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Purification of an SOS Repressor from *Bacillus subtilis*

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We have identified in *Bacillus subtilis* a DNA-binding protein that is functionally analogous to the *Escherichia coli* LexA protein. We show that the 23-kDa *B. subtilis* protein binds specifically to the consensus sequence 5′-GAACN$_4$GTTC-3′ located within the putative promoter regions of four distinct *B. subtilis* DNA damage-inducible genes: *dinA*, *dinB*, *dinC*, and *recA*. In RecA$^+$ strains, the protein's specific DNA binding activity was abolished following treatment with mitomycin C; the decrease in DNA binding activity after DNA damage had a half-life of about 5 min and was followed by an increase in SOS gene expression. There was no detectable decrease in DNA binding activity in *B. subtilis* strains deficient in RecA (*recA1*, *recA4*) or otherwise deficient in SOS induction (*recM13*) following mitomycin C treatment. The addition of purified *B. subtilis* RecA protein, activated by single-stranded DNA and dATP, abolished the specific DNA binding activity in crude extracts of RecA$^+$ strains and strains deficient in SOS induction. We purified the *B. subtilis* DNA-binding protein more than 4,000-fold, using an affinity resin in which a 199-bp DNA fragment containing the *dinC* promoter region was coupled to cellulose. We show that *B. subtilis* RecA inactivates the DNA binding activity of the purified *B. subtilis* protein in a reaction that requires single-stranded DNA and nucleoside triphosphate. By analogy with *E. coli*, our results indicate that the DNA-binding protein is the repressor of the *B. subtilis* SOS DNA repair system.

The mechanisms and regulation of inducible DNA repair systems have been extensively characterized in the bacterium *Escherichia coli*. The best understood of these is the SOS regulatory system, which comprises about 20 unlinked genes that are coordinately induced by a variety of agents that cause DNA damage (13, 32). Induction of these damage-inducible (*din*), or SOS, genes results in the pleiotropic SOS response characterized by increased DNA repair capacity, increased mutagenesis, and filamentation. Corresponding to these induced processes, SOS gene products include proteins that function in recombinational repair, excision repair, translesion bypass (SOS mutagenesis), and the inhibition of cell division. The SOS genes are regulated by two proteins, LexA and RecA, which are themselves products of SOS genes. These regulatory proteins function, respectively, as repressor and activator of SOS gene transcription. In the absence of DNA damage, the SOS genes are each directly repressed by the binding of the LexA protein to SOS operator sites; induction of the SOS response is triggered when the RecA protein is activated, by a signal generated through the processing of damaged DNA, to cause the proteolytic inactivation of LexA.

Repression of the SOS regulon by LexA is understood at the molecular level in considerable detail. Purified LexA binds to operator sites preceding the SOS genes and inhibits SOS gene transcription in vitro (3, 14). Comparison of the DNA sequences upstream of all SOS genes reveals a common consensus sequence, CTGTatatatataCAG, the so-called SOS box (32). Several lines of evidence indicate that LexA binds as a dimer to SOS operators (9, 10, 28, 29). On the basis of analyses of *recA* and *lexA* operator mutants (33), quantitative footprinting studies of LexA binding to the *recA* operator (9), and the fact that all known SOS operators contain a consensus 5′-CTGT sequence, it appears that the sequence CTGTN$_8$ACAG represents the principal recognition site for a LexA dimer.

Induction of the SOS regulon is presumably the direct result of LexA self-cleavage, which is stimulated by activated RecA protein (8, 11, 12, 30). Although RecA is present at a relatively high basal level in uninduced cells, its coproteolytic function must be activated by an inducing signal. Analyses of LexA cleavage in DNA replication mutants indicate that the primary inducing signal following UV damage is single-stranded DNA exposed by discontinuous replication past pyrimidine dimers (27). In vitro, RecA is activated for repressor (LexA and certain phage repressors) cleavage when it binds single-stranded DNA and nucleoside triphosphate (NTP) (5, 25).

SOS-like DNA repair systems in other bacterial species have been widely reported (24). In particular, the SOS response has been highly conserved in the gram-positive bacterium *Bacillus subtilis*. As in *E. coli*, a variety of DNA-damaging agents induce cellular functions in *B. subtilis* such as increased DNA repair capacity, increased mutagenesis, and inhibition of cell division (16, 23, 35). Several lines of evidence indicate that regulation of the SOS response has also been highly conserved in *B. subtilis*. The *B. subtilis* RecA protein shares about 50% homology with *E. coli* RecA (31) and catalyzes *E. coli* LexA cleavage in vitro (21) and in vivo (20, 34). The induction by DNA damage of *B. subtilis din* genes (including *recA*) is dependent on a functional RecA protein (19, 22). In the absence of a functional *B. subtilis* RecA protein, *E. coli* RecA can induce the *B. subtilis* SOS response (17). A palindromic consensus sequence, GAACN$_4$GTTC, is located within the promoter region of several distinct *B. subtilis din* genes; the locations of these sequences, coupled with deletion analyses, suggest that it could serve as an SOS repressor binding site (4, 35).

Assuming that a *B. subtilis* SOS repressor would bind specifically and tightly to the promoter regions of *din* genes, we searched for a protein that would bind these regions in mobility shift assays. We report here the purification of a *B. subtilis* protein that binds specifically to each of four *din* promoter regions, and we show that this binding is specific for the GAACN$_4$GTTC consensus sequence. We show that the level of this DNA binding activity decreases rapidly following DNA damage and that the loss of DNA binding activity requires a functional, activated RecA protein. Finally, we report that purified *B. subtilis* RecA, activated by single-

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stranded DNA and NTP, abolishes the DNA binding of the purified protein in vitro.

MATERIALS AND METHODS

Materials. The *B. subtilis* RecA (21) and *E. coli* RecA (5) proteins were purified as described previously. Polyclonal *B. subtilis* RecA antiserum was prepared by the subcutaneous injection of New Zealand White rabbits with RecA protein of greater than 95% purity. Initially, 100 μg of RecA protein suspended in 1 ml of complete Freund's adjuvant was injected, and two boosters of 100 μg of RecA suspended in 1 ml of incomplete Freund's adjuvant were injected at 3-week intervals. The antiserum was collected 10 days after the third injection. Polyclonal *E. coli* LexA antiserum was provided by M. Sassanfar. Oligonucleotides were synthesized on a Milligen Cyclone Plus DNA synthesizer. Streptavidin and biotin cellulose were obtained from Pierce. Biotin-16-dUTP was obtained from Boehringer Mannheim. Restriction enzymes, DNA-modifying enzymes, and *Taq* polymerase were purchased from New England Biolabs and Promega Corp. and used as recommended by the manufacturers. Affinity-purified goat anti-rabbit horseradish peroxidase conjugate was purchased from Bio-Rad Laboratories. Mitomycin C was purchased from Sigma Chemical Co. Nitrocellulose filters were purchased from Schleicher & Schuell.

Bacterial cultures and cell lysis. The lysates used in this study were prepared from bacterial strains cured of bacteriophage SPβ and rendered noninducible for the endogenous prophage PBSX (36). Wild-type strain YB886 (*metB5 trpC2xin*-1SPβ$^-$) and *rec* derivatives (6) YB1015 (YB886 *recA4*), YB1260 (YB886 *recA1*), and YB1017 (*recM13*) were grown in LB medium at 37°C and harvested in late log phase (optical at 600 nm = 0.8). Pelleted cells were resuspended in 5 ml of lysis buffer (20 mM Tris [pH 7.5], 10% [wt/vol] sucrose, 1 mM dithiothreitol, 0.1 mM EDTA) per liter of bacterial culture. After addition of phenylmethylsulfonyl fluoride to 0.6 mg/ml and lysozyme to 0.2 mg/ml, cells were incubated on ice for 30 min, sonicated for 1 min, and incubated at 37°C for 15 min. Debris was removed by centrifugation at 100,000 × *g* for 45 min at 4°C, and the supernatant was either used for mobility shift assays or purified as described below. Protein concentration relative to a bovine serum albumin (BSA) standard curve was determined by using the Bradford reagent.

Preparation of the *din* promoter regions for mobility shift assays. DNA probes corresponding to the *dinA* (−200 to +75), *dinB* (−155 to +70), and *recA* (−77 to +170) promoter regions were prepared by polymerase chain reaction (PCR) amplification of YB886 DNA (10 ng/ml), using synthetic oligonucleotide primers (1 μM) with a Precision Scientific Thermal Cycler. Amplified DNA was digested with the appropriate restriction enzyme (PCR primers contained restriction sites to facilitate labeling; the *recA* probe was cut at the *Sau*3A1 site at +34) and end labeled with ^{32}P by using Klenow fragment. Labeled DNA was then purified by electrophoresis on an 8% nondenaturing polyacrylamide gel. DNA containing the *dinC* promoter region was obtained either by PCR amplification or by *Eco*RI-*Cla*I digestion of cesium chloride-purified pPL603-*din17* DNA, which releases a 199-bp *dinC* promoter fragment (−130 to +69). Digested DNA was then end labeled with Klenow fragment and gel purified as described above.

Mobility shift assays. Crude extract or purified fractions were incubated with one of the radiolabeled DNA fragments for 30 min at 25°C in incubation buffer (2) containing 12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.9), 4 mM Tris-Cl (pH 7.9), 12% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 2 μg of poly(dI-dC) · poly(dI-dC), and 0.3 mg of BSA per ml. This incubation mixture (10 μl) was loaded on a 4% (acrylamide/bisacrylamide ratio of 80:1) nondenaturing polyacrylamide gel, and electrophoresis was begun immediately. The buffer within the gel and the running buffer both consisted of 25 mM Tris-Cl (pH 8.5), 250 mM glycine, and 1 mM EDTA. Samples were electrophoresed at 25 mA, and the dried gel was subjected to autoradiography. Autoradiograms were scanned with an LKB laser-enhanced densitometer to quantify percentage bound. Analyses of association and dissociation rates for the putative repressor and the *din* promoter regions used in this study, using either filter binding assays or mobility shift assays, indicate that all binding reactions reach equilibrium in less than 5 min (18). Moreover, percentage bound values determined by either method were essentially identical, indicating that autoradiographic band intensities are proportional to the amount of radioactivity and correspond to population distributions at the start of electrophoresis. K_d values for competing oligonucleotides were approximated by using the concentration that competes one-half of *dinC* binding according to the definition $K_d = [R][O]/[RO]$. Thus, for two distinct operators, each binding 50% of a fixed amount of repressor, $[RO_1]/[R] = [RO_2]/[R]$ and therefore $[O_1]/K_{d_1} = [O_2]/K_{d_2}$.

Purification of DNA binding activity. Purification of the specific DNA binding activity was achieved by using the *dinC* promoter coupled to biotin cellulose with streptavidin (2). In a typical purification the 199-bp *Eco*RI-*Cla*I fragment of pPL603b-*din17* was biotinylated with biotin-16-dUTP by using Klenow fragment. Crude extract of YB1015 (*recA4*) cells containing a threefold molar excess of DNA binding activity (determined in the mobility shift assay) was incubated with the labeled fragment under mobility shift assay conditions. (The concentration of the DNA-binding protein in the extract was determined by titrating crude extract against a known concentration of promoter fragment under conditions in which binding was essentially stoichiometric.) Following incubation at 25°C for 30 min, streptavidin was added at a fivefold molar excess relative to the biotinylated fragment and incubated for an additional 5 min. The incubation mixture was then transferred to a tube containing biotin cellulose (an amount equal to the manufacturer's suggested capacity for streptavidin), pretreated with mobility shift assay buffer, and incubated on a rotating wheel for 30 min at 25°C. Resin was pelleted by low-speed centrifugation, and supernatant was removed and assayed for protein content and DNA binding activity. Following two washes of the resin with 3 volumes of mobility shift assay buffer, the resin was resuspended in an equal volume of mobility shift assay buffer containing 1 M KCl and mixed gently on a rotating wheel for 30 min at 25°C. Resin was pelleted by centrifugation, and the supernatant was assayed for DNA binding activity and stored at −80°C. The resin was stored at 4°C and reused for subsequent purification of DNA binding activity.

In vivo quantification of *B. subtilis* RecA induction. *B. subtilis* strains were treated with 0.3 μg of mitomycin C per ml, and cell samples were analyzed by immunoblot transfer as described previously (19). Nitrocellulose membranes containing electrophoretically transferred proteins were incubated with *B. subtilis* RecA antiserum (diluted 1:5,000) and then with peroxidase-conjugated anti-rabbit antibody (diluted 1:3,000) essentially as described previously (21). Samples of purified *B. subtilis* RecA (30 to 500 ng) protein were electrophoresed alongside cell samples. Immunoblots were scanned with an LKB laser-enhanced densitometer, and the amounts of RecA were inter-

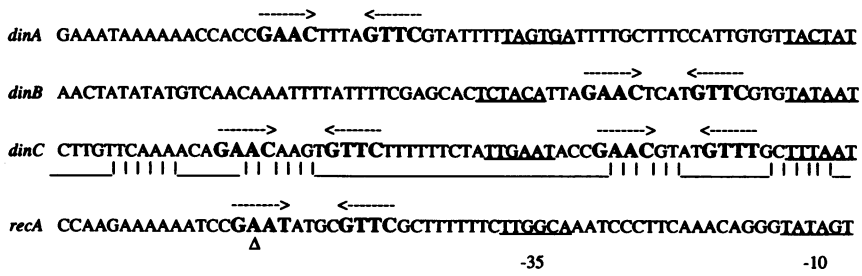

FIG. 1. Promoter regions of *B. subtilis din* genes. *din* consensus sequences are indicated in bold letters, and putative promoter elements are underlined. The results of DNase protection analysis of the *dinC* promoter region are summarized: hypersensitive sites (|) and protected regions (|__|) are indicated below the *dinC* sequence. Deletion of A (△) in *recA* consensus eliminates mobility shift.

polated from purified protein standard curves as described previously.

Gel filtration. A 100-μl sample of crude extract was applied to a Superose 12 (HR 10/30) column, equilibrated with 50 mM phosphate buffer (pH 7.0) and 150 mM NaCl, and chromatographed by using a Pharmacia FPLC (fast protein liquid chromatography) system at 0.4 ml/min. Fractions (100 μl) were collected and assayed immediately for DNA binding activity in mobility shift assays. Molecular weight standards (BSA, carbonic anhydrase, cytochrome *c*, and vitamin B_{12}) were similarly chromatographed, and fractions were analyzed by measuring A_{280}.

RESULTS

A *B. subtilis* protein binds specifically to *din* promoter regions. We conducted mobility shift assays with crude extract of *B. subtilis* cells and radiolabeled DNA probes corresponding to the putative promoter regions of four distinct *B. subtilis din* genes. The sequences of the four promoter regions are shown in Fig. 1. The palindromic SOS consensus sequence, $GAACN_4GTTC$, is located within each promoter region; two consensus sites are present in the *dinC* promoter region. In typical mobility shift assays, crude extract from *B. subtilis recA4* ($RecA^-$) cells retards the mobility of DNA fragments containing *recA* (−77 to +34), *dinB* (−155 to +70), and *dinC* (−130 to +69) promoter regions. Incubation of crude extract with a DNA fragment containing the −77 to +170 region of *recA* causes a mobility shift (see Fig. 8), but no shift was observed with *recA* DNA (+35 to +170) without the promoter region (Fig. 2A). Incubation of crude extract with either *recA*, *dinB*, or *dinA* (see Fig. 8) promoter DNA results in a distinct band with electrophoretic mobility slower than that of the probe, indicating the formation of single, higher-molecular-weight complexes for each. Consistent with the presence of two consensus sites in the *dinC* promoter region, two shifted bands were observed with *dinC* DNA at subsaturating concentrations of crude extract; at saturating concentrations of extract, only the larger complex is formed. The formation of at least two distinct protein-*dinC* complexes probably corresponds to one and two binding sites occupied. DNase footprinting analysis revealed that incubation of crude extract with the *dinC* promoter region renders two sites hypersensitive to DNase activity (Fig. 1) (18).

To determine that the same protein binds to the different promoter regions, we conducted mobility shift assays with the *dinC* promoter DNA in the presence or absence of the other promoter regions as competitors. Figure 2B shows that an excess of unlabeled plasmid DNA containing either the *recA*, *dinA*, *dinB*, or *dinC* promoter region inhibited the binding of protein to labeled *dinC* DNA. The concentrations of competing DNA were adjusted so that there was a 20-fold excess of unlabeled binding sites. A 20-fold molar excess of the plasmid vector had no effect on binding. Densitometric scanning of the autoradiogram revealed that an excess of the *dinC* or *recA* promoter DNA completely abolishes binding; excess *dinA* and *dinB* promoter DNAs inhibit binding about 90 and 30%, respectively. This result, and other results shown below, indicates that the same protein binds to all four promoter regions with approximate relative affinities as follows: *dinC* = *recA* > *dinB* > *dinA*. This order is consistent with our quantitative analyses of these binding constants (18). We observed the appearance of a faint but distinct band migrating between the two protein-*dinC* complexes in reactions containing *recA*, *dinA*, and *dinB* promoters as inhibitors (Fig. 2B, lanes b and c). We suspect that this may be due to another *dinC* binding activity in the crude extract that does not bind other *din* promoter

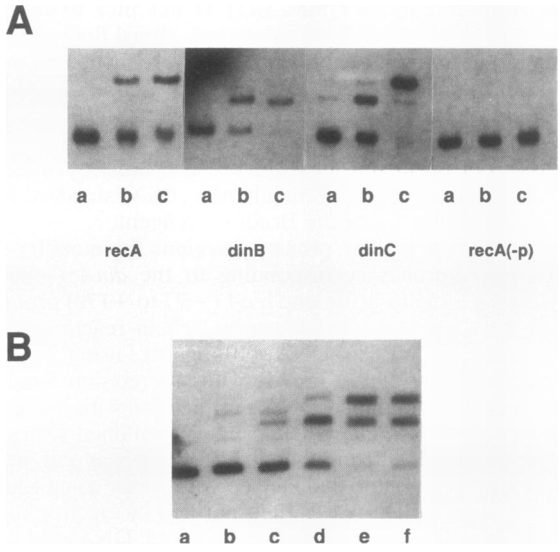

FIG. 2. Binding of a *B. subtilis* protein to *din* promoter regions. (A) Mobility shift assays were conducted with the *recA* (−77 to +34), *dinB* (−155 to +70), *dinC* (−129 to +70), and *recA* (+34 to +170) radiolabeled probes. Reactions contained 50 nM DNA, mobility shift buffer as described in Materials and Methods, and no crude extract (lanes a), 0.1 μl of YB1015 (*recA4*) crude extract (lanes b), and 1 μl of YB1015 crude extract (lanes c). (B) Mobility shift assays with 0.1 μl of YB1015 crude extract, radiolabeled *dinC* promoter DNA (10 nM), and a 10-fold molar excess of unlabeled pPL603 plasmid DNA containing promoter inserts (*dinC* [lane a], *recA* [lane b], *dinB* [lane c], and *dinA* [lane d]), no insert (lane e), and no competing DNA (lane f).

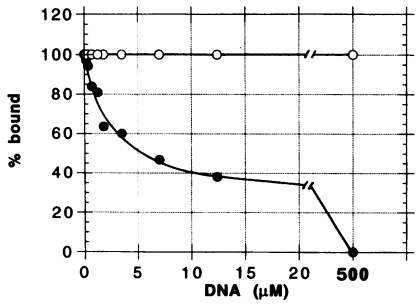

FIG. 3. Inhibition of *dinC* binding by synthetic oligomers. Mobility shift assays were conducted as described for Fig. 2 with 1 μl of YB1015 crude extract and the 199-bp *dinC* promoter fragment (100 nM) in the presence of annealed synthetic oligomers ACCGAACAATTGT TCGGT (●) and ACCGGACAATTGTCCGGT (○). Percent bound was determined by densitometric scanning of autoradiograms.

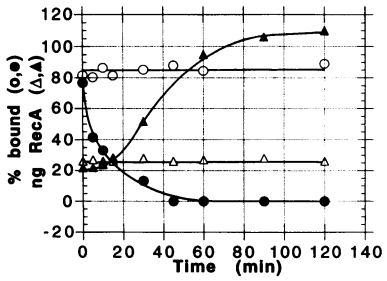

FIG. 4. *din* promoter binding activity and RecA induction following mitomycin C treatment in RecA$^+$ (●, ▲) and *recA4* (○, △) cells. Extracts from equivalent numbers of cells, harvested at indicated times after treatment, were subjected to either mobility shift assays with the 199-bp *dinC* promoter fragment or immunoblot transfer, as described in Materials and Methods, with *B. subtilis* RecA antiserum. Percent *dinC* bound and RecA levels were quantified by densitometric scanning of mobility shift autoradiograms (○) or immunoblots (△).

regions; it was observed only under conditions in which one *dinC* site was bound.

We conducted mobility shift experiments with crude extract to show that the consensus sequence is necessary and sufficient for binding by the putative regulatory protein. To show that the consensus sequence is required for binding, we altered the *recA* consensus sequence and assayed for a mobility shift. We produced the *recA* promoter region depicted in Fig. 1 by PCR amplification using 5′ primers containing various base changes within the consensus site. DNA produced by the wild-type primer, or primers with changes outside the consensus sequence, resulted in the characteristic shift, whereas the deletion of an A as indicated in Fig. 1 completely abolished the shift (data not shown).

To demonstrate that the consensus sequence is sufficient for binding, we tested the ability of a synthetic consensus sequence to compete for the DNA binding activity in mobility shift assays. We constructed a synthetic, self-complementary oligonucleotide having the sequence 5′-ACC**GAACAATTGT TC**GGT-3′. This sequence contains the consensus site (in boldface) but does not otherwise resemble any of the *din* promoter regions. When annealed, the 18-mer duplex effectively inhibited *dinC* binding (Fig. 3). Because the *dinC* concentration used in the assay was more than 10-fold greater than the apparent K_d of 7 nM (18), the K_d for the 18-mer can be approximated by using the concentration that competes for one-half of *dinC* binding in the equation $[dinC]/K_d(dinC) = [18\text{-mer}]/K_d(18\text{-mer})$. Figure 3 shows that 50% inhibition occurs at a competitor DNA concentration of about 6 μM, which yields a K_d of about 420 nM for the 18-mer, indicative of relatively tight and specific binding. By contrast, a similar experiment with the sequence 5′ ACC**GGACAATTGTCC** GGT-3′ resulted in no inhibition, even at millimolar concentrations. These results indicate that the consensus sequence GAACN$_4$GTTC contains a strong binding site for protein.

***din* promoter binding activity is abolished following DNA damage in RecA$^+$ cells.** If the specific binding to *din* promoter regions demonstrated above is due to the activity of an SOS repressor, this binding activity should be reduced in RecA$^+$ cells following treatment with a DNA-damaging agent. Moreover, the rate of decrease in binding activity should be fast enough to precede induction of SOS genes.

We measured the rate of repressor inactivation in vivo by conducting mobility shift assays with extracts from equivalent amounts of cells removed at timed intervals following the addition of mitomycin C. Figure 4 shows the results of densitometric analyses of such assays using the *dinC* promoter region with crude extract harvested from either wild-type or *recA4* cells. The same extracts were also assayed for RecA induction, which was quantified by densitometric analyses of immunoblot transfers. In wild-type cells, *dinC* promoter binding activity decreases with a half-life of less than 5 min; RecA levels begin to increase at about 10 min, in agreement with previous results (19). By contrast, there is no decrease in DNA binding activity in *recA4* cells, which is consistent with the absence of RecA induction in these cells.

Effects of *rec* mutants on *din* promoter binding activity. We examined *dinC* binding activity in crude extracts of RecA$^+$ and DNA repair-deficient cells before and 45 min after treatment with mitomycin C. The 45-min time interval was chosen on the basis of the results shown in Fig. 4 indicating no detectable DNA binding activity in wild-type cells 45 min after exposure to mitomycin C. Figure 5 shows mobility shift assays in which the *dinC* promoter region was incubated with various amounts of crude extracts from mitomycin C-induced and uninduced cells. To ensure stoichiometric binding of the regulatory protein to DNA, the concentration of *dinC* binding sites was at least 100 times greater that the apparent K_d for *dinC* binding (18). Lanes a correspond to assays with undiluted crude extracts from uninduced cells; lanes b and c show assays conducted with these extracts diluted 1:5 and 1:25, respectively. Similarly, lanes f, g, and h correspond to assays with DNA damage-induced extracts undiluted, diluted 1:5, and diluted 1:25, respectively. Before dilution, all crude extracts were adjusted to contain equivalent amounts of total protein as determined by the Bradford assay.

Comparison of lanes a and f in Fig. 5 for wild-type cells shows that specific *dinC* binding activity was significantly reduced following treatment with mitomycin C, as judged by the absence of the two bands characteristic of protein-*dinC* complexes (arrows) in lane f. By contrast, mitomycin C treatment of the *recA4*, *recA1*, and *recM13* mutants had no detectable effect on *dinC* binding activity (compare lanes a and f for each strain). All three *rec* mutants are completely deficient in RecA and *din* operon induction following treatment with mitomycin C (19). In *recA4* and *recA1* mutants, this is probably due to mutations within the *recA* structural gene that result in defective RecA protein (19). The *recM13* strain carries a mutation in the *E. coli recR* homolog, and because the cloned *recM* gene restores normal SOS induction to *recM13* cells, the RecA protein is apparently normal in this strain (1). However,

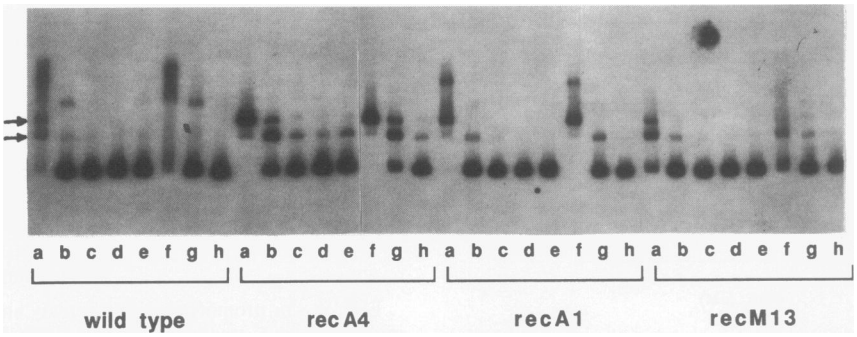

FIG. 5. *din* promoter binding activity in *rec* mutants. Mobility shift assays were conducted as described for Fig. 2 with the 199-bp *dinC* promoter fragment and crude extracts from wild-type, *recA4*, *recA1*, and *recM13* cells. Binding activity was assayed in equivalent amounts of mitomycin C-induced (lanes f to h) and uninduced (lanes a to e) *B. subtilis* extracts. Lanes: a and f, undiluted extracts; b and g, 5-fold dilutions of extracts; c and h, 25-fold dilutions of extracts; d, undiluted, uninduced extracts incubated with *B. subtilis* RecA (1 μM), single-stranded øX174 DNA (10 nM), $MgCl_2$ (5 mM), and dATP (5 mM) for 30 min at 37°C and then assayed for mobility shift; e, undiluted, uninduced extracts incubated with *E. coli* RecA (1 μM), single-stranded øX174 DNA (10 nM), $MgCl_2$ (5 mM), and dATP (5 mM) for 30 min at 37°C and then assayed for mobility shift. Arrows indicate characteristic *dinC*-protein bands.

recM13 mutants are completely deficient in *E. coli* LexA cleavage in vivo (20); thus, the deficiency in SOS induction is probably due to an inability to efficiently activate RecA following DNA damage.

Figure 5 also shows the relative basal levels of *dinC* promoter binding activity in $RecA^+$, *recA4*, *recA1*, and *recM13* cells. Although equivalent amounts of crude extract were used in these assays, there is about fivefold less binding activity (as determined by densitometric scanning) in untreated wild-type, *recA1*, and *recM13* extracts than in *recA4* extracts. This can be readily seen by comparing lane c for the *recA4* extract with lanes b for the other extracts. The higher level of binding activity is consistent with previous observations that the basal level of RecA is lower in *recA4* cells, which was attributed to reduced RecA activity in uninduced *recA4* cells (19). However, because these are crude extracts for which total protein concentration was determined, these differences may also reflect differences in the concentrations of other proteins.

Another notable difference between strains is the presence of very large DNA complexes in reactions with wild-type extracts, and to some extent in *recA1* extracts, that are not visible with the other extracts. The presence of these complexes in both induced and uninduced cells suggests that they are not related to SOS induction.

Purified RecA inactivates *din* promoter binding activity in crude extracts. We infer from the foregoing results that the loss of *dinC* binding activity in wild-type cells, following inducing treatment, results from the activation of RecA protein by the SOS-inducing signal. We have reported elsewhere that the nature of the inducing signal following different DNA-damaging treatments in *B. subtilis* is essentially the same as that in *E. coli* (20, 27). The evidence indicates that the SOS-inducing signal comprises regions of single-stranded DNA, exposed by discontinuous DNA replication (for UV-induced lesions) or generated by the enzymatic processing of lesions (e.g., cross-links or double-stranded breaks). Corresponding to its activation in vivo by the inducing signal, *E. coli* RecA is activated for repressor cleavage in vitro when it binds single-stranded DNA and NTP to form a ternary complex (5, 25). Similarly, *B. subtilis* RecA requires single-stranded DNA and NTP for *E. coli* LexA cleavage in vitro (21). Moreover, *B. subtilis* RecA cleaves *E. coli* LexA in vivo, at a rate comparable to that in vitro, following DNA-damaging treatments (20).

To further show that the loss of binding activity is dependent on RecA, we tested the ability of activated RecA protein to reduce the level of *din* promoter binding activity in the extracts of cells not treated with mitomycin C. Figure 5 shows that the addition of *B. subtilis* RecA plus single-stranded DNA and dATP (lanes d) or *E. coli* RecA plus the same cofactors (lanes e) significantly reduced DNA binding activity in extracts of all strains. By comparison with reactions containing diluted extracts, we estimate that binding activity was reduced by either RecA protein to less than 4% of its original level in all strains. It is not clear why addition of RecA also reduces the amount of larger molecular weight complexes in wild-type cells.

Purification of *din* promoter binding activity. We purified the *din* promoter binding activity from strain YB1015 (*recA4*). This strain was chosen for three reasons: (i) it has the highest level of *dinC* binding activity (Fig. 5), (ii) it displays no detectable nonspecific DNA binding activity under typical mobility shift assay conditions (Fig. 5), and (iii) it is most deficient in RecA activity (which may have affected the integrity and/or amount of the protein during purification). Purification was achieved in one step by using an affinity resin containing the 199-bp *dinC* promoter fragment. We chose the *dinC* promoter because it has the highest affinity for the DNA-binding protein (18) and because there are presumably two binding sites. After incubating a clarified extract of cells with a threefold molar excess of biotinylated *dinC* promoter DNA, we coupled the DNA-protein complex via streptavidin to biotinylated cellulose as described in Materials and Methods. Following several washes of the resin, we eluted the specific DNA binding activity from the resin with buffer containing 1 M KCl. Once the protein was eluted from the DNA, the remaining resin was effectively a specific DNA affinity resin and was reused to purify the protein at least 10 times with no apparent loss in yield, purification level, or activity.

Based on its functional analogy with *E. coli* LexA, we argue below that the putative regulatory protein that binds the *din* consensus sequence is an SOS repressor (see Discussion). The protein will hereafter be referred to as such rather than as the *din* promoter binding activity.

Figure 6 shows a mobility shift assay (Fig. 6A) and a sodium dodecyl sulfate (SDS)-polyacrylamide gel analysis (Fig. 6B) for a typical purification. Comparison of lanes b and c, corresponding to the extract applied to the resin and the protein that did not bind to the resin, respectively, reveals that while

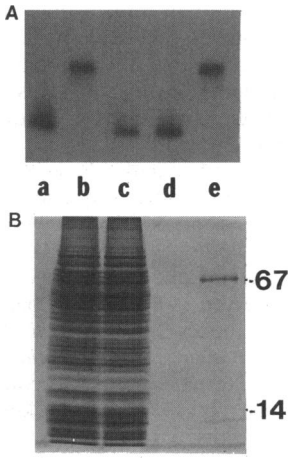

FIG. 6. Purification of *din* promoter binding activity. (A) Crude extract of YB1015 cells was purified as described in Materials and Methods, and fractions were subjected to mobility shift assays with the 100-bp *dinC* fragment. Lanes: a, 199-bp *dinC* promoter fragment; b, crude extract plus the sample in lane a; c, column flowthrough fraction plus the sample in lane a; d, first column wash plus the sample in lane a; e, concentrated and desalted 1 M KCl elution. (B) Purification fractions (lanes b to e) were subject to SDS–13% polyacrylamide gel electrophoresis as described previously (21).

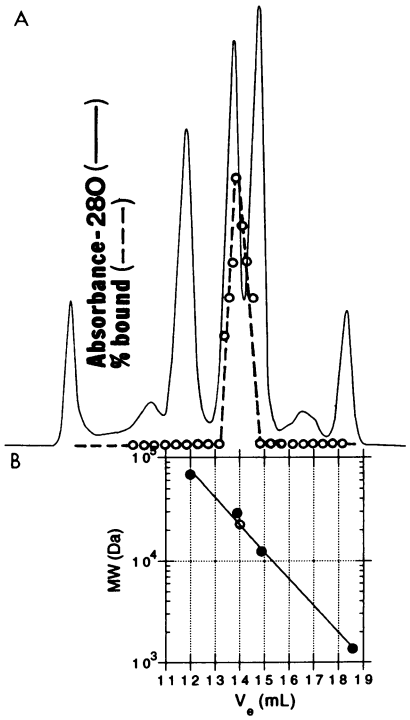

FIG. 7. Molecular weight analysis of *din* promoter binding activity by gel filtration. Samples were chromatographed on an FPLC Superose-12 column as described in Materials and Methods. (A) Chromatogram of molecular weight standards (A_{280} in relative units) and *dinC* promoter binding activity (percent bound in relative units) versus elution volume. Semilog plot of molecular weight versus elution volume for molecular weight standards (●) and *din* promoter binding activity (○).

there is no visible difference in the protein composition between the extract before and after incubation with the resin, virtually all of the DNA binding activity was lost after incubation. After washing of the resin, the active repressor was eluted with high-ionic-strength buffer in highly purified form (lane e).

Two distinct bands corresponding to molecular sizes of 67 and 13.6 kDa are visible on the stained gel. The former is BSA added to the elution buffer to stabilize the eluted protein; the 13.6-kDa protein (which is not present in the BSA preparation) could be the repressor; however, we have no other evidence for this identification. When substantially more protein from the purified fraction was loaded on an SDS-polyacrylamide gel, a large number of protein bands were observed after the gel was silver stained, but these bands were also present in the BSA preparation (data not shown). Assuming a monomeric molecular size of 13.6 kDa, we estimate that the maximum amount of regulatory protein loaded on gel would be about 50 ng (corresponding to a maximum concentration of 5 μg/ml), which would be consistent with the intensity of the band. This estimation is based on titration of crude extract with excess binding site of known concentration, assuming that the protein binds to the palindromic consensus sequence as a dimer (although we have no conclusive evidence that this is the case). Our estimation could be off by a factor of 2, considering the combined error involved in the titration. Although we cannot say with any certainty whether or not the 13.6-kDa protein is the repressor, it is clear that the binding activity has been purified at least 4,000-fold, based on an initial crude extract protein concentration of 20 mg/ml (determined by the Bradford method [2]) and a maximum purified repressor concentration of 5 μg/ml.

The molecular size of the native form of the repressor was determined by gel filtration to be 23 kDa (Fig. 7). The same result was obtained when either crude extract or purified protein was chromatographed. We detected only one peak of activity; however, fractions containing less than 10% of the maximum eluted activity would not have been detected by our assay. Thus, the predominant form of the repressor has a molecular size of about 23 kDa, which may correspond to a monomer or a multimer.

Because the resin used to purify the repressor contained only the *dinC* promoter region, it was conceivable that we purified a protein that was specific for *dinC* and not the SOS consensus sequence located within all *B. subtilis din* promoters. Figure 8 shows a mobility shift assay conducted with the four *din* promoter regions with either *recA4* crude extract (lanes b) or purified repressor (lanes c). (The extraneous bands are

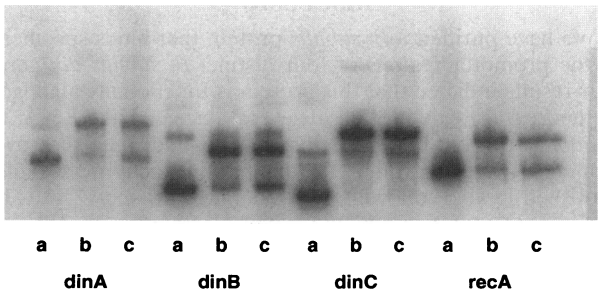

FIG. 8. *din* promoter binding activity in crude extract and purified fractions. Mobility shift assays were conducted as described for Fig. 2 with no protein (lanes a), crude extract (lanes b), and concentrated and desalted 1 M KCl elution of a *dinC* affinity column (lane c) and radiolabeled probes as described in Materials and Methods.

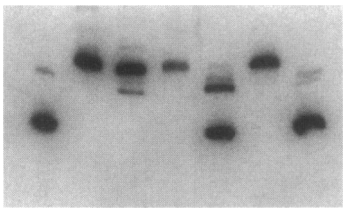

FIG. 9. RecA-mediated inactivation of purified *din* promoter binding activity. The 199-bp *dinC* promoter fragment (lane a) was subjected to mobility shift assays with equivalent amounts of the 1 M KCl elution and preincubated for 30 min at 37°C with mobility shift buffer and the following: ATP (5 mM), $MgCl_2$ (5 mM), and single-stranded øX174 DNA (10 nM) (lane c), *B. subtilis* RecA (1 μM), ATP (5 mM), and $MgCl_2$ (5 mM) (lane d), *B. subtilis* RecA (1 μM), single-stranded øX174 DNA (10 nM), ATP (5 mM), and $MgCl_2$ (5 mM) (lane e), no other components (lane f), and *B. subtilis* RecA (1 μM), single-stranded øX174 DNA (10 nM), dATP (5 mM), and $MgCl_2$ (5 mM) (lane g).

presumably minor PCR products generated during the preparation of the promoter regions.) The assay clearly indicates that the purified repressor binds specifically to each of the four *din* promoter regions, causing shifts that are visually indistinguishable from those produced with crude extract.

Purified RecA inactivates purified *din* promoter binding activity. According to the well-established *E. coli* SOS system model, RecA protein mediates the cleavage of LexA repressor (as well as certain phage repressors) at a site roughly in the middle of the repressor molecule (8). This reaction in vitro requires that RecA (*E. coli* or *B. subtilis*) be activated by binding single-stranded DNA and NTP (5, 21, 25). We found that under similar conditions, *B. subtilis* RecA abolishes *B. subtilis* repressor binding activity in vitro. Figure 9 shows that RecA, in the presence of single-stranded DNA and dATP, completely abolishes repressor binding activity (lane g). When ATP was used instead of dATP, the extent of inactivation was reduced (lane e); this is consistent with the nucleotide specificity in the RecA-mediated cleavage of LexA (21). When NTP, RecA, and single-stranded DNA were omitted from the reaction mixture (lanes b, c, and d, respectively), there was no significant decrease in repressor binding activity. Inactivation was not accompanied by detectable cleavage of the 13.6-kDa protein or any other protein as determined by silver staining of an SDS-polyacrylamide gel containing aliquots of the samples depicted in Fig. 9.

DISCUSSION

We have purified a *B. subtilis* protein that binds specifically to the promoter regions of four distinct *B. subtilis din* genes. Our results indicate that this protein is functionally analogous to the *E. coli* LexA protein in several respects: like LexA, the *B. subtilis* protein binds specifically to palindromic sites overlapping *din* gene promoters; the binding activity is rapidly abolished in vivo following DNA damage in $RecA^+$ cells but not in *recA* mutants; and the binding activity is abolished in vitro by the action of RecA, activated by single-stranded DNA and NTP. Because we have not shown directly that the protein represses *din* gene transcription, its role as the SOS repressor is inferred primarily by analogy with the *E. coli* SOS system (although the purified protein represses *dinC* transcription by *E. coli* RNA polymerase in vitro [18]). However, we have shown conclusively that the protein binds specifically to sites overlapping the promoters of four distinct *din* genes, suggesting that its binding affects the binding of RNA polymerase. Moreover, as discussed below, the binding site suggests that the protein interacts with two opposite faces of the helix, which would probably preclude simultaneous binding by RNA polymerase to *din* promoters.

We have demonstrated that the *B. subtilis* repressor binds specifically to the consensus sequence $GAACN_4GTTC$, thereby supporting the proposal by Cheo et al. that this sequence is the *B. subtilis* SOS box (4). Interestingly, this sequence bears no resemblance to the *E. coli* SOS box, which has been highly conserved in a variety of bacterial species (7). Comparison of SOS boxes from *E. coli*, *Salmonella typhimurium*, *Erwinia carotovora*, *Pseudomonas aeruginosa*, and *Pseudomonas putida* reveals that the consensus sequence $CTGN_{10}CAG$ is conserved in virtually all known LexA operators of these gram-negative bacteria. Although *B. subtilis* is the only gram-positive bacterium for which an SOS box has been identified, it appears that this is one feature of the SOS system that has not been conserved between gram-negative and gram-positive species.

An interesting feature of the *B. subtilis* SOS box is the spacing between the recognition sites. The LexA binding site presumably involves binding of two monomers to the same face of the DNA because the recognition sites are separated by roughly one complete turn of the helix. By contrast, the recognition sites within the *B. subtilis* SOS box are separated by about one-half turn of the helix, which could place two monomers (assuming the repressor binds as a dimer) on opposite faces of the helix, which, to our knowledge, is an unusual arrangement among known DNA-binding proteins.

Our results do not provide information about the identity of the *B. subtilis* SOS repressor. The likely candidate for the LexA analog is the product of the *dinR* gene, which was identified as a damage-inducible gene by transpositional mutagenesis with Tn*917-lacZ* (26). The *dinR* gene encodes a 22.8-kDa polypeptide having 34% identity and 47% similarity with the *E. coli* LexA protein. In fact, our determination of a molecular size of 23 kDa for the repressor is consistent with its being the product of the *dinR* gene. Moreover, *dinR* contains the consensus SOS box within its promoter region, and a wild-type copy of *dinR* is required for SOS-induced expression of a *dinR-lacZ* fusion in a merodiploid strain (26). However, interruption of the *dinR* gene by Tn*917-lacZ* insertion caused reduced induction of *dinC* following DNA damage, contrary to what would be expected in a repressor mutant. It is possible that the insertion, located near the 3′ end of the gene, gives rise to a truncated protein that retains its DNA binding activity but is deficient in its interaction with RecA. We are presently testing this possibility.

With the exception of *dinC*, the repressor binds to one site upstream of *din* genes. The two sites in the *dinC* promoter region are separated by 18 bp, suggesting the potential for cooperative interactions. In fact, quantitative studies of repressor binding indicate a high level of cooperativity between the two *dinC* sites (18). There are three *recA* consensus sites centered at −110, −50, and +80 (4); however, we found that only the −50 site resulted in a mobility shift with crude extract (Fig. 2 and data not shown). We do not know why the other two sites are not bound by repressor under our assay conditions, but the question is under investigation.

The rate of decrease in operator binding activity following DNA damage is very similar to the rate of *E. coli* LexA cleavage in *B. subtilis* (20), which is significantly slower than LexA cleavage in *E. coli* (25). This is consistent with our previous observation that induction of *B. subtilis* RecA is

slower than induction of its counterpart in *E. coli* (19). Specifically, there is a lag period of nearly 15 min following inducing treatment in *B. subtilis* that does not occur in *E. coli*. It is not clear whether the difference in response time to DNA damage is due to differences in the rate at which RecA is activated or differences in the rate of repressor inactivation. At the present time, we have no evidence regarding the nature of repressor inactivation in *B. subtilis*.

If the repressor is the product of the *dinR* gene, it is not unlikely that repressor is cleaved; the *dinR* sequence reveals that the LexA cleavage site has been highly conserved in the two proteins (26). In any case, the elucidation of the repressor inactivation mechanism in *B. subtilis* will be of particular interest because it will either further our understanding of the unusual autocatalytic activity of LexA or reveal a completely novel mechanism for SOS induction.

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REFERENCES

1. **Alonso, J. C., K. Shirahige, and N. Ogasawara.** 1990. Molecular cloning, genetic characterization and DNA sequence analysis of the *recM* region of *Bacillus subtilis*. Nucleic Acids Res. **18:**6771–6777.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1988. Current protocols in molecular biology. John Wiley & Sons, New York.
3. **Brent, R., and M. Ptashne.** 1981. Mechanism of action of the *lexA* gene product. Proc. Natl. Acad. Sci. USA **78:**4204–4208.
4. **Cheo, D. L., K. W. Bayles, and R. E. Yasbin.** 1991. Cloning and characterization of DNA damage-inducible promoter regions from *Bacillus subtilis*. J. Bacteriol. **173:**1696–1703.
5. **Craig, N. L., and J. W. Roberts.** 1980. *E. coli* RecA protein-directed cleavage of phage lambda repressor requires polynucleotide. Nature (London) **283:**26–30.
6. **Friedman, B., and R. E. Yasbin.** 1983. The genetics and specificity of the constitutive excision repair system of *Bacillus subtilis*. Mol. Gen. Genet. **190:**481–486.
7. **Garrig, X., S. Calero, and J. Barbe.** 1992. Nucleotide sequence analysis and comparison of the *lexA* genes from *Salmonella typhimurium*, *Erwinia carotovora*, *Pseudomonas aeruginosa*, and *Pseudomonas putida*. Mol. Gen. Genet. **236:**125–134.
8. **Horii, T., T. Ogawa, T. Nakatani, T. Hase, H. Matsubara, and H. Ogawa.** 1981. Regulation of SOS functions: purification of *E. coli* LexA protein and determination of its specific site cleaved by RecA protein. Cell **27:**515–522.
9. **Hurstel, S., M. Granger-Schnarr, M. Daune, and M. Schnarr.** 1988. In vitro binding of LexA repressor to DNA: evidence for the involvement of the amino-terminal domain. EMBO J. **5:**793–798.
10. **Kim, B., and J. W. Little.** 1992. Dimerization of a specific DNA-binding protein on the DNA. Science **255:**203–206.
11. **Little, J. W.** 1984. Autodigestion of lexA and phage lambda repressors. Proc. Natl. Acad. Sci. USA **81:**1375–1379.
12. **Little, J. W., S. H. Edmiston, L. Z. Pacelli, and D. W. Mount.** 1980. Cleavage of Escherichia coli lexA protein by the recA protease. Proc. Natl. Acad. Sci. USA **77:**3225–3229.
13. **Little, J. W., and D. W. Mount.** 1982. The SOS regulatory system of *Escherichia coli*. Cell **29:**11–22.
14. **Little, J. W., D. W. Mount, and C. R. Yanisch-Perron.** 1981. Purified LexA protein is the repressor of the *recA* and *lexA* genes. Proc. Natl. Acad. Sci. USA **78:**4199–4203.
15. **Love, P. E., M. J. Lyle, and R. E. Yasbin.** 1985. DNA damage-inducible (*din*) loci are transcriptionally activated in competent *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **82:**6201–6205.
16. **Love, P. E., and R. E. Yasbin.** 1984. Genetic characterization of the inducible SOS-like system of *Bacillus subtilis*. J. Bacteriol. **160:**910–920.
17. **Love, P. E., and R. E. Yasbin.** 1986. Induction of the *Bacillus subtilis* SOS-like response by *Escherichia coli* RecA protein. Proc. Natl. Acad. Sci. USA **83:**5204–5208.
18. **Lovett, C. M., and P. Banky.** Unpublished data.
19. **Lovett, C. M., P. E. Love, R. E. Yasbin, and J. W. Roberts.** 1988. SOS-like induction in *Bacillus subtilis*: induction of the RecA protein analog and a damage-inducible operon by DNA damage in Rec^+ and DNA repair-deficient strains. J. Bacteriol. **170:**1467–1474.
20. **Lovett, C. M., T. M. O'Gara, and J. N. Woodruff.** Submitted for publication.
21. **Lovett, C. M., and J. W. Roberts.** 1985. Purification of a RecA protein analogue from *Bacillus subtilis*. J. Biol. Chem. **260:**3305–3313.
22. **Marrero, R., and R. E. Yasbin.** 1988. Cloning of the *Bacillus subtilis* $recE^+$ gene and functional expression of $recE^+$ in *B. subtilis*. J. Bacteriol. **170:**335–344.
23. **Meihl-Lester, R.** 1985. Induced mutagenesis in *Bacillus subtilis*. Ph.D. thesis. University of Rochester, Rochester, N.Y.
24. **Miller, R. V., and T. A. Kokjohn.** 1990. General microbiology of *recA*. Environmental and evolutionary significance. Annu. Rev. Microbiol. **44:**365–394.
25. **Phizicky, E. M., and J. W. Roberts.** 1981. Induction of SOS functions: regulation of proteolytic activity of *E. coli* RecA protein by interaction with DNA and nucleoside triphosphate. Cell **25:**259–267.
26. **Raymond-Denise, A., and N. Guillen.** 1991. Identification of *dinR*, a DNA damage-inducible regulator gene of *Bacillus subtilis*. J. Bacteriol. **173:**7084–7093.
27. **Sassanfar, M., and J. W. Roberts.** 1990. Nature of the SOS-inducing signal in *Escherichia coli*. The involvement of DNA replication. J. Mol. Biol. **212:**79–96.
28. **Schnarr, M., P. Oertel-Buchheit, M. Kazmaier, and M. Granger-Schnarr.** 1991. DNA binding properties of the LexA repressor. Biochimie **73:**423–431.
29. **Schnarr, M., J. Pouyet, M. Granger-Schnarr, and M. Daune.** 1985. Large-scale purification, oligomerization equilibria, and specific interaction of the LexA repressor of *Escherichia coli*. Biochemistry **24:**2812–1818.
30. **Slilaty, S. N., and J. W. Little.** 1987. Lysine-156 and serine-119 are required for LexA repressor cleavage: a possible mechanism. Proc. Natl. Acad. Sci. USA **84:**3987–3991.
31. **Stranahan, M. C., K. W. Bayles, and R. E. Yasbin.** 1990. The nucleotide sequence of the *recE+* gene of *Bacillus subtilis*. Nucleic Acids Res. **18:**4249.
32. **Walker, G. C.** 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. Microbiol. Rev. **48:**60–93.
33. **Wertman, K. F., and D. W. Mount.** 1985. Nucleotide sequence binding specificity of the LexA repressor of *Escherichia coli* K-12. J. Bacteriol. **163:**376–384.
34. **Wojciechowski, M. F., K. R. Peterson, and P. E. Love.** 1991. Regulation of the SOS response in *Bacillus subtilis*: evidence for a LexA repressor homolog. J. Bacteriol. **173:**6475–6483.
35. **Yasbin, R. E., D. L. Cheo, and K. W. Bayles.** 1992. Inducible DNA repair and differentiation in *Bacillus subtilis*: interactions between global regions. Mol. Microbiol. **6:**1263–1270.
36. **Yasbin, R. E., P. I. Fields, and B. J. Anderson.** 1980. Properties of *Bacillus subtilis* derivatives freed of their natural prophages. Gene **12:**155–159.