels of the indicated proteins (A–D) or DNA synthesis (E).
A–D. Western blot analysis of cell lysates obtained from strains for conditional degradation. Blots were probed for the indicated replication protein and the adaptor SspB. A. DnaD-ssrA* (WKS265); B. DnaB-ssrA* (WKS649); C. DnaI-ssrA* (WKS738); D. DnaC-ssrA* (WKS66).
E. Replication rates were set at 100% for each untreated sample. Relative rate of incorporation of 3H-thymidine into TCA-precipitable counts is plotted as a function of time after expression of sspB or addition of HPUra to block replication. Error bars (standard error of the mean) fall within the symbols of the graphs and are omitted for clarity (n=3).
Figure 2. Association of DnaD, DnaB, DnaC (helicase) and Ssb depend on DnaA

dnaAts mutant cells (WKS588) were grown at permissive temperature (30°C) and shifted to
cold-temperature (52°C) for 1hr to inactivate DnaA and allow most ongoing rounds of replication to finish (light grey bars). Cells were then shifted to permissive temperature (35°C) in the presence of HPUra (dark grey bars) to allow replication to re-initiate and to trap initiation complexes at oriC. Indicated proteins were immunoprecipitated (IP’ed) from the cell lysates after crosslinking with formaldehyde. Error bars indicate the standard error of the mean (n=3). Note the different scale for the helicase IP on the right axis.
Figure 3. Order of dependence of DnaD, DnaB, and DnaC (helicase) for association with oriC

The relative enrichment of oriC after crosslinking and immunoprecipitation of the indicated proteins, DnaD (A, B), DnaB (C, D), and DnaC (E, F), was determined. Note the different scales on the y-axes. The indicated protein (x-axis) was inactivated by shift to non-permissive temperature (A, C, E) or expression of SspB to induce degradation of ssrA*-tagged proteins (B, D, F).

A, C, E. Temperature sensitive mutants included: dnaDts (KPL73), dnaBts (KPL69), and dnaIts (KPL147). Cells were grown at the permissive temperature, shifted to non-permissive (high) temperature (indicated) to inactive the mutant protein and allow ongoing rounds of replication to finish, and then shifted back to low temperature to allow replication to re-initiate. Samples were taken one hour after shift to high temperature (light gray bars) and 2 minutes after shift-down to permissive temperature (dark gray bars).

B, D, F. Conditional degradation mutants included: DnaD-ssrA* (WKS265), DnaB-ssrA* (WKS649), and DnaI-ssrA* (WKS738). Samples were taken for ChIP analyses during asynchronous exponential growth under permissive conditions, i.e., in the absence of inducer and little or no expression of SspB (black bars) and 1 hour after addition of inducer (IPTG or xylose) to induce expression of SspB and cause degradation of the indicated ssrA*-tagged protein. Error bars indicate the standard error of the mean (n=3).
Figure 4. Model for the association of helicase and helicase loader with oriC in vivo
The inner surface of the cell membrane is depicted as a gray bar across the top of each panel. DNA is depicted as a double helix with oriC bound to DnaA. Shapes with letters represent the proteins: A=DnaA (initiator), B=DnaB, D=DnaD, I=DnaI (loader ATPase), C=DnaC (helicase). Established protein-protein and protein-DNA interactions (see text) are also shown, except for self-interactions.
**Table 1**

*B. subtilis* strains used.

<table>
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<tr>
<th>Strain</th>
<th>Relevant genotype (reference)</th>
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<td>KG844</td>
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