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DNA Repair and Genome Maintenance in *Bacillus subtilis*

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INTRODUCTION

All cells must accurately copy and maintain their DNA to ensure faithful transmission of their genetic material to the next generation. Organisms ranging from bacteria to humans contain a series of DNA repair pathways dedicated to the specific recognition and repair of the myriad of DNA damage or base-pairing errors that can occur throughout the lifetime of a cell. In mammals, it has been estimated that every cell is subject to >15,000 lesions per 24-h period (128, 212). Most of these lesions are hypothesized to arise from endogenous sources, such as reactive byproducts of normal cellular metabolism (128). In higher eukaryotic systems, failures in DNA repair are often attributed to numerous disease states and/or cell death (e.g., see reference 128). In bacterial cells, DNA damage and mutation accumulation can reduce cell fitness and potentially affect viability (128, 131). Conversely, mutagenesis also provides the material for evolution, as base pair substitutions may confer a selective advantage to bacterial cells vulnerable to a changing environment (e.g., see reference 481). Therefore, transient increases in mutagenesis must be balanced carefully with high-fidelity repair to ensure genome preservation while providing the opportunity for genetic diversity (for

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reviews, see references 434 to 438). The conservation of DNA repair pathways from bacteria to humans is often very impressive; such conservation has allowed for experimentally tractable bacteria to provide important mechanistic insights into processes critical for genome maintenance in more complex systems (for a review, see reference 128). To date, the DNA repair and mutagenesis pathways in *Escherichia coli* are the best understood for a bacterial system, and this information has led to the identification of several founding members of DNA repair and damage tolerance superfamilies that show exquisite conservation across biology (e.g., see references 8, 78, 118, 119, 128, 231, and 296).

Efforts in genome sequencing and evolution have estimated that Gram-positive and Gram-negative bacteria diverged over a billion years ago (e.g., see references 67, 298, and 303). Such a long separation has allowed for many DNA repair processes in Gram-negative and Gram-positive bacteria to diverge, evolving substantial differences in both their molecular mechanisms and modes of regulation. Over the last decade, it has become increasingly clear that the DNA repair pathways of many Gram-positive bacteria can be different from those described for *E. coli.* In some cases, entire pathways exist in Gram-positive bacteria that are completely absent from the prototypical Gram-negative bacterium *E. coli*. Below, we review and discuss several pathways that are critical for genome maintenance in the Gram-positive bacterium *Bacillus subtilis*. We discuss many DNA repair, DNA damage tolerance, and DNA damage checkpoints that maintain genome integrity during vegetative growth and during the developmental program of sporulation. We also review the similarities of DNA repair and DNA replication pathways in several other bacteria, and we relate these findings to what is known for better-characterized bacterial systems.

THE SOS RESPONSE

The SOS response is a transcriptional circuit that is activated upon DNA damage, replication fork stress, and many other stresses that affect genome integrity (for a review, see references 128 and 385) (Fig. 1). The SOS response of *E. coli* has been very well characterized (for a review, see reference 385). RecA bound to single-stranded DNA (ssDNA) activates the response, whereas LexA, a transcriptional repressor, negatively regulates SOS induction. Following DNA damage from endogenous or exogenous sources, ssDNA is formed during repair or replication of damaged DNA templates. RecA binds ssDNA and polymerizes, forming a nucleoprotein filament which activates LexA for self-cleavage, inactivating LexA from binding to and repressing the transcription of genes under its control. In *E. coli*, ~56 genes are repressed by LexA, and these genes comprise the SOS regulon (for a review, see reference 385) (Table 1).

A responsive gene expression system in *B. subtilis* was suggested when it was found that lysogenic strains had reduced DNA transformation when grown to the competent state for DNA transformation (469). It was proposed that prophage expression was induced in competent cells by a process analogous to the *E. coli* SOS response (464, 465). It should be noted that prophages and several other genetic elements are often induced by DNA damage, because RecA-ssDNA will often inactivate a transcriptional repressor or, in some cases, LexA can directly repress expression of some prophage genes (41, 116, 117, 218, 320, 321). Some of the first direct evidence showing that a DNA damage-inducible system was present in *B. subtilis* came from experiments

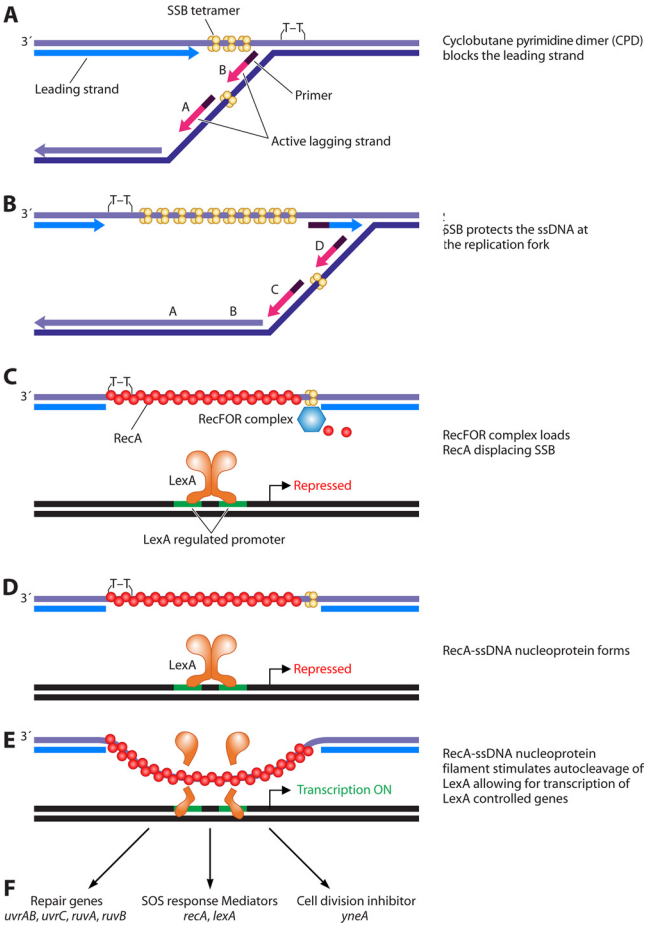

FIG 1 Model for activation of the SOS response in *B. subtilis*. (A) In this model, UV damage has created a cyclobutane pyrimidine dimer (CPD) in the leading strand template, creating a daughter strand gap after repriming and continued replication beyond the lesion. (B) SSB binds to the daughter strand gap, preserving the integrity of the DNA. (C) Recombinase mediator proteins RecF, RecO, and RecR, and possibly other accessory factors, stimulate RecA loading at the gap region as SSB is displaced from the site. (D) RecA forms a nucleoprotein filament on ssDNA. (E) The RecA-ssDNA nucleoprotein filament then interacts with LexA, activating its latent protease activity and resulting in autocleavage of LexA. Following autocleavage and inactivation of LexA, SOS gene transcription is activated, and a global transcriptional response is induced. (F) SOS-dependent changes in gene expression help *B. subtilis* to survive DNA damage by upregulating DNA repair proteins, preventing the bacterium from undergoing cell division, and finally increasing the regulatory products RecA and LexA to reset the system after repair is completed. (Adapted from reference 385.)

using random promoterless *lacZ* insertions into the *B. subtilis* chromosome to determine if DNA damage resulted in increased expression of damage-inducible (*din*) genes (134, 224, 226, 227, 468). Fifteen genes were identified as *din* genes, demonstrating that DNA damage caused by UV, mitomycin C, and ethyl methanesulfonate (EMS) resulted in an increase in gene expression (224). These observations showed the presence of an SOS-like system in *B. subtilis*. It should be noted that the SOS system of *B. subtilis* was first defined as SOB, for SOS-like system of *B. subtilis*, because it differed from the SOS response in *E. coli* (for a review, see reference 466).

The major differences between the SOS-like system of *B. subtilis* and the SOS system of *E. coli* are in the phenotypes that result

TABLE 1 LexA-regulated genes in *B. subtilis* and *E. coli*[a]

B. subtilis gene	Function or description (reference)	*E. coli* gene	Function or description (reference)
aprX	Alkaline serine protease, activity identified in culture medium (184)	*polB*	DNA polymerase involved in repair (325)
ybaK	Predicted protein of 147 amino acids; conserved domain of unknown function (DUF2521) found in *Bacillus*	*hokE*	Killing protein showing similarity to plasmid toxins (305)
cwlD	Germination, *N*-acetylmuramoyl-L-alanine amidase (cell wall hydrolase) (365)	*dinG*	DNA helicase with 5′-to-3′ polarity (431)
dinB	Nuclease inhibitor of 172 amino acids; metal binding and conserved with mycothiol maleylpyruvate isomerase N-terminal domain	*ftsK*	Chromosome segregation after septation (104)
dltA	D-Alanyl-D-alanine carrier protein ligase used for incorporation of alanine into lipoteichoic acid (162)	*dinI*	Functions to stabilize RecA-ssDNA filaments (432)
dltB	Involved in lipoteichoic acid biosynthesis (162)	*yebG*	Putative 96-amino-acid protein of the YebG superfamily, described as DNA damage-inducible small proteins of unknown function
dltD	Involved in lipoteichoic acid biosynthesis; possible carrier protein for D-alanine (162)	*sbmC*	Inhibitor of DNA gyrase (56)
dnaE	DNA polymerase III (alpha subunit); essential protein involved in primer maturation on the lagging strand (99, 352)	*recN*	SMC-like protein involved in recombination (255)
lexA	**Transcriptional repressor of the SOS regulon (264)**	***lexA***	**Transcriptional repressor of SOS regulon (217)**
pcrA	Essential ATP-dependent DNA helicase, shows similarity to *E. coli* Rep and UvrD (307, 308)	*uvrD*	DNA helicase II, involved in NER and MMR (293)
ligA	DNA ligase (183)	*molR*	Involved in molybdate transport (198)
yerH	Predicted protein of 396 amino acids, similar to putative lipoproteins	*dinF*	Putative 459-amino-acid protein predicted to be a multidrug efflux transporter
recA	**Required for homologous recombination and SOS induction (230)**	***recA***	**Required for homologous recombination and SOS induction (83)**
parE	ATP-binding subunit of DNA topoisomerase IV (16)	*dinQ*	Predicted 228-amino-acid protein of the SpoU methylase superfamily involved in rRNA methylation
parC	DNA binding and cleavage subunit of DNA topoisomerase IV (16)	*ysdAB*	Toxic protein with expression induced under stress conditions
ruvA	**DNA binding activity of the Holliday junction helicase RuvAB**	***ruvA***	**DNA binding activity of the Holliday junction helicase RuvAB (417, 418)**
ruvB	**ATP binding protein, ATPase, and helicase motor for Holliday junction helicase RuvAB**	***ruvB***	**ATP binding protein, ATPase, and helicase motor for Holliday junction helicase RuvAB (417, 418)**
queA	*S*-Adenosylmethionine tRNA ribosyltransferase-isomerase (142)	*ydjM*	Predicted to be an inner membrane protein of unknown function
tgt	Queuine tRNA-ribosyltransferase; also known as tRNA-guanine transglycosylase (326)	*dinS*	Similar to transposase; contains a helix-turn-helix motif and integrase core domain
tagC	Putative polyglycerol phosphate involved in teichoic acid biosynthesis	RmuC (*yigN*)	Contains domains conserved with RmuC superfamily of DNA recombination proteins, and the N-terminal domain is conserved with SMC proteins; this protein is predicted to function as a nuclease
uvrA	**Excinuclease ABC (subunit A)**	***uvrA***	**Excinuclease ABC (subunit A)**
uvrB	**Excinuclease ABC (subunit B)**	***uvrB***	**Excinuclease ABC (subunit B)**
uvrC	**Excinuclease ABC (subunit C)**	***ydjQ***	**UvrC ortholog (272)**
yolD	SPβ prophage protein of unknown function; 110 amino acids	*ydfE*	Predicted 306-amino-acid protein of unknown function showing similarity to phage-encoded exonucleases
uvrX	SPβ protein that shares similarity to UmuC and the Y family DNA polymerases (193)	*yjiW*	Predicted 132-amino-acid protein showing similarity to SymE toxin superfamily of endoribonucleases
xkdA	PBSX prophage, shows similarity to *yqaB* from the skin element; contains H-E-X-X-H motif suggesting Zn^{2+}-dependent peptidase activity	*borD*	Similar to prophage protein expressed during lysogeny
ydiO	Putative DNA methyltransferase, cytosine-specific	*ybiN*	Adenine-N^6-methyltransferase (366)
ydiP	Putative DNA methyltransferase subunit, cytosine specific	*grxA*	Glutoredoxin; functions as a hydrogen donor for ribonucleotide reductase (316)
yhaN (*sbcE*)	AAA^+ SMC-like protein involved in recombination (189)	*yccF*	Predicted 148-amino-acid protein of unknown function showing similarity to domain of unknown function 307 (DUF307) for small putative membrane proteins
yhaM	3′-5′ exoribonuclease; contains a C-terminal ND domain found in metal-dependent phosphohydrolases (189)	*ymfD*	Predicted 221-amino-acid protein showing similarity to SAM-dependent methyltransferases
yhaZ	Very similar to DNA alkylation repair COG4335 superfamily of proteins	*ymfE*	Predicted 234-amino-acid protein showing similarity to inner membrane proteins
yhjD	Predicted 120-amino-acid protein of unknown function with no conserved domains identified	*lit*	Protease for EF-Tu (133)

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TABLE 1 (Continued)

B. subtilis gene	Function or description (reference)	*E. coli* gene	Function or description (reference)
yhjC	Predicted 66-amino-acid protein similar to short bacterial proteins of unknown function	*intE*	Shows similarity to a prophage integrase
yjhB	Similar to sodium, proline symporter; member of the sodium solute symporter superfamily	*ymfG*	Putative 78-amino-acid protein predicated to be an e14 prophage excisionase (328)
yneA	**DNA damage-dependent inhibitor of cell division (177, 268)**	***sulA***	**Inhibitor of cell division, blocks FtsZ polymerization (132)**
yneB	Putative serine recombinase superfamily member, shows similarity to transposases and resolvases	*ymfH*	Putative 103-amino-acid e14 prophage protein predicted to be membrane associated (328)
ynzC	Predicted 77-amino-acid protein of unknown function; structure prediction suggests a nucleic acid binding domain; conserved among *Firmicutes*	*ymfI*	Putative 128-amino-acid protein of unknown function from e14 prophage
yokE	Predicted 160-amino-acid protein of unknown function with no conserved domains identified	*ymfL*	Predicted 189-amino-acid protein hypothesized to be a transcriptional regulator for e14 prophage (328)
yokF	Similar to staphylococcal nucleases	*ycgH*	Predicted 882-amino-acid protein showing similarity to a type V secretory outer membrane protein
yopT	Member of the YopT superfamily of conserved hypothetical proteins expressed in *B. subtilis*	*ydeO*	Transcriptional activator important for acid resistance (44)
yopU	Predicted 65-amino-acid protein of unknown function with no conserved domains	*ydeS*	Putative 176-amino-acid protein of the fimbrial superfamily
yopV	Predicted 64-amino-acid protein of unknown function with no conserved domains	*ydeT*	Putative 382-amino-acid protein with domains conserved with chaperone usher of type VII secretion system and a PapC C-terminal domain involved in pilus assembly
yopW	Predicted 111-amino-acid protein of unknown function with no conserved domains	*ydeR*	Putative 167-amino-acid protein predicted to function in fimbrial adhesion
yopX	Member of the YopX superfamily, a group of conserved largely α-helical proteins of unknown function; predicted to form a 12-chain α-helical structure	*arpB*	Putative 632-amino-acid protein of the toxin 15 superfamily of enterotoxins
yopY	Predicted 92-amino-acid protein of unknown function with no conserved domains identified	*yoaB*	Putative protein of 114 amino acids sharing similarity to endoribonucleases involved in the inhibition of translation
yopZ	Predicted 67-amino-acid protein of unknown function with no conserved domains identified	*ogrK*	P2 phage transcriptional regulator
yoqA	Predicted 116-amino-acid protein of unknown function with no conserved domains identified	*yqgC*	Predicted 71-amino-acid protein of unknown function with no conserved domains identified
yoqB	Predicted 112-amino-acid protein of unknown function with no conserved domains identified	*yqgD*	Predicted 83-amino-acid protein with the domain of unknown function DUF2683
yoqC	Predicted 135-amino-acid protein of unknown function with no conserved domains identified	*yhiJ*	Predicted 540-amino-acid protein with the domain of unknown function DUF4049
yoqH	Predicted 150-amino-acid protein of unknown function with no conserved domains identified	*yhiL*	Predicted 412-amino-acid protein with the domain of unknown function DUF4049
yoqI	Predicted 64-amino-acid protein of unknown function with no conserved domains identified	*glvB*	Phosphotransferase system arbutin-like IIB protein (459)
yoqJ	Putative 171-amino-acid protein in DUF1273 superfamily of conserved bacterial proteins of unknown function with approximately 180 residues	*ibpB*	Heat-inducible chaperone (247)
yoqK	Predicted 67-amino-acid protein of unknown function with no conserved domains identified	*ibpA*	Heat-inducible chaperone (247)
yoqL	SPβ phage protein conserved with the α subunit of DNA polymerase III (DnaE)	*yifL*	Putative 228-amino-acid protein showing similarity to SpoU rRNA methyltransferases
yorB	SPβ 98-amino-acid protein of unknown function with no conserved domains identified		
yorC	SPβ 126-amino-acid protein of unknown function with no conserved domains identified		
yorD	SPβ 104-amino-acid protein of unknown function with no conserved domains identified		
yorE	SPβ 123-amino-acid protein of unknown function with no conserved domains identified		
yorF	SPβ 304-amino-acid protein of unknown function with no conserved domains identified		
yorH	SPβ 156-amino-acid protein of unknown function with no conserved domains identified		

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TABLE 1 (Continued)

B. subtilis gene	Function or description (reference)	*E. coli* gene	Function or description (reference)
yorI	SPβ protein similar to replicative DNA helicase of the DnaB family		
yozL	Predicted 97-amino-acid protein of the YolD superfamily of unknown proteins, functionally predicted to be UmuD of *E. coli* DNA polymerase V		
yozK	Conserved with Y family DNA polymerases, in particular with UmuC and Pol Kappa		
yqjH	**PolY2, similar to *E. coli* DinB** (403)	***dinB***	**Y family DNA polymerase** (37, 45, 254)
yqjW	**PolY1, similar to *E. coli* UmuC** (403)	***umuCD***	**Y family DNA polymerase** (327, 409)
yqjX	112-amino-acid protein of the YolD superfamily of unknown proteins, functionally predicted to be UmuD of *E. coli* DNA polymerase V		
yqjY	Similar to *N*-acetyltransferases, contains a coenzyme A binding pocket		
yqjZ	Member of the ABM superfamily, consisting of uncharacterized proteins involved in production of extracellular polysaccharides		

[a] The table was generated based on data from references 10, 138, and 385). For genes of unknown function, we performed a BLAST search through NCBI to identify and report conserved domains and possible functions found based on homology (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Genes that are shown in bold denote shared homologs or functional analogs between *E. coli* and *B. subtilis*.

from W reactivation. W reactivation is a phenomenon where phages treated with DNA damage show increased survival when the host also experiences DNA damage (464). It was noticed that W reactivation in *B. subtilis* was specific for repair of pyrimidine dimers, W reactivation can function for an extended number of generations, and the rate of mutagenesis is low. It was also noticed that a DNA methylase encoded by the prophage SPβ was induced (291, 467). These four factors differ from the *E. coli* system, and primarily for these reasons, the SOS system of *B. subtilis* has been referred to as SOB (for a review, see reference 466). For simplicity, we refer to the response as the SOS response, because the regulation of SOS in *B. subtilis* is basically the same as that in *E. coli* and many other *recA*- and *lexA*-containing bacteria. The major differences between bacterial systems are in the gene products that are under LexA control, which show substantial variation from organism to organism (Table 1).

In *B. subtilis*, the highly conserved RecA and LexA (also known as DinR) proteins are central to regulation of the SOS transcriptional response (65, 399, 451, 452, 468) (Fig. 1). RecA (also known as RecE) is a multifunctional protein required for homologous recombination, and RecA positively regulates the SOS response as it does in *E. coli* (10, 139, 178, 227, 230, 242). RecA in complex with ssDNA forms a nucleoprotein filament required to stimulate self-cleavage of LexA, the transcriptional repressor of the SOS regulon (143, 228). LexA represses the expression of 63 genes in 26 operons by binding to their promoters and preventing their transcription (10, 139) (Table 1). The LexA consensus binding sequence has been determined for *B. subtilis* and several other bacteria (Table 2). Many of the LexA-regulated genes function in some aspect of DNA repair, DNA replication, or the inhibition of cell division (10, 139) (Table 1). LexA also directly represses genes in two prophages, i.e., SPβ and PBSX (139) (Table 1). It should be noted that approximately 25% of the RecA-regulated genes in *B. subtilis* are of unknown function (10, 139). Strikingly, in our estimation, only 10 of the genes induced in *B. subtilis* are homologous or analogous to LexA-regulated genes in *E. coli* (10, 139) (Table 1). Thus, although the regulatory mechanism controlling the SOS pathway is conserved between *B. subtilis* and *E. coli*, ~85% of the genes comprising the SOS regulon in *B. subtilis* appear distinct from those in the *E. coli* SOS regulon (10, 139) (Table 1). In addition to its participation in the SOS response, RecA is also responsible for the DNA damage-dependent alteration in gene expression of nearly 600 other genes in *B. subtilis* (139). Virtually all of these genes are encoded by phages or integrative conjugative elements that are directly or indirectly regulated by RecA bound to ssDNA. The reason that these genes are not considered part of the SOS regulon is that although their expression is regulated by RecA, these genes are not known to be repressed directly by LexA (32, 139).

It is known that SOS induction is important for *E. coli* cells to survive exogenous DNA damage (e.g., see references 354 and 453). A comparison of the percentage of cells induced to respond through the SOS system following challenge with ionizing radiation between *E. coli* and *B. subtilis* showed that *E. coli* has a much lower DNA damage threshold to elicit SOS induction (386). In addition, the study showed that a site-specific double-strand break (DSB) in *B. subtilis*, generated by the homing endonuclease I-SceI, elicited SOS induction in fewer than 5% of cells (386). Moreover, in the absence of SOS induction, *B. subtilis* is capable of surviving a higher dose of ionizing radiation than that tolerated by *E. coli*, suggesting that DNA repair in the absence of SOS induction may be more efficient than that in *E. coli* (386). An alternate approach used *tetO* arrays to block replication fork progression in *B. subtilis* in order to induce and subsequently measure the response

TABLE 2 SOS boxes of Gram-positive bacteria compared with that of *E. coli*

Organism	SOS box consensus sequence[a]	Reference
Escherichia coli	CTGT-$(AT)_4$-ACAG	205
Bacillus subtilis	CGAAC-RNRY-GTTYC	451
Staphylococcus aureus	CGAAC-AAAT-GTTCG	69
Listeria monocytogenes	AATAAGAACATATGTTCGTTT	425
Cornyebacterium glutamicum	TCGAA(A/C)ANNTGTTCGA	169

[a] These and other Gram-positive SOS boxes can be found in reference 451. R, purine; Y, pyrimidine.

to replication fork arrest (23). That study found that the SOS response in *B. subtilis* was not readily induced by a protein block to replication fork progression (23). Taking both of these studies into consideration, it seems that *B. subtilis* can efficiently repair DNA damage or tolerate perturbations to replication forks in a way that does not readily induce the SOS transcriptional response. In support of this idea, cells incapable of SOS induction due to integration of a noncleavable *lexA* variant were shown to survive a considerable amount of DNA damage, suggesting efficient repair in the absence of triggering the SOS response (386).

SOS Responses in other Gram-Positive Bacteria

The SOS response has been investigated in several pathogenic and nonpathogenic Gram-positive organisms. The opportunistic human pathogen *Staphylococcus aureus* contains the *lexA* and *recA* genes (18, 27, 163). As expected, antibiotics that damage DNA, such as fluoroquinolones (inhibitors of DNA gyrase), induced the SOS response when administered at subinhibitory concentrations (259). The genome-wide SOS response of *S. aureus* to ciprofloxacin, a fluoroquinolone which induces DSBs and stalls replication forks, was determined using microarrays (69). In that study, the responses of wild-type and noncleavable *lexA*-bearing *S. aureus* strains to ciprofloxacin were compared (69). Sixteen genes were identified as under LexA control (69). This number is small relative to the number of genes under SOS control in *B. subtilis* (10, 139). The genes that were identified as upregulated included *recA* and *lexA*, genes involved in nucleotide excision repair (NER) (*uvrA* and *uvrB*), topoisomerase IV genes (*parE* and *parC*), and nuclease genes (*sbcC* and *sbcD*) (69). The binding of *S. aureus* LexA to the promoter of *recA* has been demonstrated (27), and this result is consistent with the mode of *recA* regulation in other systems (for a review, see reference 385). Interestingly, fibronectin binding proteins produced by *S. aureus* to aid in its attachment to the extracellular matrix and the plasma membrane are induced by fluoroquinolones (27), and the promoter for the fibronectin binding protein B gene (*fnbB*) is bound by LexA. This suggests that DNA damage may affect the ability of *S. aureus* to form clumps or attach to surfaces, a feature important during infection (27).

Listeria monocytogenes also contains the *lexA* and *recA* genes (113, 425). Challenge of *Listeria* with the DNA damaging agent mitomycin C resulted in the identification of 29 genes induced from 16 operons (425). Of these genes, most are involved in DNA repair, regulation of cytokinesis, and translesion synthesis (425). In addition to these studies, the SOS regulon has also been investigated in other Gram-positive bacteria. The SOS responses in many other Gram-positive bacteria are also regulated by RecA and LexA, as expected (169, 288). Overall, the number of genes and functions of genes controlled by this response differ considerably from organism to organism. With that stated, the *recA* gene, the *lexA* gene, and a gene product important for inhibiting cell division are consistently found to be under LexA control.

HOMOLOGOUS RECOMBINATION

Homologous recombination is central to DNA repair and the integration of DNA following genetic transformation (for a review, see references 108, 109, and 394). The homologous recombination pathway in *B. subtilis* has been reviewed in greater detail elsewhere (e.g., see references 11, 347, and 348). Here we focus on an overview of the pathway, with an emphasis on the cell biology of homologous recombination. We also highlight the similarities and differences in the homologous recombination pathways of *B. subtilis* and the better-understood Gram-negative model *E. coli*. In general terms, the steps of homologous recombination are conserved throughout biology. The conserved steps during DSB repair are (i) recognition and processing of a double-stranded end; (ii) loading of a recombinase such as RecA or Rad51 onto ssDNA; (iii) pairing of ssDNA with an intact homologous DNA segment, forming a crossover junction; (iv) DNA synthesis using the 3′-OH of the invading strand; and (v) endonucleolytic resolution of the crossover junction, resulting in the formation of two intact daughter chromosomes (for a review, see reference 128) (Fig. 2). These general steps in *B. subtilis* are the same as the steps in other organisms; it is the protein assemblies required to perform each step that differ between organisms.

Cross-Link Repair

Mitomycin C is a reagent that is commonly used to damage DNA and view the organization of repair proteins fused to fluorescent proteins into foci (e.g., see references 180, 245, and 387). Before we discuss the localization of recombination proteins in this section, we discuss mitomycin C as a DNA damaging agent and the mechanism of cross-link repair, based on repair of psoralen adducts. Mitomycin C forms a mono-adduct preferentially at the N^2 or N^7 position of guanine, as well as interstrand cross-links (for a review, see reference 107). The mono-adduct comprises ~87 to 88% of the lesions, whereas the cross-link represents the minor lesion that forms when mitomycin C is assayed in chick embryos (442). Interstrand cross-link repair in *E. coli* is best understood by experiments examining repair of psoralen adducts. Repair of a psoralen interstrand cross-link requires NER to generate a nick in a single strand both 5′ and 3′ of the cross-link. NER action generates an intermediate in which the dually nicked strand remains cross-linked to the other strand (389). DNA polymerase I (Pol I) in *E. coli* generates a gap in the nicked strand, providing a substrate for homologous recombination to pair the gapped region with a homologous DNA sequence, generating a three-stranded intermediate (389). Following endonucleolytic resolution of the resulting Holliday junction, the other strand is subject to NER, releasing a cross-linked double-stranded DNA (dsDNA) fragment (389; for a review, see reference 107). The major requirement for RecA and other recombination proteins is in formation and resolution of the three-stranded intermediate. In *E. coli*, a DSB can form following mitomycin C challenge, most likely by a mechanism where the replication fork encounters a gap in the DNA following processing of the lesion by NER and Pol I. When DSBs form in *E. coli*, this type of lesion represents a minor outcome of mitomycin C challenge relative to the mono-adduct (for a review, see reference 107).

RecN

A single, unrepaired DSB is lethal (e.g., see references 127 and 215), and thus efficient identification and repair are critical for ensuring genome preservation. RecN is hypothesized to respond early following DSB formation in *B. subtilis* (180, 350).

RecN is a conserved bacterial recombination protein that is a member of the SMC family of proteins (structural maintenance of chromosomes) (for a review, see reference 141). SMC-like proteins are best known for their role in chromosome partitioning and DNA compaction in organisms ranging from bacteria to humans (e.g., see references 36, 144, and 400; for a

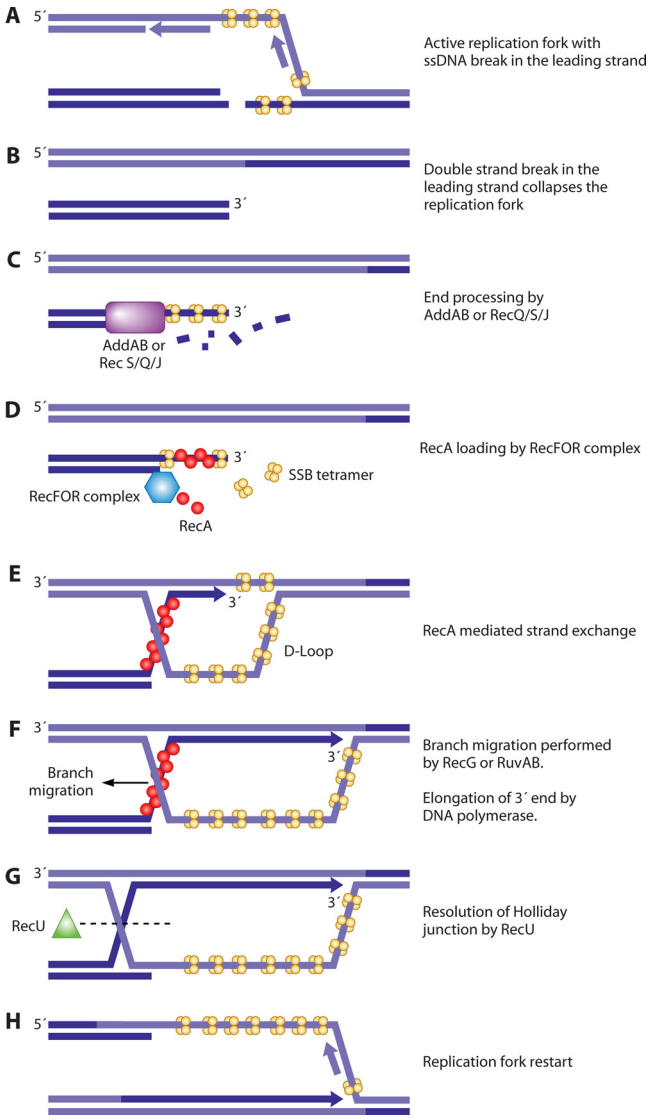

FIG 2 Model for repair of a single double-strand break by homologous recombination in *B. subtilis.* (A) Active replication fork with a single-strand nick in the leading strand template. (B) Upon the fork encountering the lesion, a DSB is produced and the replication fork collapses. (C) The double-stranded end is processed by the AddAB helicase-nuclease complex, or perhaps by RecQ or RecS helicase in combination with RecJ. In this case, AddAB degrades both the 5′ and 3′ ends until it reaches a Chi site, stimulating formation of a 3′-ssDNA tail. (D) The recombinase mediator complex RecFOR is recruited to load the recombinase RecA onto the ssDNA region. This reaction produces a ssDNA-RecA nucleoprotein filament. (E) The RecA-ssDNA filament forms a D loop, where one strand of the template DNA is displaced by the RecA-ssDNA nucleoprotein filament. (F) The 3′ end of the filament is then extended by DNA polymerase by use of the homologous strand as a template for DNA synthesis. The RecG protein or the RuvAB complex can facilitate migration of the D loop. (G) After the damaged strand is sufficiently extended, the Holliday junction is cleaved by RecU or possibly RecV (the dashed line indicates strand nicking). (H) PriA-facilitated replication restart reconstitutes replication of the lagging strand. (Adapted from reference 154 with permission from Elsevier.)

review, see reference 285). The eukaryotic protein Rad50 is a member of the SMC family and is part of the MRE11-Rad50-NBS1 (MRN) repair complex in humans, which associates with and stabilizes DNA ends at a DSB (68, 266).

Following treatment of cells with the DNA damaging agent mitomycin C, a functional fusion of RecN to green fluorescent protein (GFP) localizes and forms foci within the first 20 min after mitomycin C challenge (180). RecN-yellow fluorescent protein (RecN-YFP) focus formation is independent of RecA or DNA replication, further suggesting that RecN operates early following mitomycin C challenge and adduct formation (189). In support of a role for RecN in DSB repair, fully functional RecN-GFP was shown to form foci in response to an HO endonuclease-generated DSB (179). Consistent with an early role for RecN in repair is the observation that the *recN* gene in *B. subtilis* is not regulated by the SOS response, suggesting that this protein may be present at sufficient levels to efficiently respond to cross-linked adducts or DSBs that form *in vivo* (10). Interestingly, when X-ray challenge was used as a source to generate base damage sites, single-strand breaks, and DSBs, RecN-YFP focus formation was independent of the DNA damage dose, meaning that typically one RecN-YFP focus was observed per cell, regardless of the number of DSBs hypothesized to exist following X-ray treatment (180). This result suggests that RecN establishes a "repair center" where multiple DSBs might undergo repair in one large complex (180). Such a complex would be analogous to the DSB repair complexes in eukaryotic systems, where it has been shown that multiple breaks converge to form a single repair center (e.g., see references 9 and 215). Consistent with the cytological results indicating that RecN functions as a repair platform, biochemical characterization showed that RecN can form large oligomeric complexes in the presence or absence of DNA (345, 346). Considering the *in vitro* and *in vivo* results together, the current model is that *B. subtilis* RecN is capable of gathering multiple breaks into a complex prior to the arrival of other proteins involved in repair. In evaluating the "DNA repair center" model, it has yet to be demonstrated that multiple DNA ends originating from numerous DSBs are indeed present in a single RecN focus. Additionally, the signaling mechanism that directs RecN in response to a DSB has not been identified, and it will be interesting to uncover the mechanism that recruits RecN to a DSB(s) and to mitomycin C-generated lesions *in vivo*. Although RecN is important for DNA repair, cells can survive a site-specific I-SceI endonuclease-generated DSB in the absence of RecN, showing that RecN is not obligatory for DSB repair in *B. subtilis* (189).

In addition to a role for RecN in DSB repair, cells disrupted in *recN* are sensitive to several alkylating agents. The study of *recN*-disrupted strains has shown increased sensitivity to DNA damaging agents, including the alkylating agent methyl methansulfonate (MMS) and 4-nitroquinoline-1-oxide (4NQO), as well as a reduction in the transformation of chromosomal DNA (3, 180). Thus, the absence of *recN* has an effect on DNA repair and the integration of DNA during genetic transformation. However, *recN*-deficient strains are not nearly as sensitive to a DSB as *recA*-deficient strains, and furthermore, RecA-GFP foci are capable of forming in the absence of *recN*, as determined following mitomycin C challenge (3, 179). These results show that RecA function is not dependent on the presence of *recN* (3, 179). Taken together, the data show that RecN contributes to homologous recombination and appears to function early in the pathway of DSB repair in *B. subtilis*.

End Processing by AddAB

After a DSB is identified, the ends are processed, marking them for repair. In bacterial systems, the two enzyme classes responsible for this task are the helicase-nuclease complexes RecBCD and AddAB (for a review, see reference 471). During repair of a double-stranded end, the DNA is unwound and simultaneously digested by a nuclease, eventually generating a 3′-ssDNA overhang, which once loaded with RecA forms a nucleoprotein filament capable of undergoing strand exchange in the next step of homologous recombination (for a review, see reference 81) (Fig. 2). In *E. coli*, the major complex required for double-stranded end processing is the RecBCD helicase-nuclease complex (for a review, see reference 100). Briefly, RecBCD contains two helicase motors, RecB and RecD, with opposite polarities (29, 101, 410). The unwound ssDNA segments are then cleaved by the single RecB nuclease (388, 474, 475). The RecB nuclease is required for cleavage of both strands (388). Cleavage of the 3′ strand is more processive because the 3′ strand is channeled closer to the active site of RecB. Cleavage of the 5′ strand is less frequent, as movement of the 5′ strand is further away from the active site, making cleavage of the 5′ strand less efficient (388). The RecC protein contains a "pin" functioning to efficiently separate the duplex DNA (388). Cleavage of the 3′ strand is attenuated when the Chi sequence (crossover hot spot instigator) (5′-GCTGGTGG-3′) is encountered and bound by RecC, while cleavage of the 5′ strand continues. This overall mechanism allows for degradation of both strands until a Chi sequence is encountered, generating a 3′ overhang, a substrate appropriate for RecA binding.

B. subtilis lacks RecBCD, so the analogous functioning complex is AddAB, which performs the same overall reaction, although the mechanisms and organization of the protein complex are different (for a review, see reference 471). The AddAB complex engages in DSB end processing and is highly conserved among the *Firmicutes* (84, 148–151, 185, 257). Deletion or inactivation of the *addA* or *addB* gene causes substantial defects in DSB repair and increases the sensitivity of *B. subtilis* to a wide spectrum of DNA damaging agents (3).

AddA is both a helicase and an endonuclease. The N terminus of AddA is an SF1A family helicase, and the C-terminal domain is a RecB-type nuclease, which cuts the 3′-5′ strand (319, 338, 470). AddB does not have helicase activity; the C terminus of AddB forms a RecB-like nuclease domain which cleaves the 5′-3′ strand (319, 470). An Fe-S cluster is present in AddB, and this region has been shown to bind DNA in the crystal structure and to stabilize the protein structure (338). AddAB initiates end processing by binding to a double-stranded end, followed by processive unwinding and cleavage of both DNA strands (58; for a review, see reference 471). Degradation of both strands continues until a Chi site is encountered (58–61). Chi sites in *B. subtilis* have the short sequence 5′-AGCGG-3′ and are enriched in the chromosome (61). Chi sites are also found to generally coorient with the leading strand of replication (61). When AddAB encounters Chi, much like *E. coli* RecBCD, its nuclease activity is altered, allowing for continued unwinding and degradation of the 5′ strand downstream of Chi while interrupting the degradation of the 3′ strand downstream of Chi (59). The attenuation in nuclease activity on the 3′ strand produces a 3′-ssDNA segment that is appropriate for RecA binding (45, 46). As mentioned above, AddB is a nuclease, and the N-terminal portion of the protein shows similarity to DNA helicases, although AddB lacks the motifs required for helicase activity (338). Recent structural work has shown that the Chi sequence binds to the Chi recognition site in AddB. This binding event prevents degradation of the 3′ strand by AddAB as it is channeled through the AddAB complex (338).

Stoichiometric analysis of the AddAB complex shows that AddAB is active *in vitro* as a 1:1 heterodimer (472). The heterodimer has numerous activities, including the ability to bind a dsDNA end and to catalyze unwinding of duplex DNA (471). AddAB also has two genetically separable nuclease activities (470). As mentioned above, the AddAB helicase activity is conferred by the AddA subunit and has 3′-to-5′ polarity (472, 473). Inactivation of the ATP binding site (Walker A motif) in AddB has very little effect on the helicase activity of the AddAB complex, further demonstrating that AddA powers the helicase activity of AddAB (149–151). Unlike RecBCD, which contains two helicase activities and a single nuclease, AddAB has two nucleases that reside separately in its individual subunits, as well as a single helicase (for a review, see reference 471). In *B. subtilis* and many Gram-positive bacteria, the AddAB complex is responsible primarily for end processing during DSB repair.

RecQ, RecS, and RecJ

DNA helicases of the RecQ family are well conserved and present throughout biology (for a review, see reference 455). Humans have five RecQ helicases, and deficiencies in three have been linked to syndromes featuring a predisposition to cancer (for a review, see reference 331). Individuals with a deficiency in *WRN*, encoding a RecQ helicase, are predisposed to developing Werner's syndrome, which is characterized by a premature aging phenotype and increased incidence of mesenchymal tumor formation (for a review, see references 175 and 455). Interestingly, *E. coli* and yeast (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) both contain a single RecQ helicase homolog, as opposed to the multiple RecQ helicases found in higher eukaryotic organisms. In contrast, *B. subtilis* encodes two recently discovered RecQ helicase homologs, known as RecQ and RecS (350). Gene annotations for RecQ and RecS are different depending on the source of information. RecQ is encoded by an open reading frame named either *recQ* or *yocI*, whereas RecS is encoded by an open reading frame named either *recQ* or *ypbC*, again depending on the source used (77, 350). *B. subtilis* RecQ shares the closest sequence homology to human WRN and *E. coli* RecQ, as it contains the highly conserved helicase domain, the RecQ helicase conserved region (RecQ-Ct), and the helicase and RNase D C-terminal domains (HRDC) (Fig. 3). However, WRN contains an RNase D 3′-to-5′ exonuclease domain (281) which is absent from *B. subtilis* RecQ and RecS, *E. coli* RecQ, and *S. cerevisiae* Sgs1 (281, 284).

Recent evidence suggests that RecQ and possibly RecS can function in combination with the exonuclease RecJ in repair (350). Cells deficient in *recQ* or *recS* show almost no sensitivity to DNA damaging agents (350), but cells deficient in *addAB* in combination with *recJ* show a synergistic loss of survival, and the sensitivity of the resulting strain to alkylating agents (MMS and 4NQO) and the damaging agent mitomycin C is similar to that of a *recA* null mutant (350). Thus, in the absence of both *addAB* and *recJ*, the phenotype is the same as that of a strain lacking *recA*. Analysis of RecN-YFP localization in the absence of *addAB* and *recJ* showed that RecN-YFP foci still form in response to DNA damage, yet RecN-YFP is impaired in forming a single large focus and instead

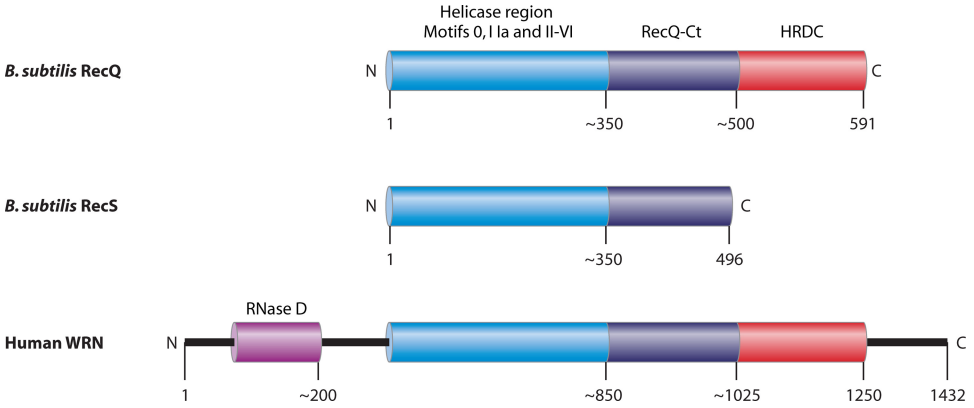

FIG 3 Schematic representation of the domain structure of *B. subtilis* DNA helicases RecQ and RecS in comparison with human WRN. The N-terminal region contains the helicase motifs (light blue); the RecQ helicase conserved region (RecQ-Ct) (dark blue/purple) and the helicase and RNase D C-terminal domains (HRDC) (red) are also shown. The human protein contains the RNase D domain N-terminal region, which contains a 3′-to-5′ exonuclease domain. (Adapted from references 24 with permission of Oxford University Press, 281 with permission from Elsevier, and 284 with permission from Macmillan Publishers Ltd.)

forms multiple small foci (350). These results have been used to conclude that end processing is required for the establishment of a single large RecN-YFP complex, with the caveat that alkylating agents were used and a DSB was not directly tested (350). Together, these data also support the model that RecN complexes form *in vivo* prior to action by AddAB or RecQ-RecS-RecJ, further supporting a role for RecN early in DNA repair.

Recent work showed that the SSB protein targets several proteins, including RecQ, RecS, and RecJ, to the replication fork (77). Proteomic analysis of the interactome of SSB revealed numerous DNA repair proteins that bind SSB *in vivo*, including but not limited to RecQ, RecS, and RecJ (77) (see Table 3 for a complete list). When YFP-RecQ was expressed ectopically with the native *recQ* gene intact, YFP-RecQ was shown to form foci that colocalized with the replisome (DnaX-cyan fluorescent protein [DnaX-CFP]) in untreated cells (197). This suggests that RecQ is constitutively associated with the DNA replication machinery and that replisome association is mediated by interaction with SSB. Strikingly, a C-terminal truncation of *ssb* (*ssbΔ35*) which reduces RecQ binding *in vitro* blocks GFP-RecQ focus formation *in vivo*, indicating that the C terminus of SSB may recruit RecQ to replication forks in *B. subtilis* (197). This observation is supported by results showing that the SSB-RecQ interaction is conserved in *E. coli* and that the SSB C terminus is required for their interaction (374).

Ectopic expression of GFP-RecJ with the native *recJ* locus intact also showed that GFP-RecJ formed foci *in vivo* (77). Focus formation by GFP-RecJ under these conditions occurred in the absence of exogenous DNA damage, suggesting that like RecQ, RecJ may

TABLE 3 Summary of SSB-interacting partners in *E. coli* and *B. subtilis*[a]

E. coli protein	*B. subtilis* protein	Function
Chi (*holC*) (+)	Absent	Pol III subunit
Pol II (*polB*) (+)	Absent	Repair DNA polymerase
Pol V (*umuC*) (+)	PolY1/YqjW (*)	Translesion DNA polymerase
Exo I (*sbcB*) (+)	Absent	3′-to-5′ exonuclease involved in MMR
Exo IX (*xni*) (+)	Absent	3′-to-5′ exonuclease and 3′ phosphodiesterase (372)
Primase (DnaG) (+)	DnaG (*)	Primase
PriA (+)	PriA (+)	Primosome assembly and DNA helicase
PriB (+)	Absent	Involved in primosome assembly
RecG (+)	RecG (+)	Repair helicase
RecJ (+)	RecJ (+)	5′-to-3′ exonuclease
RecO (+)	RecO (+)	RecA loading
RecQ (*recQ*) (+)	RecQ (*yocI*) (+)	3′-to-5′ DNA helicase
Topo III (*topB*) (+)	Topo III (*topB*) (*)	Type 1A topoisomerase
Ung (+)	Ung (+)	Uracil DNA glycosylase
DnaE (*)	DnaE (+)	Primer maturation in *B. subtilis*, catalytic replicative DNA polymerase in *E. coli*
Absent	YrrC (+)	Similar to *E. coli* RecD, the alpha subunit of exonuclease V; has a role in mismatch repair in *Bacillus anthracis* (462)
XseA (*)	XseA (+)	Large subunit of exonuclease VII
Absent	YpbB/RecS (*recQ*) (+)	RecS is a RecQ family DNA helicase, and YpbB is unknown and shows homology to RecQ-like ATP-dependent helicases
SbcC (*)	SbcC (*yirY*) (+)	RecN-like protein involved in recombination and DNA repair
RarA (*mgsA*) (*)	RarA (*mgsA*) (+)	RecA loading and chromosome partitioning

[a] This table was assembled using data from references 77, 374, and 375. (*), the protein is present in both *E. coli* and *B. subtilis* but has not been shown to interact with SSB; (+), an interaction between the indicated protein and SSB has been measured.

also routinely be positioned at active replication forks in *B. subtilis*. GFP-RecJ formed one or two foci per nucleoid, at a subcellular position similar to where the replisome would be expected to localize (77). It is worth noting that when visualized *in vivo*, functional AddA-GFP and AddB-YFP localized diffusely throughout *B. subtilis* cells and failed to organize into discrete foci in cells that were exposed to DNA damaging agents (245). Together, these results suggest that RecQ-GFP and RecJ-GFP could be positioned at the replisome, whereas AddAB does not appear to be located at the replisome as judged by fluorescence microscopy. These results suggest that the RecQ/RecJ functions could be localized to the site of DNA replication in *B. subtilis* through interaction with SSB (77).

RecS was also shown to bind *B. subtilis* SSB *in vitro*, and SSB bearing a tandem affinity purification (TAP) tag was purified from extracts with RecS associated (77). In the reciprocal experiment, TAP tag purification of RecS showed interaction with SSB but also with an unannotated protein, YpbB (77). Interestingly, RecS is cotranslated with YpbB, as the two genes slightly overlap (77). When *gfp-ypbB-recS* was expressed ectopically, the complex formed foci, but only if the SSB C terminus was intact (77). When GFP-YpbB or GFP-RecS was imaged, foci were not observed, suggesting that RecS and YpbB function together. Thus, the C terminus of SSB in *B. subtilis* is critical for DNA repair and recruitment of RecQ, RecS, and RecJ to the replication fork *in vivo*. SSB is gaining considerable attention as a protein that facilitates trafficking of replication and repair proteins to the replication fork and other ssDNA substrates in *B. subtilis* and *E. coli* (77, 197, 374, 375). The proteins that bind SSB in *B. subtilis* and *E. coli* show some overlap. However, many SSB binding partners are not shared between the two organisms (Table 2).

RecA Recruitment, Loading, and Coupling to DNA Synthesis

Processing of DNA ends, predominantly by AddAB and perhaps by RecQ-RecJ or RecS-RecJ, will result in a 3′-ssDNA suitable for RecA binding (Fig. 2 and 4). As mentioned above, *B. subtilis* also contains SSB (also termed SsbA), which is essential for DNA replication and critical for repair processes during exponential-phase growth (183). Unlike *E. coli*, *B. subtilis* contains a second SSB paralog, designated SsbB, encoded by the *ywpH* gene (213). YwpH is upregulated during the development of genetic competence and is critical for DNA transformation (213). SSB-coated ssDNA inhibits RecA filament formation (31, 187, 195, 423). At the same time, SSB can promote RecA binding by removing secondary structure from the DNA, ultimately providing a more suitable substrate for RecA filament formation (187). Even so, RecA must replace SSB on ssDNA in order to form the RecA-ssDNA nucleoprotein filament that mediates strand exchange (422). The mechanisms for RecA loading are well established for *E. coli* yet poorly understood for many other bacteria. In *E. coli*, RecBCD and the RecFOR pathways can each function in RecA loading (153, 201, 412, 422, 423). Once *E. coli* RecBCD produces a 3′-ssDNA end, RecBCD actively begins to load RecA onto ssDNA in the 5′-3′ direction, while displacing SSB (83). The RecFOR pathway functions primarily in the repair of daughter strand gaps, as well as in protection of the nascent strand following replication fork arrest in response to UV damage (66).

In *B. subtilis*, AddAB is not known to load RecA, whereas the RecFOR complex, specifically RecO, does have a RecA loading function (for a review, see references 11, 347, and 348). Mutations in *recF*, *recO*, or *recR* strongly sensitize *B. subtilis* to DNA damaging agents (MMS, EMS, and 4NQO), which primarily form daughter strand gaps (3, 4, 121). Mutations in *recF*, *recO*, and *recR* also decrease the transformation of *B. subtilis* with chromosomal DNA, providing more direct evidence that these proteins function in recombination of ssDNA entering the cell (3, 4, 121). It is not entirely clear if *B. subtilis* RecFOR functions in repair of a double-stranded end (DSE), which would be formed by ionizing radiation or through an I-SceI-induced break in the chromosome. If it does, one possibility is that RecFOR may help to load RecA onto the 3′-ssDNA tail generated following end processing by AddAB.

A critical actor in the RecFOR complex is the RecO protein. The domain organization and structure of the *E. coli* and *Deinococcus radiodurans* RecO proteins are very similar (201, 232, 337). Based on homology, *B. subtilis* RecO has a similar overall domain organization, particularly to that of *D. radiodurans* RecO (201, 232, 337). The N-terminal domain is an oligonucleotide/oligosaccharide binding fold (OB fold) characteristic of proteins that bind ssDNA and/or dsDNA. RecO contains a C-terminal domain composed of six alpha helices forming the core and a zinc binding domain (201, 232). For *D. radiodurans*, zinc binding is coordinated by four conserved cysteine residues, which are conserved in the *B. subtilis* protein (201, 232). The *E. coli* protein has one of the four cysteine residues, and the crystal structure of *E. coli* RecO lacks zinc (337). The overall fold of the "zinc binding domain" in *E. coli* RecO is very similar to that of the *D. radiodurans* protein. Thus, although sequence conservation between the *E. coli* and *D. radiodurans* RecO proteins is low (~21% identical), the overall structures are very similar (201, 232).

In addition to a role in RecA loading, RecO generally contains two conserved biochemical activities: it can anneal complementary single strands, and it helps to load and facilitate strand exchange by RecA (236, 237). For the *B. subtilis* proteins, it has been shown *in vitro* that RecO will help to load RecA onto SSB-bound ssDNA, although the mechanism of action is not clear (236, 237). A major difference in RecO function between *B. subtilis* and *E. coli* is that *B. subtilis* RecO alone is sufficient to nucleate RecA filament formation on SSB-coated ssDNA *in vitro*, while in *E. coli*, RecO and RecR are necessary, because RecO will not overcome SSB inhibition alone (422, 423).

A possible mechanism is that RecO physically binds and loads RecA onto ssDNA or that the strand annealing activity of RecO indirectly helps to stimulate RecA loading. Experiments have been performed to test for a direct interaction between *B. subtilis* RecA and RecO, but so far an interaction between these proteins has not been shown (236). However, in *E. coli*, a very weak interaction was detected between RecO and RecA by surface plasmon resonance (423). Thus, there is some evidence suggesting that RecO binds directly to RecA, although the binding appears to be very weak (423). It is also not clear whether RecF and RecR function in the loading of RecA onto ssDNA in *B. subtilis* (179). In total, the RecA loading mechanism in *B. subtilis* is unclear and will require further study in order to understand the concerted steps that result in RecA-ssDNA filaments *in vitro* and *in vivo*.

One of the requirements for homologous recombination is the presence of two chromosome copies in order to provide an identical template for repair of a DSB. By coupling homologous recombination with DNA replication status, a cell may ensure that this requirement is met. A study using a partially functional *recA-gfp* fusion allele integrated at the native *recA* locus as the only

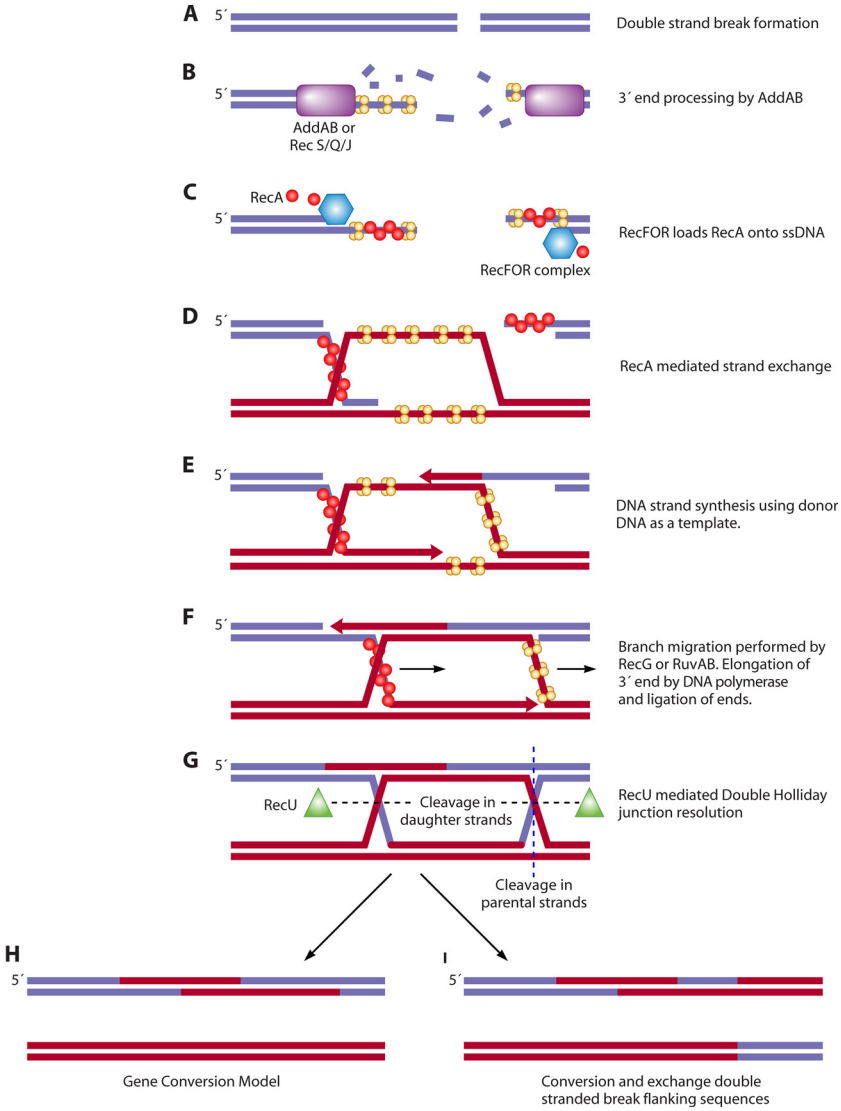

FIG 4 Model for double Holliday junction formation during homologous recombination and repair of DSBs in *B. subtilis.* (A) Ionizing radiation or an I-SceI endonuclease creates a DSB in the *B. subtilis* chromosome. (B) The ends of the DSB are processed by the AddAB helicase-nuclease complex, or perhaps by RecQ or RecS in combination with RecJ. AddAB degrades both the 5′ and 3′ ends until it encounters a Chi site (5′-AGCGG-3′), where 3′-5′ degradation is attenuated, whereas degradation of the 5′-3′ strand continues. This produces a 3′-ssDNA strand on both sides of the DSB, which is bound by SSB. (C) The recombinase mediator complex RecFOR is recruited and functions to load RecA, generating a 3′-ssDNA–RecA nucleoprotein filament. (D) One of the filaments undergoes a homology search and pairs with a template. This produces a displacement loop (D loop) where one strand of the template DNA is displaced by the RecA filament during pairing. One advantage of D loop formation is that the displaced strand can anneal to the other processed DNA, providing a template for its replication. (E) The 3′ ends of both invading strands are then extended by DNA polymerase, using the homologous strand as a template for DNA synthesis. The RecG protein or the RuvAB complex facilitates migration of the D loop, extending the degree of strand exchange. (G) Endonuclease resolution of the double Holliday junctions is facilitated by RecU or RecV, and depending on the location of the cut site, different exchanges between the two strands will be generated. (H) If the Holliday junctions are cleaved at the black dashed line, a gene conversion results in which the flanking sequences are the same as before. (I) If the Holliday junctions are cleaved at the blue dashed line, the downstream sequence flanking the site of damage is exchanged between the two strands. (Adapted from reference 154 with permission from Elsevier.)

source of RecA activity in the cell showed that ongoing DNA replication was necessary for RecA-GFP to form foci in response to single-strand gaps or an I-SceI-induced DSB *in vivo* (387). In this study, a DSB was generated and RecA-GFP failed to organize into a focus when DNA replication initiation was blocked (387). It is also worth noting that DNA replication has previously been shown to be necessary for SOS induction in *E. coli* (357). In that work, the LexA cleavage and degradation following UV irradiation were shown to be dependent on active DNA replication (357). The dependence on DNA replication may be due to the production of a significant amount of ssDNA at collapsed replication forks or perhaps to the presence of recombination proteins at the replisome. As discussed above, the SSB C-terminal tail is able to recruit proteins that might stimulate RecA loading at stalled or collapsed replication forks in *B. subtilis*, providing a possible platform for coupling between DNA replication and recombination (77). For example, the RecA loading protein RecO fused to GFP does not localize in cells deleted for the C-terminal 35 amino acids

of SSB (77). Furthermore, RecO colocalizes predominantly with the replisome, suggesting that RecO is staged at the replication forks through SSB (77, 197). Thus, RecA-GFP focus formation is dependent on ongoing DNA synthesis. Interestingly, this feature is conserved in *S. cerevisiae*. Rad52, the *S. cerevisiae* analog of bacterial RecO, was found to form foci only during S phase (216). These data show a distinct coupling of Rad52 to DNA replication status in eukaryotes (216).

RecA-Catalyzed Strand Exchange

After RecA has been recruited into a complex with ssDNA, it must then search for an intact homologous sequence and catalyze strand exchange. *In vitro*, it has been shown that *B. subtilis* RecA bound to ssDNA is sufficient to catalyze strand exchange, forming recombinational intermediates (49, 54, 230, 397). The RecA/Rad51/DCM1/RadA superfamily is comprised of RecA-like recombinases that bind ATP and contain the conserved GXXXXGKT ATP binding motif (e.g., see reference 457). RecA binds to and hydrolyzes ATP and dATP (54, 230). Once the RecA-ssDNA nucleoprotein filament is formed, RecA-dATP hydrolysis in the presence of SSB stimulates strand exchange (54). RecA complexed with ATP can still catalyze strand exchange, but the level of RecA-ATP required to achieve this reaction is 10-fold higher than the necessary level of RecA-dATP (54). It has been proposed that the formation of the RecA nucleoprotein filament is regulated by dATP and through interaction with SSB (54). In Gram-negative bacteria, many proteins have been identified to modulate RecA activity, and most of these proteins are not present in *B. subtilis* (for a review, see reference 82). However, in *B. subtilis*, the Holliday junction endonuclease RecU modulates RecA activity (12, 46, 47, 50). RecU shows some stimulation of RecA binding to ssDNA, yet it inhibits its ssDNA-dependent dATPase activity (49). In support of this idea, RecU mutants have been isolated that are no longer capable of modulating RecA activities yet remain capable of Holliday junction cleavage (46, 47, 50). Cells with a *recU* deletion are sensitive to DNA damage and have a substantially reduced capability for transformation with plasmid and chromosomal DNAs (51, 52). *In vitro* cross-linking results show a weak but direct interaction between RecA and RecU in the absence of DNA (46). These results suggest that RecA and RecU interact in a step preceding Holliday junction cleavage. Taken together, the data show that RecA and RecU bind in a defined system and that RecU has the ability to regulate the role of RecA in homologous recombination.

Branch Migration and Holliday Junction Resolution

In *E. coli*, the RuvABC complex is involved in branch migration and Holliday junction cleavage (for a review, see reference 446). RuvA binds Holliday junctions and recruits the helicase RuvB to undertake branch migration (for a review, see reference 446). RuvC is an endonuclease that ultimately cleaves the Holliday junction. *B. subtilis* contains homologs of RuvA and RuvB but not RuvC. It is hypothesized that RecU provides the function of *E. coli* RuvC in *B. subtilis* and many other Gram-positive bacteria, as RecU appears to be absent from most or all Gram-negative bacteria (250). As mentioned above, RecU can cleave four-way junctions, and *recU*-deficient cells are sensitive to DNA damaging agents and have a reduced capability for transformation of *B. subtilis* with chromosomal DNA (122, 250) (Fig. 4). The crystal structure of RecU shows that RecU is similar to type II restriction endonucleases and a Holliday junction endonuclease (Hjc) from the archaeal organism *Sulfolobus solfataricus* (30, 250, 289). The overall RecU fold has been described as a "mushroom" with a cap and a stalk (250). The cap contains the catalytic residues important for Holliday junction cleavage (250) (Fig. 5). The stalk region is important for Holliday junction binding and for inhibiting the dATPase activity of RecA (47). Cytological experiments with a functional RecU-GFP fusion showed that RecU forms discrete foci, though in a small percentage of damaged cells and very late in the process of repair (351). Cells deficient for *ruvAB* do not support RecU-GFP focus formation, suggesting that RuvAB may recruit RecU *in vivo* (351).

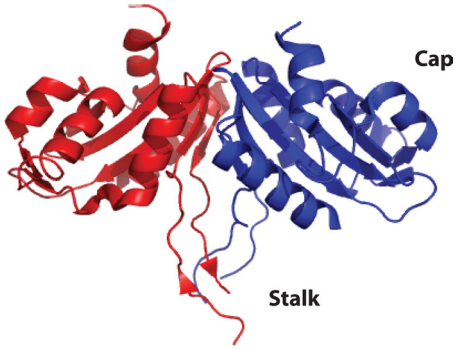

FIG 5 Crystal structure of Holliday junction resolvase RecU of *B. subtilis*. (Adapted from reference 250 with permission from Elsevier.) RecU has been described as a "mushroom"-like protein with a "stalk" and "cap" as indicated in the figure. The catalytic residues critical for Holliday junction cleavage are located in the cap, whereas the stalk interacts with RecA and modulates RecA activity. The Protein Data Bank accession number for the RecU structure is 1ZP7. This image was generated using Pymol (www.pymol.org/).

For *B. subtilis*, RecV has also been proposed to cleave Holliday junctions (349). The RecV protein is largely uncharacterized; however, it contains motifs that are similar to those of known Holliday junction endonucleases (349), and the *recV41* allele results in a similar level of sensitivity to DNA damage to that for a strain deleted for *recU* (349). Because deletion of *ruvAB* does not completely disable homologous recombination, it is possible that RecG catalyzes branch migration along with RecU or RecV to cleave and thus resolve recombinational intermediates. The current data argue against RecV working together with RecG, as a double mutant combining the *recV41* and Δ*recG* alleles is much more sensitive to DNA damage caused by alkylating agents than the corresponding single mutants (349). Very little is known about RecV, and more experiments are necessary to understand the physiological and biochemical roles of this protein in homologous recombination in Gram-positive bacteria.

Primosome Assembly

The primosome has been well characterized for *E. coli*, and we direct readers to recent reviews on this subject (e.g., see references 238 to 240). The *E. coli* primosome is composed of PriA, PriB, PriC, DnaT, DnaB, DnaC, and DnaG (for a review, see reference 241). Briefly, PriA is an SF2 helicase with 3′-to-5′ polarity (199). During replication fork restart, PriA recognizes D loops and can reestablish replication forks at a D loop or free 3′-OH, where it can facilitate the loading and reassembly of the replisome (e.g., see references 219, 220, 249, and 292). In *E. coli*, PriA, PriB, and DnaT

are dedicated to the primosome and assist with replication restart, yet they do not function in replication initiation from *oriC*. In *B. subtilis*, the primosome has the same overall function as in *E. coli* with respect to replication fork restart, but the composition of the primosome is different. *B. subtilis* contains the highly conserved PriA protein but lacks orthologs for PriB, PriC, and DnaT (193). Instead, *B. subtilis* uses components that normally function in replication initiation from *oriC* to fulfill the role of PriB, PriC, and DnaT in primosome assembly and replication fork restart (38, 243, 315). DnaD, DnaB (helicase loader in *B. subtilis*), and DnaI function in the loading of the *B. subtilis* replicative helicase DnaC at recombinational intermediates and *oriC* (38, 243, 315, 393). In *B. subtilis*, *priA*-deficient strains have very slow growth and are extremely sensitive to DNA damage, and their cells filament and show aberrant nucleoid morphology, demonstrating a critical need for PriA during vegetative growth (38). Mutations in the helicase loading protein of *B. subtilis* (DnaB) rapidly accumulate to suppress the growth phenotype resulting from deprivation of *priA* (38). This observation is very similar to the case for *E. coli*, as a *priA* deficiency is suppressed by mutations in the helicase loader encoded by *dnaC* (353).

Analysis of DnaD and DnaB has shown that these two proteins are related even though their sequence similarity is low (244). Both proteins contain a conserved YXXXIXXXW motif critical for DNA binding (244), and both proteins are capable of binding ssDNA and dsDNA. DnaD is a scaffold protein that remodels DNA, resulting in untwisting of a plasmid substrate. In contrast, DnaB remodels DNA by compaction, and thus the two proteins have opposing effects (48, 361, 477). For DnaD, the N-terminal domain forms oligomers, and structural analysis has shown a winged helix domain important for dimer and tetramer formation (361). The N-terminal domain of DnaD forms oligomers in the absence of DNA, while the C-terminal domain harbors DNA binding activity and a DNA-dependent oligomerization activity (48). Atomic force microscopy shows that DnaB forms a tetramer with a central hole (476). DnaB binds and condenses DNA, suggesting a more global role in DNA organization *in vivo*.

Ectopically expressed GFP-PriA shows constitutive association with the replisome, suggesting that it is poised to aid in reestablishment of replication forks that collapse during vegetative growth (197). Consistent with this hypothesis, PriA interacts with the C terminus of SSB. Thus, SSB probably functions to help recruit PriA to sites of replication fork arrest (197). Experiments *in vivo* show that the primosome proteins DnaD and DnaB associate with genomic regions experiencing high transcriptional activity, causing conflicts with DNA replication (258). To minimize such conflicts, transcription and DNA replication are cooriented in *B. subtilis in vivo*, but conflicts arise nevertheless (396). In particular, DnaD and DnaB associate with rRNA genes or the promoter of an integrative conjugative element, causing codirectional or head-on collisions with DNA replication, respectively (258). Association of DnaD and DnaB with rRNA genes requires *priA* and is independent of the replication initiation protein DnaA (258). Taken together, the data show that the composition of the primosome in *B. subtilis* is different from that in *E. coli*. It is not clear how the primosome functions at the biochemical level; however, it is known that it functions to reestablish collapsed replication forks and that the pathway is critical for *B. subtilis* during vegetative growth.

Potential Roles for Other SMC-Like Proteins in DSB Repair

In eukaryotic organisms, the SMC family of proteins has many functions in genome maintenance: Smc1 and Smc3 function in sister chromatid cohesion, while Smc5 and Smc6 are involved in replication fork stabilization, the DNA damage checkpoint response, and homologous recombination (6, 76, 97, 155, 156, 398). In addition, Smc2 and Smc4 are involved in X chromosome gene dosage compensation and mitotic chromosome condensation (e.g., see references 85 to 87). Chromosome organization and segregation in *B. subtilis* are accomplished by the SMC complex, which includes SMC, ScpA (kleisin), and ScpB (35, 36, 144, 214, 246, 395, 400). In addition to SMC, *B. subtilis* contains three other SMC-like proteins (RecN, SbcC, and YhaN [now known as SbcE]) (for a review, see reference 141). A yeast two-hybrid analysis showed that ScpA interacts with AddB, a nuclease subunit of the end processing helicase-nuclease AddAB complex and a critical component of DSB repair in Gram-positive bacteria (discussed above) (98). In support of a role for ScpA in homologous recombination, point mutations in ScpA were identified that rendered cells sensitive to mitomycin C challenge (98). Overexpression of *addBA* within these mutant backgrounds suppressed this phenotype, suggesting that ScpA interacts with AddB during some step of the repair process (74). Deletion of *scpAB* or *smc* also rendered cells sensitive to mitomycin C, although the sensitivity was not suppressed by increased expression of *addBA*, showing that suppression of *scpA* was specific (98). The sensitivity of *scpA*- and *smc*-deleted cells to mitomycin C does not implicate the corresponding proteins in a direct role for DSB repair *in vivo*; however, the observations that ScpA binds AddB and that *addBA* overexpression suppresses the sensitivity of mutant alleles of *scpA* to DNA damage do suggest a role for at least ScpA in DSB repair. It has yet to be determined if the role of ScpA in DNA repair takes place while ScpA is in complex with SMC and ScpB or if ScpA has a role in repair on its own. Because eukaryotic Smc5 and Smc6 have established roles in DSB repair (6, 76, 97, 130, 200), it is tempting to speculate that the SMC complex in *B. subtilis* may also have a role in DSB repair. One possibility is that ScpA in complex with SMC and ScpB functions to direct the end processing reaction of AddAB to break sites *in vivo*.

In *S. cerevisiae*, Rad50 is an SMC-like protein that forms a complex with Mre11 and Xrs2, forming the MRX complex (for a review, see reference 265). MRX with Sae2 functions to catalyze the first step in end processing of DSBs by catalyzing DNA end resection, a step that facilitates repair by homologous recombination while inhibiting nonhomologous end joining (NHEJ) (266, 480). In bacterial systems, the closest orthologs of Rad50 and Mre11 are the SMC-like protein SbcC and the endonuclease SbcD (373). In *E. coli*, SbcCD proteins are able to cleave certain DNA structures, such as hairpins (75). In *B. subtilis*, cells disrupted for *sbcC* show only a slight decrease in survival to DNA damage by ionizing radiation and mitomycin C challenge, yet a fully functional SbcC-YFP fusion forms faint foci *in vivo* during exponential-phase growth, an effect exacerbated following challenge with mitomycin C (245). Colocalization experiments showed that SbcC-YFP foci were coincident with the replisome, as detected with the DnaX-CFP fusion protein (245). Consistent with this finding, SbcC was shown to interact with SSB in *B. subtilis* as part of the SSB interactome (77). Furthermore, SbcC and the SbcD homolog YhaO were shown to interact with the *B. subtilis* primase (DnaG) in a

yeast two-hybrid analysis (245). Based on these results, it seems that the SbcCD complex may act at the site of DNA synthesis, perhaps through coupling to primase. This could aid *in vivo* in the repair of DNA damage such as interstrand cross-links at replication forks, as already suggested. However, the overall roles of these proteins in repair and genome maintenance are still unclear (387).

SbcE (YhaN) was recently identified as an SMC family protein in *B. subtilis* which has a role in DNA repair (189). Cells disrupted for *sbcE* show ~30% survival following introduction of an I-SceI-induced DSB (189). The gene downstream of *sbcE* shows homology to *sbcD* and has been named *sbcF* (*yhaO*) (189). SbcF and SbcE have been shown to interact by yeast two-hybrid analysis (290). Fully functional SbcE-YFP forms foci during vegetative growth, with slightly more foci in cells exposed to DNA damage (189). Like the case for RecN, SbcE-YFP foci form in cells experiencing DNA damage induced by mitomycin C in the absence of DNA replication. SbcE has also been shown to function during competence. Cells deficient for *sbcE* have a reduced capability for transformation, maintaining a transformation efficiency of only ~5% relative to the wild-type level (189). Epistasis analysis places SbcE in a pathway parallel to that of RecN, because *sbcE* and *recN* double mutants are more sensitive than the single mutants (189, 290). Taken together, the data indicate that SbcE is an SMC-like protein that contributes to DSB repair and transformation of *B. subtilis* cells.

NONHOMOLOGOUS END JOINING

NHEJ is a low-fidelity DSB repair pathway that catalyzes end joining of two broken DNA ends, using minimal or no sequence homology (for a review, see references 34, 89, 206, 207, and 380). Although it is mutagenic, NHEJ can repair DSBs when a homologous chromosome is not available. NHEJ was identified and studied in eukaryotic systems based on studies of viral integration (450). It was later found that NHEJ is important for the repair of DSBs (for a review, see references 34, 89, 206, 207, and 380). NHEJ is commonly used in mammals to repair DSBs, while the pathway is much more restricted in *S. cerevisiae* (174). In eukaryotes, the Ku70/80 heterodimer is responsible for binding broken DNA ends (439), followed by recruitment of other proteins responsible for repair (e.g., see reference 188). In 2001, it became apparent that some prokaryotes contain Ku-like proteins and several ATP-dependent ligases that are necessary to perform NHEJ (7, 102, 444, 447). Currently, our understanding of the bacterial NHEJ pathway is based largely on biochemical analysis of the proteins from *Mycobacterium tuberculosis* (for a review, see references 34 and 380).

Unlike eukaryotic NHEJ, bacterial NHEJ is a two-component DNA repair pathway consisting of a Ku homolog and an ATP-dependent ligase (96, 137, 445). Bacterial Ku functions as a homodimer that preferentially binds double-stranded DNA ends, both protecting them from nuclease attack and facilitating repair (445). Ku recruits the ATP-dependent ligase to the DSB, where it facilitates end processing, polymerization, and ligation in order to repair the broken segment (137, 445). Using *Mycobacterium smegmatis* as a genetic system, it was shown that ATP-dependent LigD is required to ligate a blunt-ended or 5′-cohesive-ended plasmid *in vivo* (137). Sequencing of the repair junctions showed that NHEJ is error prone, demonstrating that repair of the DNA ends is accompanied by nucleotide insertions or deletions (137). In the absence of *ligD*, NHEJ is dependent on the ligase LigC (137). LigD is a multidomain protein harboring an N-terminal polymerase domain, a central domain with 3′-5′ exonuclease activity, and a C-terminal domain with ATP-dependent ligase activity (313). The Pol domain belongs to the X family of DNA polymerases, whose members include Pol μ and Pol λ, which function during NHEJ in mammalian cells (34). In all, LigD has consolidated three processing activities into one protein, allowing for NHEJ to repair a wide range of DNA breaks without the need for other proteins.

B. subtilis also utilizes an NHEJ pathway that has been characterized almost exclusively *in vivo*. The *B. subtilis* Ku and LigD-like gene products are encoded in an operon, by the *ykoV* and *ykoU* genes, respectively (103). Deletion of *ykoV* or *ykoU* sensitizes *B. subtilis* strains to ionizing radiation in stationary phase (445) and sensitizes spores to X-ray and high-energy charged particle challenge (270). These results are consistent with a role of Ku and LigD in DSB repair. The *B. subtilis* proteins have not been examined biochemically, because both YkoV and YkoU have been reported to be insoluble (445).

What is the function of such a specialized DSB repair pathway in *B. subtilis?* Results defining the forespore-specific σ^G regulon of *B. subtilis* have provided a clue (440). The sigma factor σ^G directs transcription of genes in the forespore during development (401). This observation is striking because asymmetric septation during sporulation would physically separate each chromosome from its homolog by a membrane barrier, preventing homologous recombination (for a review, see reference 335). The physical compartmentalization and separation of the two chromosomes during this growth phase would be ideal for NHEJ to repair DSBs that might occur once a chromosome has been partitioned into the developing forespore. In addition, NHEJ has been associated with prokaryotes that undergo extended periods with one genome copy as part of their life-style, whether during sporulation or during extended periods in stationary phase, in the case of *M. tuberculosis* (34, 96, 137, 444, 445). These observations support the idea that NHEJ in *B. subtilis* contributes to genome maintenance during stationary phase and during germination of *B. subtilis* spores, when *B. subtilis* has predominantly 1C chromosomal DNA content per cell (270, 440, 445).

NUCLEOTIDE EXCISION REPAIR

NER is an important DNA repair pathway that allows for the high-fidelity repair of a variety of drug- and UV-induced lesions (for a review, see reference 128). The NER pathway is conserved from prokaryotes to eukaryotes, and defects in NER in humans are associated with many diseases, including xeroderma pigmentosum, which contributes to a high risk for developing skin cancer (71, 72). The highly conserved UvrABC excinuclease complex is required for recognition and excision of the damaged base (for a review, see reference 128). *B. subtilis* contains homologs of the Uvr system, including UvrA, UvrB, and UvrC (193). The *uvrBA* genes are in the same operon, and their expression is regulated by the SOS response (10, 65). Disruption of *uvrA* renders cells highly sensitive to a variety of DNA damaging agents, including UV, 4NQO, and mitomycin C (129). The *uvrC* gene is located in a different position on the chromosome from *uvrBA* and shows very low levels of SOS induction (10, 193). In *E. coli*, the $UvrA_2B$ complex is responsible for identifying the damaged base (159, 248; for a review, see references 342 to 344). UvrA dissociates, leaving UvrB to recruit UvrC to the site of damage (300–302). UvrC in cooperation with UvrB removes approximately 10 to 15 nucleo-

tides surrounding and encompassing the noncoding base (208, 209, 301). The dually nicked strand containing the noncoding base is then removed by DNA helicase II (UvrD), followed by gap filling with DNA polymerase I for the *E. coli* pathway (128). The resulting nick is then sealed by DNA ligase to finish the repair process (342, 381). Most of the biochemical experiments on NER have been performed with the *E. coli* proteins. It has been shown that *B. subtilis uvrC* will complement an *E. coli uvrC* deletion and that purified UvrC protein from *B. subtilis* will replace *E. coli* UvrC in a repair reaction reconstituted with purified components (210). Localization experiments using a functional UvrA-GFP fusion showed that UvrA-GFP was coincident with the nucleoid and that UvrA-GFP fluorescence was enriched on the nucleoid following UV damage (390). It has been proposed that UvrA may scan the chromosome to identify damaged bases, and the subcellular localization of UvrA-GFP is consistent with this hypothesis (390).

Transcription-Coupled Repair

The mutation frequency decline (Mfd) protein, also known as transcription repair coupling factor (TRCF), is a 133-kDa protein that dislodges stalled RNA polymerase from the transcribed strand when a lesion is encountered, targeting repair to the transcribed strand (123, 124). Deletion of *mfd* causes an increase in mutagenesis during vegetative growth, and interestingly, an *mfd* deficiency substantially reduces "adaptive" or "stationary-phase" mutagenesis in *B. subtilis* (330). Stationary-phase mutagenesis refers to the formation of mutations under nonlethal selection when cells are not growing, including conditions of nutrient limitation (402, 403). The effect of Mfd on DNA damage-inducible mutation frequency requires the presence of a functional excision repair pathway (124). *B. subtilis* Mfd has been purified and shown to displace RNA polymerase stalled at a cross-linked lesion (13). In addition, Mfd-deficient strains have a reduced capacity for supporting homologous recombination as measured by plasmid and chromosomal DNA transformation (13). These data show that an *mfd* deficiency results in 2- and 2.6-fold reductions in transformation with plasmid and chromosomal DNAs, respectively (13). When an *mfd* deficiency is combined with other gene defects in homologous recombination, transformation is further reduced (13). A synergistic decrease in plasmid and chromosomal DNA transformation was measured when an *mfd* disruption was combined with deficiencies in *recH*, *recP*, *recB*, and *recG* with respect to chromosomal DNA transformation (13). The mechanism underlying this observation is unknown. Because the functions of *recH* and *recP* are not clear and deficiencies in these genes cause the most striking effects with loss of *mfd*, elucidating the role of *mfd* in homologous recombination has been difficult (13). It has been suggested that Mfd may bind a recombination protein such as RecG, recruiting it to sites of homologous recombination (13). Mfd has not been studied in great detail in Gram-positive systems, and thus more work is necessary to understand the mechanism and possible role of Mfd in transcription-coupled repair and homologous recombination.

BASE EXCISION REPAIR

Base excision repair (BER) is a pathway that specializes in the repair of nonbulky lesions in DNA. This is in contrast to NER, which functions on the nucleotide/oligonucleotide level to repair bulky lesions (for a review, see reference 90). Nonbulky lesions are caused by numerous chemical assaults, including alkylation, oxidation, depurination/depyrimidination, deamination, and dUTP incorporation during DNA replication (for a review, see references 90 and 128). With many sources of potential damage, BER is considered the most frequently used DNA repair pathway *in vivo* (for a review, see references 28 and 191). The general process begins with detection of the lesion by a glycosylase, which hydrolyzes the N-glycosidic bond, removing the damaged base. This produces an apurinic/apyrimidinic or abasic site (referred to as an AP site henceforth). These sites are highly mutagenic and have the potential to cause single-stranded DNA breaks (for a review, see references 17, 90, and 128). AP endonucleases and AP lyases nick sites 5′ and 3′ of the AP site, respectively, which allows subsequent processing of the AP site by an exonuclease or a deoxyribophosphodiesterase (dRpase). The small gap is then closed by a repair polymerase, such as Pol I, and ligated, restoring the site to its undamaged form (for a review, see references 17, 90, and 128).

The "GO" System

The "GO" system is part of the BER pathway dedicated to the repair of oxidized guanines, such as 7,8-dihydro-8-oxoguanine (8-oxo-G), as well as opened guanine imidazole rings, referred to as formamidopyrimidines (FaPy-G) (176, 411). 8-oxo-G is the result of the oxidation of guanine at carbon 8, while FaPy-G is caused by ionizing radiation, methylation of N^7, or oxidative damage (128). DNA damage caused by oxidation can originate from exogenous sources; however, most damage is due to endogenous reactive oxygen species (ROS) originating from cellular metabolism (176). 8-oxo-G is the most prevalent form of oxidative damage in DNA, and if left unrepaired, it can be mutagenic because replicative polymerases can pair dATP opposite the damaged base during DNA synthesis (160, 233, 377). This produces an 8-oxo-G–A mismatch. Failure to repair this mismatch before the next replication cycle will create a GC → TA transversion (128, 260–263). In *E. coli*, the glycosylases MutM (*fpg*) and MutY reduce the mutagenic potential of 8-oxo-G in DNA by removing 8-oxo-G (MutM) or the mismatched adenine (MutY) from the 8-oxo-G–A mispair. Specifically, MutM selectively removes 8-oxo-G, in addition to producing a nick 3′ of the AP site with its AP lyase activity (e.g., see references 317 and 318). This occurs before an 8-oxo-G–A mismatch is produced during the next replication cycle. To provide an additional layer of protection from G → T transversions, MutY recognizes the 8-oxo-G–A mismatches and selectively removes the adenine base. MutY also has 3′ AP lyase activity, allowing for MutY to nick the DNA 3′ of the AP site, followed by repair synthesis with Pol I (415, 416). MutY therefore provides the cell with another replication cycle for MutM to remove 8-oxo-G before a mutation can occur (190, 448, 449).

Detection and excision of 8-oxo-G lesions and mismatches in *B. subtilis* are performed by homologs of *E. coli* MutM and MutY. In *B. subtilis*, a *mutM*-deficient strain has a slight increase in spontaneous mutagenesis (~5-fold), whereas *mutY* knockout confers a much larger increase in frequency of mutant occurrence (~100-fold). Characteristic of the GO pathway, a double knockout (*mutM mutY*) which prevents removal of both 8-oxo-G lesions and 8-oxo-G–A mismatches is synergistic, with a mutation frequency of ~1,000-fold (355).

Another target of oxidation is the nucleotide pool. For example (d)GTP can be oxidized to form 8-oxo-(d)GTP (26, 80). Oxidized nucleotides remain competent for incorporation into DNA during replication, with a high mutagenic potential (233). When

8-oxo-dGTP is incorporated into DNA, it can pair with adenine, producing an AT → CG transversion (233). Thus, the GO system has a third component responsible for cleansing nucleotide pools and preventing 8-oxo-dGTP incorporation into DNA. In *E. coli*, MutT provides this function (e.g., see references 26 and 80). Cells deficient for *mutT* confer a large increase in spontaneous mutation frequency (up to 10,000-fold) (79), with a mutation spectrum strongly biased toward AT → CG transversions (358). MutT is a nucleoside triphosphatase that sanitizes the nucleotide pools by selectively hydrolyzing 8-oxo-dGTP to 8-oxo-dGMP, with release of pyrophosphate, producing a nucleotide that cannot be incorporated into DNA during replication (26).

A clear functional analog for MutT in *B. subtilis* has not been identified. Analysis of the sequenced *B. subtilis* genome revealed a putative *mutT* gene (with 27.4% amino acid sequence homology) as well as two other possible orthologs (*yvcI* and *yjhB*) (193). These three genes, along with two more (*ytkD* and *nudF*), carry a conserved array of amino acids called the Nudix box (nucleoside diphosphates linked to some other moiety X), to which MutT's ability to hydrolyze 8-oxo-(d)GTP is attributed (25). Cells deficient for *mutT*, *yvcI*, or *yjhB* showed virtually no change in mutation frequency relative to a wild-type strain; a similar result occurred when all three genes were disrupted (355, 356). There is evidence suggesting that the *ytkD* gene may encode an activity that is similar to that of *E. coli* MutT (55, 324, 428). It was shown that YtkD can specifically hydrolyze 8-oxo-(d)GTP, as well as complement a *mutT* deficiency in *E. coli*. It was also shown that cells deficient for *ytkD* are more susceptible to oxidative DNA damage (55, 324, 428). However, others have presented evidence that YtkD fails to hydrolyze 8-oxo-dGTP selectively over dGTP (458). These results, in addition to the low frequency of occurrence of mutants observed with a *ytkD* knockout (~4-fold higher than that of the wild type), suggest that YtkD does not provide a role analogous to that of *E. coli* MutT but may function as a general nucleotide hydrolase turning over the nucleotide pool, indirectly reducing the amount of oxidized nucleotides within the pool. Another possibility is that with the lack of a clear *B. subtilis* ortholog of *E. coli mutT*, removal of oxidized nucleotides from the deoxynucleoside triphosphate (dNTP) pools is due to ribo- and deoxyribonucleoside tri- and diphosphatases that remain unidentified.

Uracil Glycosylases

Base excision repair is also responsible for reducing mutagenic potential from the introduction of UMP into the chromosome (111). Uracil is integrated into dsDNA through incorporation of dUMP opposite dAMP by the replicative DNA polymerase (419). dUMP incorporation can lead to GC → AT transition mutations (407, 408, 420). dUMP incorporation is rare because the dUTP pool is relatively small compared to that of dTTP; however, the kinetics of incorporation are similar to those in *E. coli*, and any increase in the dUTP/dTTP ratio will increase the likelihood of dUMP incorporation into DNA (379, 421). Another mechanism for dUMP incorporation into chromosomal DNA is through deamination of dCMP to dUMP. This process can occur spontaneously but may also be exacerbated by the presence of hydroxyl radicals or other DNA damaging agents (128). To counteract dUMP incorporation, *B. subtilis* possesses a uracil-DNA glycosylase (UDG; encoded by *ung*) which specifically removes uracil from DNA (74).

Interestingly, the PBS1 and PBS2 phages of *B. subtilis* have dUMP substituted for dTMP in their genomes (406). These phages contain Ung or UDG inhibitors (Ugi) that prevent Ung from removing dUMP from their DNA (20). Overexpression of Ugi causes an increase in mutagenesis in *E. coli*, demonstrating the effect of this protein as a Ung/UDG inhibitor *in vitro* and *in vivo* (441).

Processing of Apurinic/Apyrimidinic Sites

After removal of the damaged nitrogenous base by the appropriate glycosylase, a noncoding 2-deoxyribose-phosphate or AP site remains present within the DNA (211). AP sites also arise spontaneously following breakage of the N-glycosidic bond under physiological conditions (90). AP sites can damage DNA through mutagenesis or stalling of DNA replication and transcription complexes and can lead to chromosomal breakage (145, 191). In addition, when RNA polymerase encounters either ssDNA breaks or AP sites during transcription, it may incorporate ATP by default as a mechanism to ensure continued transcription, potentially producing base substitutions in the RNA transcript (70, 479). Therefore, it is important for the cell to process and correctly repair AP sites in order to prevent phenotypic consequences (211).

Both glycosylase and spontaneously produced AP sites are processed by AP endonucleases, which yield a 3′-OH group (128). *B. subtilis* contains three AP endonuclease genes: *yqfS*, *exoA*, and *yshC* (14, 193). All three AP endonucleases nick the phosphate sugar backbone 5′ of the AP site, in a metal-dependent manner, producing a replication-competent 3′-OH and a 5′ deoxyribose phosphate terminus (5′-dRp) (339, 378). Differences arise between the three AP endonucleases in terms of their spatial localization, temporal expression, and enzymatic activities. YqfS is a member of the Nfo family (endonuclease IV) of related AP endonucleases (339). Members of this family possess both AP endonuclease and 3′-phosphatase activities yet lack 3′-5′ exonuclease function and 5′-phosphatase activities (90). Importantly, when expressed from a plasmid, *B. subtilis yqfS* can complement an *E. coli* strain lacking its 2 major AP endonuclease genes (*nfo* and *xthA*) (339). In *B. subtilis*, *yqfS* expression is under the control of the σ^G transcription factor and is therefore expressed late during sporulation (339). This provides the forespore with its own AP endonuclease to repair AP sites formed in the forespore chromosome during development. In addition, His_6-YqfS protein is detected in mature spores, suggesting that this protein could repair AP sites during spore germination (339). In contrast to *E. coli nfo*, *yqfS* is not induced by the presence of superoxide radicals, which are generated following challenge of cells with paraquat and hydrogen peroxide (424). *B. subtilis* also has an AP endonuclease gene of the exonuclease III class, called *exoA*, which is conserved throughout biology, with members including *E. coli xthA* and human *APEX1* and *APEX2*. Northern blot analysis indicates that *exoA* is expressed during vegetative growth in *B. subtilis* (378). ExoA, much like *E. coli* XthA, possesses AP endonuclease, 3′-5′ exonuclease, and 3′-phospho-monoesterase activities (378). Surprisingly, an *exoA yqfS* double mutant fails to display a reduction in tolerance to AP sites, and its mutation frequency does not increase, suggesting that another AP endonuclease may be present in *B. subtilis* (424).

B. subtilis contains a homolog of *E. coli* Nth (endonuclease III) (73). Interestingly, the *nth* gene is located directly downstream of the *dnaD* gene. As discussed above, DnaD is involved in loading of

the replicative DNA helicase at *oriC* and at sites of replication fork restart (73). In addition, it has been proposed that DnaD provides a general role in nucleoid organization, as it untwists DNA and has DNA remodeling activities (48). Nth has received very little attention in *B. subtilis*; however, it has been shown to nick substrates with AP sites (73). DnaD, YonN, and HBsu showed stimulation of Nth nicking of AP site-containing substrates *in vitro*. A direct physical interaction between DnaD and Nth has not been shown, suggesting that DnaD, YonN, and HBsu are capable of remodeling DNA in a way that allows for efficient access of Nth to AP sites (73). Activation of the *nth* gene in *B. subtilis* confers sensitivity to hydrogen peroxide, consistent with the idea that Nth recognizes oxidized derivatives, including thymine glycol (443).

A newly discovered AP endonuclease activity was found for the *B. subtilis* DNA polymerase X (*yshC*) (14). PolX is a DNA polymerase that possesses low processivity and slow polymerization. PolX also acts preferentially on gaps of one to a few nucleotides in length (14). Polymerase activity is increased by the presence of a phosphate group on the 5′ terminus 1 to 5 nucleotides downstream of a 3′-OH (14). In addition, it was shown that PolX is able to nick a DNA substrate containing a nucleotide analog that mimics an AP site (15). The combination of AP endonuclease activity and the preference of its polymerase activity for small gapped substrates strongly suggests that PolX may be the primary polymerase that repairs small lesions, such as those produced by BER. Furthermore, PolX possesses a 3′-5′ exonuclease activity which was shown to process AP sites that were cleaved on the 3′ side by an AP lyase (15). Lyase cleavage produces a 3′-phospho-α,β-unsaturated aldehyde (3′-PUA), a group that blocks replication unless it is processed further (15). Therefore, PolX 3′-5′ exonuclease activity will process 3′-PUA by hydrolyzing the phosphodiester bond, leaving a 3′-OH from which PolX can extend (15). Interestingly, the exonuclease and AP endonuclease activities could not be separated by mutational analysis, and the presence of an AP site appears to inhibit the exonuclease activity of PolX, thereby favoring the AP endonuclease activity when an AP site is present (15).

PolX has been well characterized *in vitro*; however, the *in vivo* effects of deleting PolX are unknown. Because an *exoA* deficiency *in vivo* has very little effect on the repair of AP sites, it is hypothesized that PolX, jointly with ExoA, may provide an overlapping strategy for *B. subtilis* to repair AP sites during vegetative growth (15).

ALKYLATION DAMAGE

Bacteria occupy a variety of niches, from aqueous environments to mammalian and plant hosts. DNA damage by alkylating and methylating agents is common, and therefore the repair of alkylation and methylation damage is critical for survival of many bacterial species. Soil bacteria, including *B. subtilis*, are frequently exposed to environmental alkylating agents, including methyl chloride and methyl bromide as well as many naturally produced antibiotics (for a review, see reference 364). Alkylating agents react with many sites on DNA bases, causing numerous forms of DNA damage. Some DNA modifications are benign, whereas others can be toxic (for a review, see reference 90). These agents are divided into two categories based on their modes of substitution: S_N1 and S_N2 (for a review, see references 105 and 363). The S_N2 class of alkylating agents, such as the methyl halides, react selectively with nitrogen within the base ring not bound to hydrogen (for a review, see references 105 and 363). The main targets (and products) of S_N2 alkylation are the 7th nitrogen of both the guanine (7-meG) and adenine (7-meA) rings as well as the 3rd nitrogen of adenine (3-meA) (for a review, see references 105 and 363). S_N1-type alkylating agents, such as the lab reagent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), react with the same nitrogen moieties as S_N2 alkylating agents, while also reacting with the exocyclic oxygen sites primarily of guanine and thymine, yielding O^6-meG and O^4-meT (for a review, see references 105 and 363). 3-meA and 3-meG lesions can block the cellular replicase, leading to a loss of viability. O^6-meG and O^4-meT lesions have the capacity to cause mispairing and the formation of GC → AT transitions *in vivo* (for a review, see references 105 and 363).

Methyl and Alkyl Glycosylases

Bacteria such as those from the genus *Bacillus* have developed both constitutive and inducible pathways for repair of lesions resulting from DNA alkylation (146, 274–276, 278–280). The AdaA pathway is inducible and relies on a combination of methyltransferases and 3-methyl glycosylases to repair alkylation damage to DNA. Part of the constitutive pathway relies on the presence of numerous methyl and alkyl glycosylases encoded within the *Bacillus* genome (193). We focus most of our attention on the inducible Ada response. The AdaA response includes induction of a 3-methyl glycosylase called AlkA, encoded by the *alkA* gene (for a review, see reference 90). AlkA functions predominantly in the removal of lesions formed by alkylating agents that target nitrogen moieties, specifically 3-meA, 3-meG, and 7-meG (277). In addition to AlkA, the *B. subtilis* genome encodes at least four other glycosylases, which have redundant substrate repair activities as well as the ability to recognize other alkylation-based lesions (274–280). *B. subtilis* Aag (*yxlJ*) is a member of the mammalian AAG family, which is composed of 3-methyladenine DNA glycosylases (1). *B. subtilis* Aag is able to excise 3-meA and 3-meG but lacks the ability to excise 7-meG *in vitro* (1). In addition, *B. subtilis* contains two putative alkyl glycosylases (encoded by *yfjP* and *yhaZ*), although their functions and substrate recognition are unknown (193).

The bacterium *Bacillus cereus* also possesses three known 3-meA DNA glycosylases: AlkC, AlkD, and AlkE (5). The *alkE* gene is predicted to produce a protein that shares 26% sequence identity with *E. coli* AlkA, and it has been shown to complement an *E. coli* strain deficient in both *alkA* and *tag*, encoding the two glycosylases able to remove 3-meA (5). Therefore, AlkE is probably the AlkA ortholog in *B. cereus*. Conversely, AlkC and AlkD of *B. cereus* may represent a novel protein superfamily of 3-meA glycosylases (5). Both AlkC and AlkD have been shown to remove S_N2 lesions, whereas AlkD has also been shown to remove bulky lesions *in vitro* (5, 334). Recently, it was shown that AlkD bends alkylated dsDNA without intercalating between the bases. Strikingly, AlkD causes hydrolysis of the N-glycosidic bond without direct chemical attack from the enzyme itself, thus distinguishing its mechanism of action from the base-flipping mechanism used by most other glycosylases (334).

Alkyltransferases and the Ada Response

Upon alkylation of DNA, enzymes called alkyltransferases catalyze the transfer of the alkyl group from the DNA onto a cysteine residue (for a review, see reference 128). This process repairs the DNA by accepting the alkyl group while consuming its enzymatic activity in the reaction (128). The expensive activity of removing

TABLE 4 Comparison of mismatch repair proteins between *E. coli* and *B. subtilis*

E. coli protein(s)	Function	*B. subtilis* protein
MutS	Mismatch recognition	MutS
MutL	Protein "matchmaker"	MutL (endonuclease activity)
MutH	Methylation-directed endonuclease	Absent
Dam	Methylates adenine in d(GATC) sequences	Absent
Exonucleases I, X, and VII, RecJ	Exonucleases for degradation of the mismatch-bearing strand	Yet to be established in mismatch repair[a]
UvrD	DNA helicase	YrrC[b]

[a] Exonucleases I and X are not present in *B. subtilis*; Exo VII and RecJ are present in *B. subtilis*, but it is not known if these proteins function in mismatch repair.
[b] YrrC may function as a mismatch repair helicase in Gram-positive systems. YrrC is a RecD2 ortholog with 5′-to-3′ DNA helicase polarity, based on studies of the *D. radiodurans* RecD2 protein (462).

alkylation damage via a suicidal action is a highly conserved process, and unlike glycosylases, this process does not produce mutagenic AP sites (for a review, see reference 306). The *B. subtilis* genome carries at least five separate alkyltransferase genes: *adaA*, *adaB*, *dat*, *ydiO*, and *ydiP* (193).

The Ada response is a semiconserved response to alkylation agents designed to increase defenses against further alkylation (167, 168, 359). For a complete and comprehensive review of the *E. coli* Ada response, we direct readers to previously published reviews (128, 376, 430). The Ada response occurs in many Gram-negative bacteria as well as some Gram-positive bacteria, including but not limited to *B. subtilis*, *Bacillus thuringiensis*, *M. tuberculosis*, and *Micrococcus luteus* (340, 341, 463). The Ada response is a transcriptional response that was originally referred to as "the adaptive response to alkylation damage," because pretreatment of *E. coli* cells with small doses of alkylating agents guarded cells against the effects of higher doses (128, 167, 168, 359, 376, 430). Nonadapted (i.e., nonpretreated) *B. subtilis* cells displayed higher mutation frequencies and lower viability upon exposure to alkylating agents (274–280). The *B. subtilis adaAB* operon encodes two separate alkyltransferases with different substrate specificities. The AdaA protein catalyzes the selective removal of the *S* stereoisomer of methylphosphotriesters from the sugar-phosphate backbone of DNA (274). This irreversible transfer of the methyl group from the methylphosphotriester to AdaA elicits a conformational change which activates a latent transcriptional activator activity inducing the Ada response (274–280). Thus, repair of methylphosphotriesters is used to upregulate AdaA target genes, including the *adaAB* operon and *alkA*. The second gene in the operon, *adaB*, is hypothesized to encode a protein catalyzing the removal of O^6-methylguanine (274–280). In doing so, AdaB protects the chromosome from the deleterious effects of alkylation agents.

MISMATCH REPAIR

Mismatch repair (MMR) is a process used to correct replication errors introduced during DNA synthesis (for a review, see references 192 and 362). In *E. coli*, MutS binds a mismatch, followed by recruitment of MutL (for a review, see references 192 and 362). MutL recruits and activates the MutH endonuclease, causing MutH to nick the unmethylated and thus nascent strand bearing the mismatch (for a review, see references 192 and 362). The helicase UvrD is then loaded at the site of the nick, where it unwinds the DNA strand containing the error (for a review, see references 192 and 362). The error-containing strand is then degraded by one of many exonucleases, depending on the polarity of the excised strand (429). The resulting gap is synthesized by Pol III, and the nick is sealed by DNA ligase (for a review, see references 192 and 362). The MMR pathway in *E. coli* requires MutS, MutL, MutH, UvrD, and Dam methylase to methylate adenine in the d(GATC) sequence, allowing for identification of the newly replicated, mismatch-bearing strand (Table 4).

B. subtilis contains the highly conserved MutS and MutL proteins (135); however, it lacks MutH, Dam, and a clear UvrD ortholog known to function in mismatch repair (88, 118, 119). MutS is the "sensor" that recognizes the mismatch (384), whereas MutL is the "linker," which functions to link the remaining proteins in the pathway together to allow for efficient repair of replication errors in *B. subtilis* (for a review, see references 192 and 362). Because neither *dam* nor *mutH* is present within the *B. subtilis* genome, it has been hypothesized that *B. subtilis* uses a methylation-independent mismatch repair pathway, unlike the mismatch repair pathway characterized for *E. coli*. Consistent with this idea, d(GATC) sequences in *B. subtilis* and *S. aureus* are not methylated, suggesting that a functional analog of Dam is not present in these organisms (106). It is also important that the methylation-directed mismatch repair pathway characterized for *E. coli* is absent from most bacteria, and it is hypothesized that most bacteria utilize a methylation-independent mismatch repair system (310), just as in eukaryotic systems it is hypothesized that identifying the newly replicated strand in *B. subtilis* and other organisms that lack a methylation-directed signal relies on strand discontinuities located at or near DNA replication forks *in vivo* (for a review, see reference 194).

B. subtilis MutL is a Latent Endonuclease

One of the major differences between *E. coli* and *B. subtilis* is that *B. subtilis* MutL is an endonuclease and *E. coli* MutL is not (171, 310). In the *E. coli* methylation-directed mismatch repair system, MutH endonuclease activity is required for mismatch correction (2, 152). The endonuclease active site in *B. subtilis* MutL is identical to the active site in human and *S. cerevisiae* MutLα, suggesting a strong conservation in mechanism between *B. subtilis* and eukaryotic organisms (171). Recently, the crystal structure of the endonuclease domain of *B. subtilis* MutL was solved (310). Two critical observations came from this work. First, MutL contains a zinc-binding loop, and mutations that abolish zinc binding inactivate mismatch repair *in vivo* (310). The zinc-binding loop is hypothesized to play a structural role in allowing for the proper positioning of DNA into the active site for subsequent cleavage (310). Second, a β clamp-binding site with the sequence ^{487}QEMIVP^{492} was identified in the C-terminal domain of MutL (310), and this site was shown to indeed bind the β clamp (311). One favored mode of action is that the β clamp binds to the C

terminus of MutL, opening the active site and allowing for DNA cleavage during repair (311). Interaction between MutL and the β clamp may function to both position and activate MutL for cleavage of the mismatch-containing strand. In support of this idea, mutation of the β clamp-binding motif blocks β clamp binding *in vitro* and prevents mismatch repair *in vivo* (310, 311). In contrast, mutations to the homologous site in *E. coli* MutL have a less pronounced effect on mismatch repair *in vivo* (311). Thus, it is attractive to consider that the β clamp may help to orient MutL to the nascent strand by directing MutL endonuclease activity. Once the newly synthesized strand is nicked, the nick site could then serve as an entry point for other repair proteins involved in mismatch correction in *B. subtilis*. Although this is an attractive model, these steps have yet to be shown experimentally. We provide a current model for mismatch repair in *B. subtilis* in Fig. 6.

Mismatch Repair Proteins Are Coupled to DNA Synthesis

Several lines of evidence show that mismatch repair proteins assemble into complexes at the site of DNA synthesis (181, 384, 391). Many replication proteins localize in cells as discrete foci marking the site of DNA synthesis (21, 22, 99, 203, 256). Some of the first evidence suggesting that mismatch repair is coupled to DNA synthesis came from visualizing the formation of mismatch repair foci in human cell culture and in live *B. subtilis* cells (181, 384, 391). In *B. subtilis*, MutS-GFP and MutL-GFP fusion alleles expressed from their native promoter were shown to form foci in a small proportion of cells (~5 to 10% of cells) during exponential-phase growth, and this proportion was increased when cells were challenged with the mismatch-forming agent 2-aminopurine (2-AP) (384, 391). It should be noted that the *mutS-gfp* allele is functional and the *mutL-gfp* allele is nonfunctional with respect to mutant occurrence; however, the MutL-GFP fusion protein does form foci in response to mismatches, suggesting active recruitment in response to replication errors (391). The focus formation response by MutS-GFP and MutL-GFP requires ongoing DNA replication in *B. subtilis* (391). When MutS-GFP and MutL-GFP form foci, they preferentially localize to the midcell area, the site in the cell where DNA synthesis occurs (391). Moreover, MutS-YFP foci colocalize with the replisome (DnaX-CFP) in ~48% of live cells (391). These data suggest that mismatch repair proteins are coupled to or function near the site of DNA replication in *B. subtilis*.

In support of a mechanism that couples mismatch repair to DNA synthesis in *B. subtilis*, it was recently shown that MutS alters the subcellular localization of the essential DNA polymerase DnaE in response to replication errors (182). In this work, ectopically expressed DnaE-GFP foci decreased in cells challenged with 2-AP or in cells that bore a proofreading-deficient *polC* allele (*mut-1*) (182). The decrease in DnaE-GFP foci required MutS, suggesting that the effect takes place at the step of mismatch recognition. Protein far-Western blot experiments demonstrated that both mismatch repair proteins MutS and MutL directly bind DnaE, suggesting that a strong interaction between these proteins may exist *in vivo* (182). Thus, MutS detection of mismatches affects the subcellular localization of an essential DNA polymerase in *B. subtilis*, suggesting that MutS is able to signal to or perturb the replication complex following mismatch identification in live cells (182). One candidate protein for recruiting MutS to active replication forks is the processivity β clamp, discussed below.

Recent work in *S. cerevisiae* made use of a functional fusion of the MutS homolog MSH6 to an S-phase-specific cyclin to restrict MSH6 protein expression to S phase. In this experiment, the MSH6–S-phase cyclin fusion protein conferred wild-type levels of mismatch repair (158). In contrast, when MSH6 expression was limited to G_2/M by fusion to a G_2/M-specific cyclin, mismatch repair was nonfunctional (158). In addition, live-cell imaging using fully functional fluorescent fusions to MSH6 showed colocalization with the replication machinery in live *S. cerevisiae* cells (157). Colocalization of MSH6 to replication forks corresponded

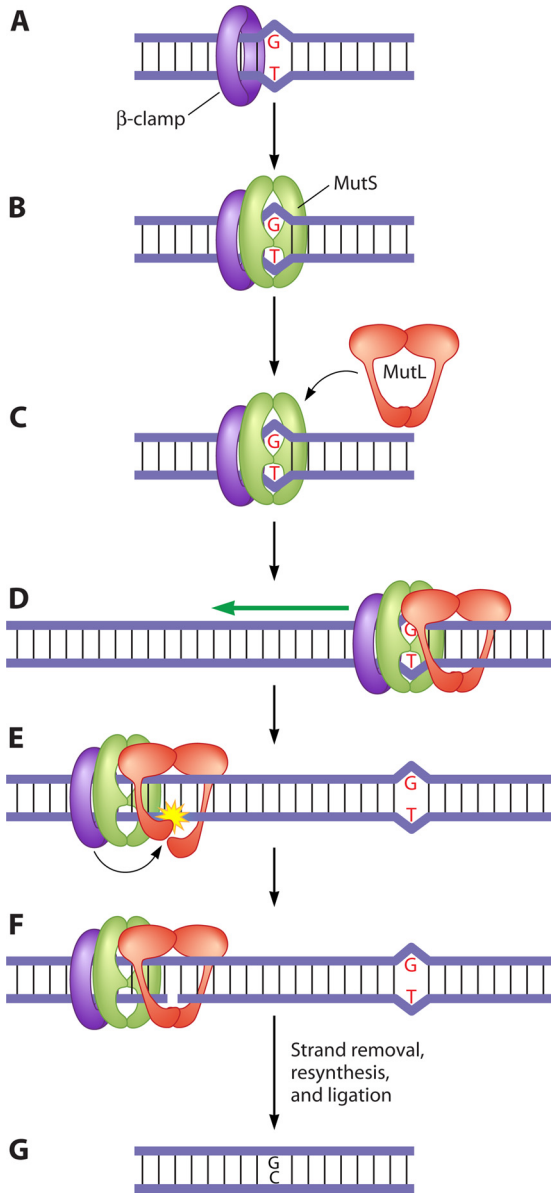

FIG 6 Model for mismatch repair in *B. subtilis*. (A and B) The β clamp directs MutS to the DNA to aid in identification of a mismatch. (C) MutS recruits MutL to the site of the mismatch. (D to F) We speculate that the complex slides along the DNA until the latent endonuclease activity of MutL is stimulated, possibly through interaction with the β clamp, causing MutL to nick the nascent strand. (F) The error-containing strand is then unwound, perhaps by RecD2 helicase, and degraded by an exonuclease. New homoduplex DNA is synthesized in the gap, and the new strand is ligated to complete mismatch correction. (Adapted from reference 310 with permission.)

to ~15% of MMR in *S. cerevisiae*, and interestingly, it was required for functional mismatch repair in the absence of ExoI (157). This work shows that mismatch repair is coupled to DNA replication in *S. cerevisiae*, demonstrating that the coupling of mismatch repair to DNA replication is conserved in higher organisms and is not unique to *Bacillus* (157, 158).

Involvement of β Clamp in Mismatch Repair

The DNA replication processivity factor β clamp is important for linking mismatch repair foci to the DNA replication status (112, 384). *B. subtilis* MutS contains a five-amino-acid motif originally identified through bioinformatics analysis as a putative β clamp-binding site (91). Residues ^{810}QLSFF^{814} provide a mostly hydrophobic plug that fits into a hydrophobic cleft on the β clamp (42, 91). Replacement of this motif with five alanine residues caused an ~40-fold increase in measurements of mutant frequency, and a mutant form of MutS fused to GFP and bearing a replacement of QLSFF with five alanine residues was reduced ~3-fold for focus formation in response to 2-AP challenge (384). Biochemical analysis shows that the β clamp binds to peptides bearing the QLSFF residues (384). Therefore, the MutS QLSFF motif is critical for interaction between MutS and the β clamp for efficient mismatch repair in *B. subtilis* (384).

Although mutation of the β clamp-binding motif in MutS increased the frequency of mutants of live cells, deletion of the 58-residue unstructured C-terminal region bearing this motif (resulting in the MutS800 protein) nearly abolished the interaction between *B. subtilis* MutS and the β clamp *in vitro* (384). Importantly, purified MutS800 protein bound to a mismatch at the same level as wild-type MutS *in vitro*, suggesting that removal of the C-terminal 58 amino acids does not diminish mismatch binding (384). Although it was expressed at wild-type levels, the *mutS800* allele conferred a spontaneous mutant frequency close to that for a strain disrupted in the *mutS* gene. MutS800 fused with GFP failed to localize as a focus in response to 2-AP, and YFP fused to only the C-terminal 58 amino acids of MutS bearing the β clamp-binding motif was sufficient to target YFP for localization when the fusion was overexpressed (384). These data support the hypothesis that the β clamp aids in the formation of MutS-GFP foci in response to mismatches *in vivo* (384). This work also found that a conditional allele of *dnaN* (coding for the β clamp) which caused a partial defect in mismatch repair had a reduced capacity for supporting MutS-GFP focus formation *in vivo* (384). In addition, intragenic suppressors of the temperature-sensitive phenotype caused by this β clamp allele (*dnaN5*) maintained defects in mismatch repair while rescuing the DNA replication defect conferred by this allele. These data further support a role for the β clamp in mismatch repair in *B. subtilis* (112).

An important role for the β clamp (DnaN) in mismatch repair is strongly supported by studies of *Bacillus anthracis*. Interestingly, *B. anthracis* contains two *dnaN* genes: *dnaN1* and *dnaN2* (460). Both *dnaN*-encoded clamps support growth *in vivo*, yet deletion of *dnaN1* confers a rate of spontaneous mutagenesis that is identical to that of *B. anthracis* cells disrupted for mismatch repair (460). These results show that both *dnaN* genes allow for proper DNA synthesis; however, only *dnaN1* is capable of functioning in mismatch repair (460). These data further establish a role for the β clamp in correction of DNA replication errors in the genus *Bacillus* (460). The data from *B. subtilis* and *B. anthracis* show that MutS interaction with the β clamp is crucial for mismatch repair in some Gram-positive organisms, and it may be found to be important in other organisms that lack a *dam*-directed repair pathway, although this remains to be established. The involvement of the β clamp in mismatch repair in *dam*-directed systems such as that of *E. coli* is unclear and requires further study (221–223, 314).

RecD2 Is a Possible Mismatch Repair Helicase in *Bacillus*

UvrD (DNA helicase II) is a 3′-5′ DNA helicase that functions during methylation-directed mismatch repair in *E. coli* (for a review, see references 192 and 362). *B. subtilis* contains two proteins showing homology to UvrD, namely, PcrA and YjcD (53, 307). PcrA is an essential helicase in *B. subtilis*, and the *pcrA* gene is able to complement the UV sensitivity conferred on *E. coli* lacking *uvrD* (307). It has been reported that a strain deficient in *yjcD* confers a wild-type level of mutagenesis (307). Thus, it is possible that PcrA functions in mismatch repair. Another possibility is that *B. subtilis* does not have a helicase that participates in mismatch repair and that mismatch correction is more similar to the process in eukaryotes, where an exonuclease (Exo I) is likely to function in the absence of a DNA helicase (for a review, see references 192 and 362). Recently, a screen for transposon insertions in *B. anthracis* that result in colony papillation revealed several genes that increase the rate of mutagenesis (461). Of these, a *recD2* ortholog was identified, and this work showed that a *recD2* deficiency in *B. anthracis* conferred a spontaneous mutation frequency and a mutation spectrum similar to those seen with a mismatch repair defect (461). Epistasis analysis of the *recD2*-deficient allele in combination with a *mutS*-defective allele showed a spontaneous mutation frequency that was similar to that of a strain bearing only a *mutS* deficiency, suggesting a role for RecD2 in mismatch repair in *B. anthracis* (462). RecD2 is similar to RecD1, a component of the RecBCD complex involved in end processing during DSB repair (e.g., see reference 462). RecD2 homologs are conserved in the *Firmicutes* and in Gram-positive bacteria that lack the RecBCD recombination complex (462). Based on these data, it is tempting to conclude that RecD2 helicases function in mismatch repair in many Gram-positive bacteria.

YshD, a MutS Paralog

In rare fashion, *B. subtilis* encodes MutS and a paralog, MutS2 (*yshD*). The *mutS2* gene is transcribed during normal growth, and transcription continues until cells reach stationary phase (332). Disruption of *mutS* causes the substantial increase in mutagenesis expected for a gene product involved in mismatch repair (112, 135, 384). Disruption of *mutS2* (*yshD*) has no observable effect on mutant frequency (332); however, analysis of the mutation spectrum in the *rpoB* genes of *mutS2*-deficient cells showed an unusual increase in transversion mutations (332). It has been suggested that such an increase could be attributed to inefficient repair of oxidative DNA damage; however, it is not known if *mutS2*-deficient strains are sensitive to oxidative damage (14). Analysis of the MutS2 protein in the Gram-negative organism *Helicobacter pylori* showed that MutS2 is an endonuclease involved in homologous recombination (312). Although the role for MutS2 in *B. subtilis* is unknown, based on conservation and analogy with other systems, *B. subtilis* MutS2 is probably an endonuclease involved in some unknown aspect of genome maintenance.

DNA DAMAGE TOLERANCE AND TRANSLESION SYNTHESIS

Translesion DNA polymerases are so named because they are capable of replicative bypass over noncoding bases that would normally block progression of a replicative DNA polymerase (for a review, see references 404 and 405). *B. subtilis* and many other Gram-positive bacteria contain two Y family DNA polymerases, termed PolY1 (YqjW) and PolY2 (YqjH) (403). PolY1 shows the highest sequence identity to *E. coli* UmuC, and PolY2 most closely resembles *E. coli* DinB (Pol IV) (403). In *E. coli*, UmuC cooperates with a posttranslationally modified version of UmuD called UmuD′ (for a review, see reference 297). This protein lacks the N-terminal 24 amino acids of UmuD, and two UmuD′ monomers dimerize and associate with UmuC to form DNA polymerase V (UmuD′$_2$C) (327, 409). UmuD is not well conserved outside bacteria closely related to *E. coli* (119). Thus, a protein analogous to *E. coli* UmuD has not been identified in *B. subtilis*, though not without attempts (110). Therefore, we hypothesize that PolY1 of *B. subtilis* acts in the absence of an accessory factor analogous to *E. coli* UmuD. Both PolY1 and PolY2 in *B. subtilis* were originally identified through sequence comparisons to the *E. coli* Y family DNA polymerases and later in a genome-wide yeast two-hybrid assay aimed at identifying *B. subtilis* proteins that interact with the replication processivity β clamp (DnaN) (290, 403). Like the case with their *E. coli* counterparts, expression of PolY1 or PolY2 from an ectopic locus induces mutation frequency on undamaged DNA, but not to the same extent (110, 403).

It should be noted that *E. coli* cells deficient in *dinB* were originally identified as causing a minor decrease in untargeted UV mutagenesis of λ phage and later shown to have a more striking role in adaptive mutagenesis (37, 45, 254). *B. subtilis* PolY2 also has a role in adaptive or stationary-phase mutagenesis, as *polY2* (*yqjH*)-deficient cells show a significant decease in stationary-phase *his*$^+$ reversion (403). Thus, both *E. coli* DinB and *B. subtilis* PolY2 have roles in adaptive mutagenesis.

DnaE-Induced Mutagenesis

Translesion synthesis is not limited to DNA polymerases of the Y family, because C family DNA polymerases can also have roles in lesion bypass and mutagenesis in several Gram-positive bacteria. DnaE is the replicative polymerase in *E. coli* and other Gram-negative organisms (for a review, see references 170, 251, and 253). *B. subtilis* and many low-G+C Gram-positive bacteria contain two C family DNA polymerases, i.e., PolC and DnaE (186, 478). The composition of the replicative DNA polymerase in low-G+C Gram-positive bacteria is different from that of the Pol III characterized for *E. coli*. For readers interested in these differences, we suggest a series of reviews and research articles that have recently been published (40, 98, 186, 196, 251–253, 352, 392, 427, 478). In the case of *B. subtilis*, *Streptomyces coelicolor*, *Streptococcus pyogenes*, *S. aureus*, and *Enterococcus faecalis*, the *dnaE* gene is essential (99, 125, 126, 164). The essential role of DnaE in *B. subtilis* is due to the requirement for utilization of RNA primers on the lagging strand during replication (352). DnaE can efficiently extend from an RNA primer, and PolC cannot (352). DnaE in Gram-negative organisms has an associated proofreading ε subunit (DnaQ) which provides the 3′-5′ exonuclease activity, allowing for proofreading and thus high-fidelity DNA replication (115, 360). Searches of *B. subtilis* coding sequences have failed to identify an ε subunit that would be available to associate with DnaE (data not shown). Extensive biochemical characterization of *S. pyogenes* DnaE showed that this protein is error prone, can easily bypass abasic sites, and can efficiently extend from a mismatched primer terminus (39). All of this evidence points to DnaE of *S. pyogenes* providing a role in mutagenesis and translesion synthesis (39).

As mentioned above, the *dnaE* gene product in *B. subtilis* is essential and required for maturation of the lagging strand *in vitro* (352). DnaE protein levels in *B. subtilis* have been shown to increase following DNA damage, and DnaE protein levels are elevated in cells deficient for *lexA* (196). In addition, the *dnaE* gene has a LexA binding site located upstream, and *dnaE* transcripts are induced ~11-fold in response to DNA damage (10). These data support the hypothesis that DnaE could have a role in DNA repair or lesion bypass, in addition to its essential role in chromosomal DNA replication. Consistent with a role for DnaE in lesion bypass, *B. subtilis* DnaE has been shown to bypass N^2-acetylaminofluorene guanine and AP sites with low efficiency, although DnaE was unable to bypass benzo-(*a*)-pyrene adducts or 6-4 TT photoproducts (196). It was also recently shown that the percentage of cells with DnaE-GFP foci was elevated in cells challenged with the DNA damaging agent mitomycin C, further suggesting a role for DnaE in repair or translesion synthesis across from mitomycin C adducts (182). Together, the data indicate that *dnaE* encodes an SOS-inducible DNA polymerase capable of lesion bypass *in vitro* and that DnaE is essential for chromosomal replication *in vivo*.

In some Gram-positive bacteria, typified by *M. tuberculosis*, PolC is absent, and *M. tuberculosis* instead contains two *dnaE* genes (*dnaE1* and *dnaE2*) (33). The *dnaE1* gene is essential, and deletion of *dnaE2* abolishes induced mutagenesis (33). It seems that bacteria containing two DnaE-type DNA polymerases use the *dnaE2* gene product for lesion bypass *in vivo*.

DNA INTEGRITY REGULATES BACTERIAL CELL DIVISION

When bacterial cells experience DNA damage or replication fork stress during vegetative growth, a response is activated to prevent cell division (57, 161, 177, 269, 294). A block to cell division, either transient or prolonged, allows for repair of damaged DNA, ensuring that a broken or otherwise damaged chromosome is not segregated into a new cell. Cells experiencing a sustained block to cell division show filamentation, meaning that cells continue to grow in length without a cell division event. For example, in *E. coli*, inhibition of the cell cycle is accomplished through the SOS-induced expression of SulA (*sfiA* or *sulA*). SulA prevents cell division by inhibiting polymerization of FtsZ, the tubulin ortholog responsible for cytokinesis (for a review, see references 147 and 283). After the SOS response is turned off, SulA is rapidly degraded by Lon protease, allowing for cell division to resume, and cell cycle progression continues (267). In *B. subtilis*, the *yneA* gene is SOS regulated, and expression of *yneA* inhibits cell division. We discuss YneA in greater detail below.

RecA-Dependent Regulation of Cell Division

When *B. subtilis* is challenged with DNA damage, cells filament in a *recA*-dependent manner (177, 225). The *yneA* gene is transcribed divergently from the *lexA* gene, and the *yneA* promoter region contains three LexA binding sites (10, 177). Inactivation of the *yneA* gene prevents DNA damage-inducible cell filamentation (177). YneA is a small protein secreted by the Sec pathway (232). It has a single transmembrane segment and a C-terminal pepti-

doglycan-binding LysM domain separated by a predicted protease cleavage site (268). YneA shares no sequence similarity to *E. coli* SulA (177). If *yneA* is under the control of an inducible promoter, expression of *yneA* is sufficient to prevent cell division, with cells growing to a mean length of about 5.5 μm (177). Upon subsequent depletion of inducer and repression of *yneA* expression, filamenting cells rapidly engage in cell division, and the average cell length returns to ~3 μm in as little as 20 min (268). These data show that cell division resumes efficiently after *yneA* expression is blocked, indicating that the YneA protein is rapidly cleared (268). Mutational analysis indicates that full-length YneA is necessary to inhibit cell division. Upon signal peptide cleavage, the LysM domain of YneA is released into the culture medium, inactivating YneA, which is followed by rapid degradation by extracellular proteases (268). It should be noted that overexpression of *yneA* also appears to impair separation of daughter cells following cell division, suggesting an additional role of YneA in cell wall remodeling (268). Furthermore, *yneA* is upregulated by the competence transcription factor ComK, suggesting a potential *in vivo* role in daughter cell separation following entry into stationary phase (295).

Currently, the mechanism that YneA uses to inhibit cell division in *B. subtilis* is unknown. Ectopic expression of *yneA* or expression of *yneA* through disruption of *lexA* caused a reduction or slight loss of FtsZ ring formation (177, 268). Yeast two-hybrid analysis failed to detect an interaction between YneA and FtsZ (177). It has been proposed that YneA may interact with a component of the cell division apparatus, probably through its transmembrane domain (268). Consistent with this idea, mutations in the transmembrane domain of YneA inactivate its ability to block cytokinesis (268). Furthermore, these mutations cluster to a single face of the transmembrane helix, adding evidence that the transmembrane domain may facilitate checkpoint activation through interaction with another protein, or perhaps through direct contact and inhibition of the cell division apparatus (268). Transmembrane domains have previously been shown to facilitate formation of hetero- and homo-oligomers during complex formation (202, 414). Interestingly, the newly discovered *Caulobacter crescentus* SOS-induced inhibitor of cell division A (SidA) was shown to prevent cell division by blocking the final stages of cytokinesis through inhibiting formation of the FtsWIN subcomplex (269). The interaction between SidA and FtsW occurs partly through the transmembrane domain, and mutations localized to the transmembrane domain abolish SidA-dependent blocks to cell division (269).

Blocking of cytokinesis is widely conserved among bacterial checkpoint proteins, as exemplified by YneA gene orthologs present in other Gram-positive bacteria, including *L. monocytogenes* (*yneA*), *M. tuberculosis* (*Rv2719c*), and *Corynebacterium glutamicum* (*divS*) (57, 294). Like *B. subtilis yneA*, these genes all maintain LexA-dependent repression, have a conserved genomic position adjacent to *lexA*, and are sufficient to prevent cell division when expressed (57, 294). Challenge of *M. tuberculosis* with cell wall antibiotics or the DNA damaging agent mitomycin C induces expression of Rv2719c and results in a block to cell division resulting in cell filamentation (57). Deletion of Rv2719c renders cells highly sensitive to DNA damaging agents, indicative of a checkpoint-like function (57). The DivS protein of *C. glutamicum* does not show sequence similarity to YneA or *M. tuberculosis* Rv2719c, but this protein does appear to have a single-pass transmembrane domain (294). It also appears that peptidoglycan synthesis at midcell and, to some extent, FtsZ ring assembly are altered upon *divS* induction following mitomycin C challenge (294). It is not known whether DivS, YneA, and Rv2719c directly inhibit Z ring assembly, like SulA, or whether they do so indirectly, regulating a late stage in cell division, like *Caulobacter* SidA. It should be noted that all known functional analogs of *B. subtilis* YneA aside from *E. coli* SulA, which is cytoplasmic, possess a single transmembrane domain. YneA may therefore represent a more widely conserved mode of inhibiting cell division than the SulA-type mechanism present in *E. coli* (57, 268, 269, 294).

RecA-Independent and DnaA-Dependent Regulation of Cell Division

B. subtilis regulates DNA replication initiation through regulating the activity of the initiation protein DnaA at the origin, *oriC* (for a review, see reference 273). The formation of a nucleoprotein complex between DnaA and *oriC* marks the start of replication initiation, a process that ultimately facilitates local chromosome unwinding and the assembly of the replication apparatus at the origin (e.g., see references 172, 173, and 282). DnaA is essential and is required for replication initiation in virtually all free-living bacteria (for a review, see reference 172). Although the essential function of DnaA is in DNA replication initiation, we focus on other roles for DnaA here and refer readers to several comprehensive reviews of DnaA and its role in chromosomal DNA replication initiation (e.g., see references 172, 173, and 282).

In addition to the well-defined roles for DnaA in replication initiation, DnaA has also been shown to function as a transcription factor that activates or represses transcription of a variety of genes, including its own (138). Results from gene profiling studies performed with *B. subtilis* show that DnaA controls a regulon comprising 56 genes in 20 operons (138). Among these DnaA-regulated genes, putative DnaA binding sites were identified upstream of 19 of the 20 operons (138). Furthermore, DnaA has been shown to bind a number of these sites *in vivo*, as determined by chromatin immunoprecipitation (138).

A striking finding was that DnaA is capable of sensing DNA replication fork perturbations, resulting in alterations to the expression of DnaA-regulated genes (138). Upon closer analysis of the DnaA regulon, two genes of particular interest were found: *sda* and *ftsL* (Fig. 7). Suppressor of *dnaA* (Sda) is a sporulation checkpoint protein and is described in detail below; however, we focus here on *ftsL* and the regulation of cell division. In contrast to the case for *yneA*, which is SOS regulated, *ftsL* regulation of cell division is *recA* independent (138). Upon encountering DNA replication stress, DnaA binds to the promoter region of the *yllB-ylxA-ftsL-pbpB* operon, downregulating transcription 2- to 4-fold and ultimately preventing additional rounds of cell division (138).

The penicillin-binding protein 2B gene (*pbpB*) and *ftsL* are essential genes required for cell division (183). FtsL has an unknown function but is known to form an oligomeric complex with DivIB and DivIC preceding cell division, and this recruitment is dependent on FtsZ ring formation (94, 382, 383). PbpB synthesizes peptidoglycan that eventually constitutes the new poles of daughter cells (120, 136). PbpB is a stable septal protein, whereas FtsL has been shown to be unstable, making *ftsL* an ideal target for regulating a cell division checkpoint (92, 138). Depletion of FtsL independent of *pbpB* results in substantial cell filamentation, as cell division is arrested at a step preceding septum formation and

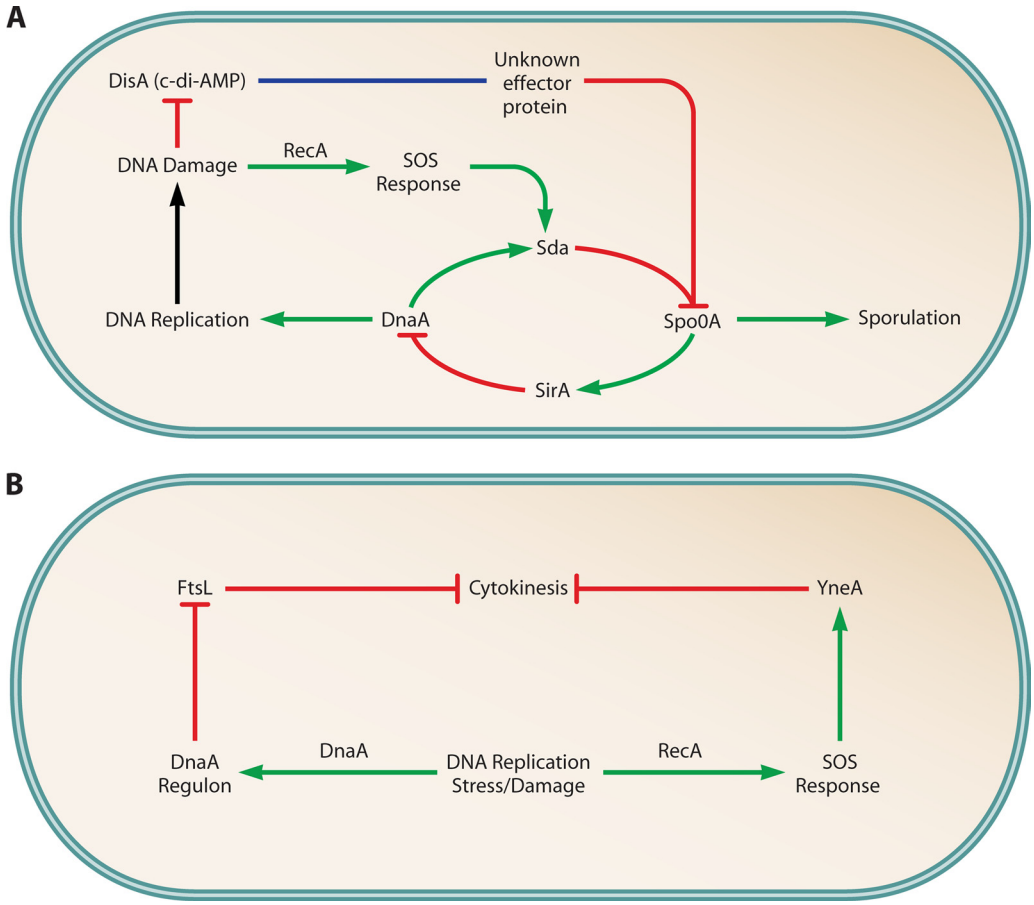

FIG 7 Schematic diagram of the genome maintenance checkpoints in *B. subtilis.* (A) The interplay between DNA replication and entry into the sporulation program is carefully regulated. The cell cycle regulator protein Sda prevents sporulation when DNA replication is ongoing. In contrast, SirA is activated upon entry into sporulation to prevent new rounds of DNA replication. In addition, entry into sporulation is regulated by at least two separate pathways in response to DNA damage. Sda is upregulated by the SOS response, and DisA senses DNA repair intermediates. Both Sda and DisA inhibit Spo0A phosphorylation, delaying progression of sporulation. (B) *B. subtilis* has mechanisms that prevent cell division when damaged chromosomes are detected. DnaA regulates cell division by decreasing levels of FtsL, and YneA is an SOS-regulated gene which blocks cell division through an unknown mechanism. These cell cycle checkpoints prevent septum formation and provide cells with a transient period to repair their DNA prior to cytokinesis. Green arrows represent activation events, red line segments represent repression or inactivation, and the black arrow indicates that DNA damage has occurred. (Adapted from reference 433 with permission of the publisher.)

membrane invagination (93). Following a perturbation to the DNA replication fork, DnaA binds to and represses expression of the operon containing *ftsL* (138). Due to the instability of FtsL, the steady-state level drops, causing a block to cell division. The FtsL cell division checkpoint may prevent cytokinesis over unsegregated chromosomes arrested for DNA synthesis. Interestingly, it appears that the regulation of *ftsL* by DnaA is broadly conserved (138). The mechanism used by DnaA to sense a perturbation to DNA replication is unknown.

GENOME INTEGRITY AND CHECKPOINT CONTROL DURING DEVELOPMENT

The developmental program of sporulation ensues when nutrients become limiting (for a review, see references 95, 114, 304, and 309). Nutrient limitation triggers the histidine kinases KinA, KinB, and KinC to produce a phosphorylation cascade that phosphorylates the master regulator of sporulation, the transcription factor Spo0A (making Spo0A~P). Spo0A~P regulates the transcription of genes whose products initiate the developmental program (271). As the program progresses, an asymmetric septum forms and separates two distinct compartments: the mother cell and the developing forespore. At the beginning of sporulation, cells contain 2 copies (2C) of the chromosome; one remains in the mother cell, while the other is translocated into the developing forespore (456).

Coupling Development to DNA Replication

B. subtilis has mechanisms to ensure a 2C chromosome copy number as it enters sporulation. If the cell enters sporulation with fewer than 2 chromosomal copies, the cell risks producing an anucleate endospore. In contrast, if the cell enters the sporulation program with more than 2 chromosomal copies, it may produce spores with multiple chromosomes or with twin spores, with one spore located at each cell pole (426). To avoid these outcomes, *B. subtilis* couples the process of sporulation to DNA replication (234, 235). Such coupling provides a brief window during the cell cycle in which sporulation can be initiated. If at any point during the cell cycle DNA damage or replicative stress occurs, the sporulation

program will be inhibited to allow for repair or continued DNA replication. By inhibiting DNA replication elongation with the PolC-specific drug HPURa [6-(*p*-hydroxyphenylazo)uracil], it was shown that inhibition of sporulation was *recA* dependent (166). Experiments using alleles that are temperature sensitive for replication initiation (*dnaA1*, *dnaB19*, and *dnaD23*) showed that sporulation was inhibited when replication initiation was inhibited (165, 204). This pathway used a *recA*-independent mechanism to delay sporulation. In both the *recA*-dependent and *recA*-independent pathways, sporulation is halted by inhibiting formation of Spo0A∼P. An allele of *spo0A* (*rvtA11*) that permits its activation directly by KinC bypassed the replication inhibition defect (165, 166, 204). These data indicate that the DNA replication status during sporulation is sensed and that this information is used to regulate formation of Spo0A∼P.

Sda

The source of coupling between the *B. subtilis* replication cycle and its entry into sporulation is *sda*. Mutations in the *sda* locus allowed *B. subtilis* to sporulate in a *dnaA1* temperature-sensitive mutant at the restrictive temperature in the absence of DNA replication initiation (43). Sda inhibits KinA and KinB, such that phosphorylation of Spo0A no longer efficiently occurs when levels of Sda are elevated upon inhibition of DNA replication (34). In this way, Sda inhibits sporulation by limiting Spo0A∼P levels (34). *In vitro* studies have confirmed that Sda inhibits KinA by binding its autokinase domain, preventing autophosphorylation and subsequent activation (333).

Sda protein levels are correlated directly with stages of the cell cycle and rely on cell cycle-dependent gene transcription, as well as the instability of Sda, to transiently prevent entry into sporulation (426). The *sda* promoter contains 5 *dnaA* boxes that allow regulation of *sda* transcription at the beginning of each cell cycle through binding of DnaA (43, 138, 426). Reductions in Sda levels correlate with the end of DNA replication, ensuring that a 2C DNA content is present before sporulation to help ensure viable spores. However, to enter into the sporulation program, the cell must reduce the amount of Sda in the cell to allow for Spo0A activation at the appropriate time. This process is performed by ClpXP-mediated proteolysis. *In vivo*, Sda maintains a half-life on the order of minutes (336, 426) due to residues at its C terminus that target Sda for degradation (V-S-S-COOH) (140, 336).

In addition to *dnaA*-dependent regulation of the *sda* locus, the SOS response can also mediate an increase in Sda levels in response to DNA damage or stalled replication forks (10, 336). The *sda* promoter contains a putative LexA binding site that has been shown to be responsive to UV damage and HPURa treatments (10, 166, 336). DNA damage-dependent upregulation of *sda* prevents entry into sporulation when chromosomal DNA is damaged.

SirA

As an additional measure to maintain the proper chromosome number during sporulation, *B. subtilis* can inhibit DNA replication initiation through the action of the checkpoint protein SirA (sporulation inhibitor of replication A) (*yneE*) (322, 323, 433). Using this checkpoint, the cell ensures that the 2C DNA content is maintained (322, 323, 433). The *sirA* gene was identified in the Spo0A regulon and found to inhibit DNA replication initiation (322, 323, 433). In addition, it was shown that artificial expression of *sirA* during vegetative growth caused a block in DNA replication initiation (322, 433). Conversely, sporulating cells deficient in *sirA* overreplicate, as assayed by observing genome equivalents or the origin copy number during sporulation (322, 433).

SirA prevents DNA replication initiation by binding DnaA and sequestering it from *oriC* (322, 433). In addition, cells that lack *oriC* and initiate replication through an ectopically integrated origin (*oriN*) that does not require DnaA for replication initiation are refractory to the inhibitory action of SirA (433). Depletion of DnaA blocks GFP-SirA focus formation, and expression of SirA causes DnaA-GFP foci to disperse (433). It was further shown that SirA binds to a group of amino acids (N44, F46, and V47) residing in domain 1 of DnaA, presumably preventing DnaA from establishing an active initiation complex at *oriC* (323). Mutation of these DnaA residues causes the cell to be unaffected by the inhibitory action of SirA (323).

Together, Sda and SirA maintain the 2C DNA content as cells enter sporulation. Sda prevents sporulation from occurring too early in the replication cycle, guarding against 1C bacteria undergoing development. Conversely, SirA expression is activated by Spo0A∼P upon entrance of *B. subtilis* into sporulation, thus preventing overreplication during development and preventing an increase in chromosomal copy number, which can reduce spore viability (426) (Fig. 7).

DisA

In addition to the importance of entering sporulation with two copies of the chromosome, *B. subtilis* must also ensure that genome integrity is maintained. One approach is *recA*-dependent transcription of *sda* as discussed above, and the other involves *disA* (19). DisA (DNA integrity scanning protein A) is a checkpoint protein that mediates a delay in sporulation following detection of DNA damage (19). In the absence of *disA*, the checkpoint fails and cells with damaged chromosomes progress into sporulation, producing nonviable spores (19). The *disA*-dependent checkpoint appears to attenuate phosphorylation of the master regulator Spo0A, preventing cells from entering sporulation following the detection of DNA damage (19).

How does DisA monitor genome integrity? DisA moves throughout the cell, scanning the chromosome for intermediates of DNA repair. DisA fused to GFP forms a dynamic complex that actively moves throughout the cell and pauses at sites of DNA damage (19). DisA movement requires that the cell make ATP and is independent of the cytoskeletal proteins MreB and Mbl (19). DisA-GFP also forms a mobile complex in the absence of DNA, suggesting that its movement is DNA independent (19).

Structural determination of a DisA homolog from *Thermotoga maritima* found that DisA has diadenylate cyclase activity (454). Specifically, this activity allows DisA to synthesize the second messenger c-di-AMP [bis-(3′,5′)-cyclic dimeric AMP] (454), and diadenylate cyclase activity is important for DisA-GFP focus movement *in vivo* (299). Interestingly, DisA diadenylate cyclase activity is regulated by different DNA substrates (454). DisA is unaffected by a ssDNA oligonucleotide or a blunt dsDNA substrate (454). However, branched DNA substrates that mimic intermediates of recombination, including 5′- and 3′-flapped structures or three- and four-way junctions, modulate diadenylate cyclase activity of DisA (454). These substrates reduce the rate of c-di-AMP production, which suggests that DisA does not scan for DNA damage *per se* but instead identifies intermediates of DNA repair, such as Hol-

liday junctions. This model was also supported *in vivo* by monitoring a *disA*-dependent increase in endogenous levels of c-di-AMP (299). Following challenge of cells with DNA damaging agents, c-di-AMP levels dropped, supporting the idea that DisA signals that genome integrity is compromised through a decrease in c-di-AMP production (299). To further support this model, the *B. subtilis* protein YybT was shown to ensure a constant turnover of c-di-AMP *in vivo* by degrading the signaling molecule and allowing for a quick and efficient decrease following the DNA damage-dependent attenuation of c-di-AMP production by DisA (299).

The current model invokes a mechanism where DisA scans the *B. subtilis* chromosome for intermediates of DNA repair. Upon identifying a Holliday junction or other DNA repair intermediate, DisA diadenylate cyclase activity is attenuated. The reduction in c-di-AMP may act as a signal to pause entry into sporulation by preventing an increase in Spo0A~P levels *in vivo* (Fig. 7).

CONCLUDING REMARKS

DNA replication, repair, and mutagenesis have been studied in *B. subtilis* and other Gram-positive bacteria for over 40 years. During this time, we have begun to appreciate that the mechanisms allowing for genome replication and repair are often different from the pathways described for the bacterial prototype, *E. coli*. During the last 10 years, researchers in the field have discovered new proteins and pathways with intricate mechanisms of regulation, and we have witnessed a spectacular increase in our understanding of genome maintenance in *B. subtilis* and other Gram-positive bacteria. This has allowed for a greater appreciation of the differences in the pathways of different bacteria and has provided a platform for understanding the molecular details of genome maintenance. We look forward to learning the mechanistic details of these pathways in years to come.

There are several other areas under active investigation that have contributed significantly to our knowledge of genome stability in *B. subtilis* and other Gram-positive bacteria. These include but are not limited to stationary-phase mutagenesis, natural genetic transformation, and the mechanisms that allow for spores to be resistant to genotoxic stress. We chose not to discuss these topics because several excellent review articles have been published on these subjects, and we direct readers interested in these subjects to those comprehensive reviews (62–64, 108, 109, 286, 287, 304, 329, 367–371, 413).

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