Interaction of the *Bacillus subtilis* *glnRA* Repressor with Operator and Promoter Sequences In Vivo†

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In vivo dimethyl sulfate footprinting of the *Bacillus subtilis* *glnRA* regulatory region under repressing and derepressing conditions demonstrated that the GlnR protein, encoded by *glnR*, interacts with two sites situated within and adjacent to the *glnRA* promoter. One site, *glnRAo*₁, between positions –40 and –60 relative to the start point of transcription, is a 21-bp symmetrical element that has been identified as essential for *glnRA* regulation (H. J. Schreier, C. A. Rostkowski, J. F. Nomellini, and K. D. Hirschi, J. Mol. Biol. 220:241–253, 1991). The second site, *glnRAo*₂, is a quasisymmetrical element having partial homology to *glnRAo*₁ and is located within the promoter between positions –17 and –37. The symmetry and extent of modifications observed for each site during repression and derepression indicated that GlnR interacts with the *glnRA* regulatory region by binding to both sites in approximately the same manner. Experiments using potassium permanganate to probe open complex formation by RNA polymerase demonstrated that transcriptional initiation is inhibited by GlnR. Furthermore, distortion of the DNA helix within *glnRAo*₂ occurred upon GlnR binding. While glutamine synthetase, encoded by *glnA*, has been implicated in controlling *glnRA* expression, analyses with dimethyl sulfate and potassium permanganate ruled out a role for glutamine synthetase in directly influencing transcription by binding to operator and promoter regions. Our results suggested that inhibition of transcription from the *glnRA* promoter involves GlnR occupancy at both *glnRAo*₁ and *glnRAo*₂. In addition, modification of bases within the *glnRAo*₂ operator indicated that control of *glnRA* expression under nitrogen-limiting (derepressing) conditions included the involvement of a factor(s) other than GlnR.

Upon nutrient deprivation, vegetative cells of *Bacillus subtilis* differentiate into metabolically dormant spores. While the sporulation process itself has been the focus of a considerable amount of work, relatively little is known regarding mechanisms related to sporulation initiation (13, 40). One condition that stimulates spore production is exhaustion of the nitrogen source in the medium (41). An understanding of the details by which nitrogen assimilation is regulated may, therefore, provide some insight into how the organism recognizes that it is starved for nitrogen. In bacteria, a pivotal step in nitrogen metabolism is formation of glutamine by glutamine synthetase (GS). Since most of the organism's nitrogen-containing compounds are synthesized from glutamine, glutamine production is potentially an important control point.

In *Escherichia coli* and other enteric bacteria, GS activity is controlled by the nitrogen state of the cell, being regulated at the transcriptional and posttranslational levels (for reviews, see references 18, 19, and 27). Furthermore, control of GS is coupled to the Ntr system, a global regulatory network which activates the expression of genes involved in the utilization of nitrogen-containing compounds. Under nitrogen-limiting conditions, transcription of these genes, as well as *glnA*, the GS structural gene, requires RNA polymerase σ^{54} ($E\sigma^{54}$) and the *ntrC* gene product, NR_I, in its phosphorylated form (18). For *glnA*, NR_I-phosphate binds to as many as five enhancerlike elements upstream of the *glnAp*₂ promoter (15, 22). The protein then catalyzes the

isomerization of a pre-existing $E\sigma^{54}$ -promoter closed complex to the open form (25, 31), which is assisted by formation of a DNA loop (44, 45). For some other $E\sigma^{54}$ -dependent promoters, such as *glnHp*₂ in *E. coli* or those in the *nif* operon of *Klebsiella pneumoniae*, interactions of NR_I-phosphate with $E\sigma^{54}$ through loop formation requires integration host factor protein (5, 44).

Like the enteric bacteria, *B. subtilis* regulates GS expression as a function of the nitrogen source used for growth (11, 24, 26, 39). Levels are at their lowest when cells are grown in the presence of a preferred nitrogen source, such as glutamine. However, unlike in *E. coli*, the enzyme is not regulated by posttranslational modification (9, 12). Furthermore, transcription of the *glnA* gene, which is in the *glnRA* operon, requires only the vegetative RNA polymerase ($E\sigma^A$) (4, 34, 43) and is controlled in a negative manner (35, 36, 39). Regulation of operon expression involves the action of both gene products—GlnR, encoded by *glnR*, and GS. Mutations in either gene result in constitutive expression (8, 35, 36, 39).

Recent studies have provided evidence that GlnR is the *glnRA* repressor (4, 35, 38). The protein appears to be a homodimer in solution, having a subunit molecular weight of approximately 16,000 (4, 21, 23). It shares characteristics with the class of DNA-binding proteins containing the α -helix–turn– α -helix motif (35). Dimethyl sulfate (DMS), DNase, and restriction enzyme protection studies, as well as electrophoretic mobility shift experiments, have shown that GlnR binds to sequences in the vicinity of the *glnRA* promoter (4, 38). Moreover, purified GlnR is capable of repressing *glnRA* transcription in vitro (4).

The mechanism by which GlnR activity is controlled has not been established, although there is evidence that covalent modification is not involved (23, 37). The responsive-

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ness of GlnR to the availability of rapidly utilizable nitrogen sources may be mediated by some nitrogen-dependent factor(s) that is dependent, in part, on the activity of GS, since certain *glnA* regulatory mutations are located within regions involved in enzymatic activity (46). However, the specific factors involved have not been identified and the relationship between GS and GlnR is not understood.

We have recently demonstrated that sequences adjacent to the *glnRA* promoter, upstream of position -35, are necessary for regulation and have suggested that the *glnRA* operator is a 21-bp sequence that extends from positions -40 to -60 and contains twofold nucleotide symmetry (38). Our study could not rule out the possibility that sequences within the promoter region (between positions -10 and -35) are also involved in repressor binding in vivo. In this study, we began to address the nature of repressor-DNA interactions by examining patterns of DNA modification in vivo. We found that patterns obtained for GlnR binding in vivo were comparable to those observed in vitro. Furthermore, we showed that the protein not only binds to the upstream operator element but also interacts with promoter sequences.

MATERIALS AND METHODS

Bacterial strains. *E. coli* YMC11 (*endA1 hsdR17 thi-1 supE44 ΔlacU169 hutC* *Klebsiella* *ΔglnAntrBC2000*) (2) and *B. subtilis* SMY (wild type) and HJS31 (*ΔglnR57*) (35) have been described previously. Competent cells of *E. coli* and *B. subtilis* were prepared by the method of Cohen et al. (6) and Sonenshein et al. (42), respectively.

Plasmids. Purification of plasmids by cesium density centrifugation and small-scale plasmid preparations were done as described by Sambrook et al. (28). Restriction maps of the *glnRA* DNA region cloned in plasmids used in this study are shown diagrammatically in Fig. 1. Plasmids pSF14, pJCG10, pJCG11, pJCG12, and pJCG14 are derivatives of plasmid pJH101 (10). Plasmid pSF14 (*glnR*⁺ *glnA*⁺ Tet^r Cam^r) contains a 2.7-kb *B. subtilis* DNA insert harboring the *glnRA* operon and was described previously (11). Plasmid pJCG10 (*glnR*⁺ *ΔglnA56* Tet^r Cam^r) was constructed by deleting the 800-bp *EcoRI* fragment within *glnA*. Plasmid pJCG11 (*ΔglnR57 glnA*⁺ Tet^r Cam^r) is a pSF14 derivative containing a 270-bp in-frame deletion within *glnR*. The plasmid was constructed by replacing the *SacI*-*AvaI* Tet^r fragment of plasmid pJH557 (35) with the *SacI*-*AvaI* Tet^r fragment from plasmid pSF14. Plasmid pJCG12 (*ΔglnR54 glnA*⁺ Tet^r Cam^r) is a derivative of plasmid pSF14 containing a 71-bp in-frame deletion within *glnR* and was constructed by replacing the *PstI*-*AvaI* Tet^r fragment of plasmid pJH554Bam (35) with the *PstI*-*AvaI* Tet^r fragment of plasmid pSF14. Plasmid pJCG14 (*ΔglnRA14* Tet^r Cam^r) was constructed by deleting the 1,180-bp *HpaI* fragment of pSF14, removing approximately 80 and 50% of the GlnR C-terminal and GS N-terminal coding regions, respectively.

Plasmid pIDG1 is a derivative of pHP13 (14) that harbors the 700-bp *glnRAop*-containing *HindIII* fragment from plasmid pJH5104 (39). Plasmid pHP13 and its derivatives replicate in *E. coli* and *B. subtilis*, having a low copy number in the latter organism (14).

Media and growth. Growth of *E. coli* in minimal medium containing glutamine and ammonia (excess nitrogen) or glutamine (limiting nitrogen) was done as described previously (36). *B. subtilis* cultures were grown in TSS medium containing either glutamine (a preferred nitrogen source) or glutamate as the nitrogen source (39). Tetracycline and

FIG. 1. Abbreviated restriction maps of plasmids used in this study. The top line shows a 3.2-kb *HindIII* region of *B. subtilis* DNA containing the entire *glnRA* operon. The map indicates the relative positions of the GlnR (medium bar) and GS (heavy bar) coding regions and the location of the start point for transcription (arrow). Below are shown the DNA contents of plasmids carrying portions of this region. Also shown are the *glnR* and *glnA* genotypes of each plasmid; M refers to the *glnR54* mutation found on plasmid pJCG12. Regulation (Reg): GS levels were regulated (+), partially regulated (±), or not regulated (-) when plasmids containing the indicated genotypes were present in *E. coli* YMC11, as described previously (35, 36). Restriction sites that were altered during cloning are in parentheses. Abbreviations of restriction sites: Ah, *AhaIII*; Bm, *BamHI*; E, *EcoRI*; H, *HindIII*; Hp, *HpaI*; Sc, *SacI*.

chloramphenicol were added to final concentrations of 10 and 5 μg/ml, respectively.

In vivo modification. When midexponential growth was attained, modification of DNA with permanganate was done as described previously (30-32) in the following manner. Potassium permanganate was added to 10 ml of culture to a final concentration of 10 mM. After being shaken for 2 min at 30°C, the cultures were poured into prechilled tubes and collected by centrifugation at 3,000 × *g* and 4°C for 5 min. Plasmid DNA was then isolated as described by Holmes and Quigley (16). After isopropanol precipitation, the pellets were suspended in 200 μl of TE80 (10 mM Tris-HCl [pH 8.0], 1 mM disodium EDTA) and treated with RNase (final concentration, 250 μg/ml). After phenol extraction and ethanol precipitation, DNA was suspended in 100 μl of H₂O and passed through a Sephadex G50-80 column (Pharmacia) previously equilibrated with distilled H₂O. The preparation was then used for primer extension reactions. For studies done in the presence of rifampin, rifampin was added to a final concentration of 200 μg/ml for 5 min prior to permanganate addition.

In principle, modification with DMS was done as described by Sasse-Dwight and Gralla (30), with the following alterations. DMS was added to mid-exponential-phase cultures to a final concentration of 4.2 mM, and the cultures were incubated for 4 min at 30°C for *E. coli* and at 37°C for *B. subtilis*. After harvesting, plasmid DNA was extracted by alkaline sodium dodecyl sulfate treatment (28). For *B. subtilis*, plasmids were isolated as for *E. coli*, except that lysis was done at 37°C with lysozyme at a final concentration of 10 mg/ml. After precipitation, DNA was extracted three times with buffered phenol and once with chloroform-isoamyl alcohol (24:1) and precipitated with 0.1 volume of 4 M sodium acetate and 2.5 volumes of ethanol. The DNA was then treated with 1 M piperidine for 30 min at 90°C and

lyophilized to dryness. After suspension in 25 μ l of H₂O, the preparations were spun through 0.6-ml columns of Sephadex G25-50.

In vitro modification. Purified GlnR protein (final concentration, 6 ng/ μ l), kindly provided by J. F. Nomellini, was added to 1 μ g of plasmid DNA in GlnR binding buffer (35) to a total volume of 50 μ l at 4°C for 5 min. For methylation experiments, 5 μ l of a 105 mM stock of DMS was added and the samples were kept at 37°C for exactly 4 min. For permanganate modification experiments, 5 μ l of a 100 μ M potassium permanganate stock was added and incubation was continued for 2 min at 37°C. Both modification reactions were quenched by addition of 100 μ l of 2.5 M ammonium acetate, 2.5 μ l of 1 M β -mercaptoethanol, 5 μ l of 250 mM disodium EDTA, and 2 μ l of yeast tRNA (1 mg/ml). After phenol-chloroform extraction, DNA was precipitated by addition of 2.5 volumes of ethanol. Samples were then processed in the same manner as in vivo-treated DNA samples.

Primer extensions. Oligonucleotide primers annealing to the top and bottom strands of DNA flanking the *glnRA* promoter region were 5' end labeled with [γ -³²P]ATP with the DuPont labeling system as recommended by the manufacturer. Primer specific activity was usually 150,000 dpm/pmol in a total volume of 25 μ l. Primer CS-6, 5'-TCGCAT GTGAAGAATG-3', contains the top strand sequence from positions -146 to -131; extension from this primer approaches the -35 region from upstream sequences. Primer CS-112, 5'-CTGCATGACAATTCC-3', contains the bottom strand sequence from positions +82 to +68; extension from this primer approaches the -10 region from the downstream direction. For permanganate-treated DNA samples, primer extension reactions were performed as described previously (30). Generally, 1 μ l of ³²P-labeled primer and 4 μ l of 10 mM NaOH were added to 35 μ l of permanganate-treated DNA. The samples were incubated at 80°C for 2 min to allow for denaturation and then kept at 4°C for 5 min. Five microliters of a 10 $\times$ hybridization buffer (0.5 M Tris · HCl [pH 7.2], 0.1 M MgSO₄, 2 mM dithiothreitol) was then added, and hybridization was done at 45°C for 3 min, after which the samples were kept at 4°C. Following hybridization, 5 μ l of a 5 mM deoxynucleoside triphosphate (dNTP) stock solution was added and extension was done at 50°C for 20 min after addition of 2.5 U of Klenow. The samples were then precipitated and suspended in a formamide dye mixture, and electrophoresis was done with a 6% urea-polyacrylamide gel. DNA fragments were visualized by autoradiography using Kodak XAR-5 film.

Denaturation of DMS-treated DNA isolated from *E. coli* was done by treating 8- μ l samples with 2 μ l of 0.2 N NaOH for 5 min at room temperature. The DNA was then precipitated by addition of 2 μ l of 4 M sodium acetate, 8 μ l of H₂O, and 75 μ l of ethanol. The washed and dried DNA pellet was then suspended in 1 μ l of ³²P-labeled primer, 1 μ l of 10 $\times$ hybridization buffer, and 6 μ l of H₂O and kept at 65°C for 2 min. The samples were then allowed to cool slowly to below 35°C, and extension was initiated by addition of 2 μ l of a solution containing dNTPs (each at 5 mM), dithiothreitol (1 mM), and Klenow (2 U). After incubation at 37°C for 15 min, DNA was precipitated, suspended in a formamide dye mixture, and subjected to polyacrylamide gel electrophoresis. Modification patterns were scanned by using a Bio-Rad 620 video densitometer and analyzed either by hand or on an IBM AT with Bio-Rad integration software. Bands corresponding to G residues at +2 and +7 in the top strand and

-68 and -70 in the bottom strand, which were unaltered in response to the nitrogen source, were used for reference.

Primer extension reactions using DMS-treated plasmids extracted from *B. subtilis* were done by using a modification of the polymerase chain reaction described by Sasse-Dwight and Gralla (33). DNA (1 μ g) was mixed with 10 μ l of 10 $\times$ *Taq* polymerase buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], 15 mM MgCl₂, 0.1% [wt/vol] gelatin, 1% [wt/vol] Triton X-100 [Promega Corp.]), 4 μ l of a 5 mM dNTP mixture, 1 μ l of a ³²P-labeled primer, 0.5 μ l of *Taq* polymerase (5 U/ μ l), and H₂O to a total volume of 100 μ l and covered with 100 μ l of mineral oil. The samples were then denatured at 94°C for 1.5 min, hybridized for 2 min at 45°C, and extended for 3 min at 72°C. This cycle was repeated an additional 16 times, with the length of denaturation reduced to 1 min and the extension time for the final cycle allowed to continue for 10 min instead of 3. The samples were then removed from the mineral oil, extracted with chloroform-isoamyl alcohol, precipitated with one-third of a volume of 4 M ammonium acetate-20 mM disodium EDTA and subjected to electrophoresis as described above.

Sanger dideoxy sequencing (29) of plasmid pSF14 with primers used for the extension reactions was done to assign base positions for both DMS- and potassium permanganate-treated samples.

Enzyme assays. GS levels were determined in cells made permeable by treatment with hexadecyltrimethylammonium bromide and washed and assayed as described previously (39). Protein determinations were done by the method of Lowry et al. (17).

Materials. All reagents were of the highest purity available. Restriction endonucleases and DNA modification enzymes were purchased from Boehringer Mannheim, Promega, or New England Biolabs. Klenow was obtained from New England Biolabs. Electrophoretically pure glutamine was from Calbiochem. DNA oligonucleotides were synthesized by Promega Corp.

RESULTS

Modification of the *glnRA* operator by DMS in vitro. Our previous studies showed that the DNA region necessary for nitrogen-dependent control included a 21-bp symmetrical sequence extending from positions -40 to -60 relative to the start point of transcription (38). To examine whether GlnR interacts with these sequences in vitro, we treated plasmid pSF14 with DMS in the absence or presence of purified GlnR. By the primer extension approach (see Materials and Methods), downstream and upstream ³²P-labeled primers were used to analyze modification along top and bottom strands, respectively. Results from these studies are shown in Fig. 2A (top strand) and B (bottom strand), lanes 9 (without GlnR) and 10 (with GlnR). The patterns of modification obtained in the presence of GlnR demonstrated that the protein interacted with sequences within the -40 to -60 region. Protection from DMS modification was observed at G residues at positions -57 in the top strand (compare lanes 9 and 10, Fig. 2A) and -43 in the bottom strand (compare lanes 9 and 10, Fig. 2B). Hypermethylation of G at positions -52 in the top strand and -48 in the bottom strand was also found.

In addition to binding to operator site sequences, GlnR also interacted with sequences within the *glnRA* promoter region. Protection from methylation was observed for G at -34 in the top strand (lanes 9 and 10, Fig. 2A) and -20 in the bottom strand (lanes 9 and 10, Fig. 2B). Hypermethylation

FIG. 2. In vivo DMS modification patterns of the *glnRA* regulatory region along top (A) and bottom (B) strands. The plasmids

occurred at G residues at -32 and -8 in the bottom strand and at a T at position -14 in the top strand. Methylation of thymines by DMS has been previously described by Sasse-Dwight and Gralla (30), but the chemistry of modification is unknown.

These results showed that in vitro, the GlnR-binding site included the operator as well as sequences within the *glnRA* promoter region, between positions -8 and -35. Recently, Brown and Sonenshein (4) examined GlnR binding by DNase protection and DMS modification studies in vitro and found that the protein associated with sequences extending from positions -14 to -66. Their study used linearized *glnRA* DNA fragments to probe GlnR interactions and could not, therefore, determine any contribution that DNA conformation may have on GlnR binding. However, the patterns of protection and enhancement from DMS modification described by Brown and Sonenshein (4) were very similar to those described above, which were obtained by using supercoiled plasmid DNA, suggesting that in vitro, binding of GlnR to both operator and promoter regions is not dependent on DNA conformation.

Analysis of DMS modification in vivo. To study repressor binding in vivo, we grew *E. coli* YMC11, harboring various *B. subtilis glnRA* plasmid constructions, under nitrogen excess (glutamine and ammonia) and limiting (glutamine) conditions and treated with DMS. Plasmids were extracted, and modification was analyzed by primer extension as described in Materials and Methods.

Expression of GS in *E. coli* YMC11(pSF14) (*glnR*⁺ *glnA*⁺) is regulated by the nitrogen source, being repressed approximately 10-fold under nitrogen excess conditions (11, 36). Figure 2A (top strand) and B (bottom strand) show results obtained from strain YMC11(pSF14) grown under repressing (lane 1) conditions. With two exceptions, the pattern of modification observed in vivo was similar to that obtained in vitro in the presence of GlnR (Fig. 2, lane 10). Thus, G residues at positions -57 and -34 in the top strand and -43 and -20 in the bottom strand were protected from modification. Modification was enhanced at positions -48, -32, and -8 in the bottom strand. The exceptions were seen at positions -52 (G) and -14 (T) in the top strand, which were hypermodified in vitro but not in vivo. Furthermore, T residues at positions -72 and -73 in the top strand (Fig. 2A, lane 1) were protected from methylation in vivo but not in vitro.

Results obtained from strain YMC11(pSF14) grown under nitrogen-limiting (derepressing) conditions are shown in Fig. 2A and B, lanes 2. Under these conditions, the extent of modification of bases at most positions was similar to that observed for strain YMC11(pSF14) grown under repressing conditions (Fig. 2A and B, lanes 1). However, differences in

harbored by *E. coli* YMC11 were pSF14 (lanes 1 and 2), pJCG10 (lanes 3 and 4), pJCG11 (lanes 5 and 6), and pJCG14 (lanes 7 and 9). Modification of plasmid pSF14 in vitro in the absence (lane 9) or presence (lane 10) of GlnR was done as described in Materials and Methods by using Klenow for the primer extension reaction. The arrowheads indicate bases at which protection was observed; the diamonds indicate bases that exhibited hyperreactivity to DMS. Not indicated are T residues modified by DMS (see Results). Assignments were made on the basis of densitometry scans as described in Materials and Methods. Glutamine (G) and glutamine-ammonia (G + N) cultures were limiting- and excess-nitrogen conditions, respectively. The brackets to the sides delineate the *glnRA* operator from -40 to -60.

the intensity of modification were observed at positions within operator and promoter regions compared with bases outside these regions. Densitometric analysis (data not shown) revealed that G residues at -34 and -57 in the top strand and -20 and -43 in the bottom strand were partially protected from methylation compared with patterns obtained under repressing conditions (Fig. 2A and B, lanes 1). Similarly, methylation enhancement at positions -32 and -48 in the top strand was reduced in the derepressed condition (Fig. 2B, lane 2). Partial protection from modification or decreased hypermodification observed under nitrogen-limiting conditions is explained by the presence of some repressor activity and is consistent with previous results (35). Under derepressing conditions, GS levels from strain YMC11(pSF14) are approximately two- to threefold lower than the maximum derepressed levels obtained, for instance, from a strain containing a *glnR* mutation (35).

The nitrogen-dependent changes observed in modification patterns at sites shown to be utilized for GlnR binding in vitro provide further evidence that these positions participate in GlnR binding in vivo. A GlnR-dependent modification that was not found to vary with the nitrogen source was the hypermethylation at position -8 in the bottom strand (Fig. 2B, lanes 1 and 2). On the other hand, modification of T residues at positions -72 and -73 in the top strand, which were not protected by GlnR in vitro, was found to be regulated by the nitrogen source in vivo (compare lanes 1 and 2, Fig. 2A).

To analyze modification patterns obtained in the absence of GlnR in vivo, we examined patterns from strain YMC11(pJCG11) grown under excess- and limiting-nitrogen conditions. Plasmid pJCG11 ($\Delta glnR57 glnA^+$) carries the *glnR57* allele, which results in constitutive GS expression (35). The inability to detect GlnR activity under nitrogen excess conditions was demonstrated by the absence of protections or enhancements within the -20 to -60 region of both strands (Fig. 2A and B, lanes 5 and 6) and, at most positions, were similar to the results obtained in vitro in the absence of GlnR. Comparable results were found for the patterns from strain YMC11(pJCG14) ($\Delta glnRA14$) (Fig. 2A and B, lanes 7 and 8). Enhanced modification at position -8 in the bottom strand, however, was observed in both strains, regardless of the condition of growth.

Strain YMC11(pJCG10) contains the $\Delta glnA56$ allele, an 800-bp deletion within *glnA*, and cannot produce GS (36). It has previously been shown that *E. coli* host strains harboring this mutation have lost the ability to regulate *glnRA* expression (35). Under both limiting- and excess-nitrogen conditions, transcription from the *glnRA* promoter was derepressed, although levels were not as high as those found in a *glnR* mutant background (35). Thus, any remaining repression was due to some GlnR contribution. Modification patterns from YMC11(pJCG10) grown under repressing and derepressing conditions are shown in Fig. 2A and B, lanes 3 and 4. In the operator and promoter regions, we found patterns similar to those obtained from YMC11(pSF14) grown under repressing conditions (Fig. 2A and B, lane 1), indicating the presence of GlnR activity under both conditions. The lack of regulation observed within these regions indicated that GlnR had lost the ability to recognize nitrogen-limiting conditions and is consistent with a role for GS in control. Interestingly, modification of T residues upstream of the operator, at positions -72 and -73 in the top strand, occurred only under nitrogen excess conditions (Fig. 2A, lane 4). The absence of protection at these positions under

TABLE 1. GS activities of *B. subtilis* strains grown in the presence of various nitrogen sources^a

Strain	Relevant genotype	GS act (mean $\pm$ SD) ^b		Repression ratio
		Glutamine	Glutamate	
SMY	<i>glnRA</i> ⁺	0.40 $\pm$ 0.10	3.3 $\pm$ 1.0	8.8
HJS31	$\Delta glnR57$	5.5 $\pm$ 0.49	6.4 $\pm$ 1.4	1.2
SMY(pIDG1)	<i>glnRA</i> ⁺	0.28 $\pm$ 0.10	2.4 $\pm$ 1.0	8.6
HJS31(pIDG1)	$\Delta glnR57$	5.0 $\pm$ 1.2	5.0 $\pm$ 1.8	1.0

^aGS was measured in *B. subtilis* strains grown to the exponential phase in TSS medium containing the indicated nitrogen source (0.2%) and harvested and permeabilized as described in Materials and Methods. Repression ratios were calculated by dividing the respective enzyme activities found in glutamate cultures by the glutamine culture values. The results shown are averages of duplicate analyses of permeabilized cells prepared from at least two separate sets of cultures.

^bGS activity was assayed by the Mn²⁺-dependent reverse transferase assay as described in Materials and Methods. The data are expressed as micromoles per minute per milligram of protein.

nitrogen-limiting growth suggested that GlnR interactions within this region, in vivo, were not dependent on GS.

In vivo DMS modification in *B. subtilis*. To determine whether the results obtained with *E. coli* were relevant to the mode of action of GlnR in *B. subtilis*, we grew *B. subtilis* SMY(pIDG1) (*glnR*⁺ *glnA*⁺) and HJS31(pIDG1) ($\Delta glnR57 glnA^+$) under repressing (glutamine culture) and derepressing (glutamate culture) conditions and treated with DMS. The plasmid harbored by these strains, pIDG1, is derived from pHP13 (14) and contains a 700-bp DNA fragment that includes *glnRA* promoter and operator regions (see Materials and Methods). Plasmid pHP13 and its derivatives are low copy in *B. subtilis*, and we chose pIDG1 to avoid gene dosage effects due to having multiple copies of the *glnRA* regulatory region (34). The presence of plasmid pIDG1 in strain SMY had no effect on GS regulation. GS levels in strain SMY(pIDG1) were regulated in a manner similar to that of strains SMY (Table 1) and SMY(pHP13) (data not shown), differing 8.6-fold under nitrogen excess and limiting conditions. On the other hand, because of the *glnR57* deletion, GS levels in strain HJS31(pIDG1) were not regulated by the nitrogen source, being derepressed under both growth conditions (Table 1).

Primer extension analysis of modified plasmids obtained from strains SMY(pIDG1) and HJS31(pIDG1) were done by using the polymerase chain reaction protocol described by Sasse-Dwight and Gralla (33). Since the quality and low yield of modified plasmid preparations obtained from *B. subtilis* did not allow us to use the Klenow-based protocol employed for the *E. coli* study, the polymerase chain reaction approach was warranted. Although *Taq* polymerase is less discriminating in its ability to replicate modified DNA than is Klenow (28), the patterns obtained with the two enzymes were comparable. Thus, as shown in Fig. 3A (top strand) and B (bottom strand), G residue protection and enhancement patterns in the absence (lane 5) or presence (lane 6) of GlnR in vitro were comparable to those obtained by the Klenow method. However, the presence of a double band at some positions, e.g., -39 of the top strand, and -32 of the bottom strand, were likely due to products of chain termination at and before the modified base. Furthermore, modifications at most T, A, and C bases, which resulted in Klenow extension termination, were not detected by extension with *Taq* polymerase, as evidenced by the absence of bands at these positions.

Results obtained with *B. subtilis* plasmid-carrying strains

FIG. 3. In vivo DMS modification patterns obtained from *B. subtilis* strains carrying plasmid pIDG1. Strains SMY(pIDG1) (lanes 1 and 2) and HJS31(pIDG1) (lanes 3 and 4) were grown in minimal medium containing glutamine (Gln; excess nitrogen) or glutamate (Glt; limiting nitrogen) as the sole nitrogen source and treated with DMS as described in Materials and Methods. Extensions along top (A) and bottom (B) strands were done by using *Taq* polymerase (see Materials and Methods). Modification of plasmid pIDG1 in vitro in the absence (lane 5) or presence (lane 6) of GlnR was done as described in Materials and Methods by using *Taq* polymerase for the primer extension reaction. Base assignments and symbols are as in Fig. 2.

are shown in Fig. 3A (top strand) and B (bottom strand). For strain SMY(pIDG1) (*glnR*⁺) grown under repressing conditions (Fig. 3A and B, lanes 1), G residues within the operator region, at positions -57 in the top strand (Fig. 3A) and -43 in the bottom strand (Fig. 3B), were protected from DMS attack. Enhanced methylation was found at positions -48 in the bottom strand and -52 in the top strand. These patterns paralleled those observed in vitro in the presence of GlnR (Fig. 3A and B, lanes 6) and, with the exception of hypermodification at position -52 in the top strand, were similar to patterns obtained with *glnR*⁺ strains of *E. coli* grown under repressing conditions.

Under nitrogen-limiting conditions (Fig. 3A and B, lanes 2), relief of repression by GlnR in strain SMY(pIDG1) was

accompanied by absence of protections or enhancements within the operator region, between positions -40 and -60. These results corresponded to those obtained with strain HJS31(pIDG1) (Δ *glnR57*) grown under both repressing and derepressing conditions (Fig. 3A and B, lanes 3 and 4) as well as those observed in vitro in the absence of GlnR (compare Fig. 3A and B, lanes 5). Thus, within the operator region, modification by DMS was found to be affected by GlnR in a nitrogen-dependent manner.

In contrast to results obtained for the operator region, modification of certain bases within the promoter region did not vary in a nitrogen-dependent manner. While the G at position -34 in the top strand (Fig. 3A, lane 2) was protected from modification only under repressing conditions in strain

SMY(pIDG1), protection at -20 and hypermodification at -32 occurred under both growth conditions (compare lanes 1 and 2, Fig. 3B). Comparison of patterns obtained from *glnR*⁺ wild-type and *glnR* mutant strains revealed that the observed modifications in nitrogen-limited cultures of SMY(pIDG1) were not due to GlnR activity. Under derepressing conditions, strains SMY(pIDG1) and HJS31(pIDG1) showed identical modification patterns at -20 and -32 in the bottom strand (compare lanes 2 and 4, Fig. 3B). On the other hand, modification protection and enhancement at positions -20 and -32, respectively, were absent in strain HJS31(pIDG1) grown under repressing conditions, and methylation of bases at other positions in this *glnR* mutant strain was indicative of that observed in vitro, in the absence of GlnR (compare lanes 3 and 5, Fig. 3B). The occurrence of GlnR-like modifications in HJS31(pIDG1) indicated that some factor(s) other than GlnR interacted within *glnRA* promoter sequences under nitrogen-limiting conditions.

Modification of plasmid DNA by potassium permanganate. Potassium permanganate preferentially oxidizes pyrimidines and, to a lesser extent, purines in single-stranded DNA (3, 32). Studies have shown that when DNA-binding proteins are bound to DNA, bases within the binding area become sensitive to attack. Because the above-described DMS studies showed that GlnR was capable of binding to a large region of DNA, we used the permanganate reagent to examine whether any structural distortions occurred as a consequence of GlnR binding. *E. coli* strains harboring various *glnRA* plasmids were treated with potassium permanganate, and modification was analyzed by the primer extension approach described in Materials and Methods.

Results obtained by treating plasmid pSF14 with potassium permanganate in the presence (lane 17) or absence (lane 18) of GlnR in vitro are shown in Fig. 4A (top strand) and B (bottom strand). In the presence of GlnR, bases within the region extending from -27 to -38 became reactive to potassium permanganate. These included, in the top strand, residues at positions -27 (A), -28 (T), -37 (A), and -38 (T) and, in the bottom strand, -33 (T), -34 (C), -37 (T), and -38 (A). Modification of bases within the *glnRA* promoter -35 region indicates that GlnR binding induces single-stranded characteristics within this region.

To analyze permanganate modification of plasmid DNA in vivo, it was necessary to determine contributions to the modification pattern that were due to RNA polymerase. Therefore, *E. coli* strains were treated with potassium permanganate in the absence or presence of rifampin. Rifampin inhibits transcription initiation and effectively traps RNA polymerase in the open complex. When grown under derepressing conditions, plasmids from strain YMC11(pSF14) (*glnR*⁺ *glnA*⁺) showed extensive modification in the regions from +6 to -2 and -6 to -13 in the top strand and -10 to -13 in the bottom strand compared with strains not treated with rifampin (compare lanes 8 [repressing condition] and 16 [derepressing condition], Fig. 4A and B). Thus, modifications within these regions were due to RNA polymerase initiation and defined bases involved in open complex formation.

Under repressing conditions, modification at positions -27 and -28 in the top strand and -33, -34, -37, and -38 in the bottom strand were evident in strain YMC11(pSF14) and were prominent in both rifampin-treated (Fig. 4A and B, lanes 12) and untreated (Fig. 4A and B, lanes 4) cultures. The modifications in and around the promoter -35 region on both strands were similar to those observed in vitro in the presence of GlnR, and the results obtained with *glnR* mutant

strains indicated that GlnR was responsible for modifications within this region. These modifications were either greatly diminished (in the case of the -33 to -38 region in the bottom strand) or absent in Δ *glnR57* *glnA*⁺ strain YMC11(pJCG11) (Fig. 4A and B, lanes 2 and 6 [without rifampin] and 10 and 14 [with rifampin]) and Δ *glnRA14* mutant strain YMC11(pJCG14) (data not shown). Therefore, distortion of the helix in the promoter -35 region detected in the presence of GlnR in vitro occurred in vivo as well.

Strain YMC11(pJCG10) yielded the same GlnR-dependent pattern when grown in repressing medium (Fig. 4A and B, lanes 1 [without rifampin] and 9 [with rifampin]) as it did when grown in derepressing medium (Fig. 4A and B, lanes 5 [without rifampin] and 13 [with rifampin]). This was consistent with the results obtained in the DMS experiments described above with this strain, which showed binding of GlnR to operator and promoter regions under both growth conditions.

Plasmid pJCG12 harbors the *glnR54* allele, which is a 71-bp deletion-frameshift mutation in the C-terminal portion of the *glnR* gene (35). This mutation causes a *trans*-dominant low-level constitutive phenotype, and GS levels in strain YMC11(pJCG12) are partially repressed under limiting- or excess-nitrogen conditions (35). This phenotype was apparent from the modification patterns obtained from limiting- and excess-nitrogen cultures (Fig. 4A and B, lanes 3 and 7 [without rifampin] and 11 and 15 [with rifampin]), which showed modifications attributable to GlnR and RNA polymerase that were approximately halfway between *glnR* mutant strain YMC11(pJCG11) and fully repressed *glnR*⁺ strain YMC11(pSF14). Similar results were obtained when we examined modified plasmids from excess- and limiting-nitrogen cultures of strain YMC11(pJCG12) that had been treated with DMS (data not shown).

Modification by permanganate allowed us to examine the relationship between transcriptional initiation and GlnR activity. Initiation by RNA polymerase was evident by the presence of open complexes in wild-type strain YMC11(pSF14) grown under derepressing conditions (Fig. 4A and B, lanes 16) or in the *glnR* mutant background of YMC11(pJCG11) (Fig. 4A and B, lanes 10 and 14). Under these conditions, GlnR-dependent modifications within the -35 region were either low or absent. On the other hand, open complex formation was minor compared with the modifications induced by GlnR in *glnR*⁺ strain YMC11(pSF14) grown under repressing conditions (Fig. 4A and B, lanes 12) or in *glnA* mutant strain YMC11(pJCG10) (Fig. 4A and B, lanes 9 and 13). For *glnR54* mutant strain YMC11(pJCG12), intermediate levels of repression and transcription were reflected by the presence of both open complex formation and GlnR-dependent modifications. These results reveal that an inverse relationship exists between GlnR binding and association of RNA polymerase with the *glnRA* promoter and indicate that GlnR functions by occupying operator and promoter regions, thereby inhibiting RNA polymerase from binding and/or initiating transcription.

DISCUSSION

Results obtained from in vivo modification studies done with *E. coli* and *B. subtilis* are summarized in Fig. 5. The patterns obtained under repressing conditions from both organisms were similar. Small variations at some positions, e.g., the hypermethylation at position -57, which was present in *B. subtilis* and absent in *E. coli*, could be explained by differences in extension procedures or effects on

FIG. 4. Potassium permanganate modification patterns of the *glnRA* regulatory region along top (A) and bottom (B) strands in vivo. The plasmids harbored by strain YMC11 were pJCG10 (lanes 1, 5, 9, and 13), pJCG11 (lanes 2, 6, 10, and 14), pJCG12 (lanes 3, 7, 11, and 15), and pSF14 (lanes 4, 8, 12, and 16). Modification of plasmid pSF14 in vitro in the presence (lane 17) or absence (lane 18) of GlnR was done as described in Materials and Methods. Glutamine (G) and glutamine-ammonia (G + N) cultures were limiting- and excess-nitrogen conditions, respectively. In some cases, cultures were treated with rifampin (+). Open complex formation detected in the promoter -10 and -35 regions is shown by brackets.

FIG. 5. Summary of in vivo modification studies. Shown are the *glnRA* promoter and operator regions from positions -71 to $+5$ relative to the start point of transcription (43). Not shown is the hyperreactivity of the G at -8 in the bottom strand or the T at -14 in the top strand, which did not vary with the nitrogen source. Protection of T residues at positions -72 and -73 is also not shown because the chemistry of their modification by DMS is unknown. In vitro, GlnR did not protect these T residues from modification. Arrowheads and diamonds indicate bases at which protection from and enhanced susceptibility to DMS, respectively, were detected in vivo. The small open squares indicate bases that were modified by potassium permanganate in the presence of GlnR in vivo.

GlnR binding due to dissimilarities in the host's internal environment. Nevertheless, GlnR was found to influence methylation of bases within the 37-bp region extending from positions -20 to -57 relative to the start point of transcription. This region contains the *glnRA* operator (*glnRAo*₁), a symmetrical sequence between positions -40 and -60 (arrows in Fig. 5) previously shown to be necessary for nitrogen-dependent control (38). The symmetry of methylation in this region—enhancement and protection of G residues 2 and 7 bp from the dyad axis, respectively—is consistent with the observation that the protein associates as a dimer in solution (21, 23). Thus, each arm of the dyad would be bound by one subunit. Our in vivo results were similar to those obtained in vitro with supercoiled plasmids and were also consistent with those of Brown and Sonenshein's study using linear DNA fragments (4).

In addition to the binding of GlnR at sequences upstream from the promoter, our in vivo studies also showed that GlnR interacted with bases within the *glnRA* promoter, between the -10 and -35 regions, which had been shown to occur in vitro (this study and reference 4). The region from -35 to -17 contains a quasisymmetrical element sharing homology with *glnRAo*₁ (Fig. 5). As was observed for *glnRAo*₁, G residues residing 7 bp from the dyad axis on both arms were protected from methylation in vivo as well as in vitro. This pattern suggests that a second GlnR dimer binds to bases in the promoter region in much the same fashion as it does to the upstream operator. Thus, a second operator site, *glnRAo*₂, found between positions -37 and -17 , is utilized in regulating *glnRA* expression.

Sequences upstream of *glnRAo*₁ may also be involved in GlnR-DNA interactions in vivo. Protection of T residues at positions -72 and -73 in the top strand was observed in *glnR*⁺ *E. coli* strains only under repressing conditions and occurred even in a *glnA* mutant background. GlnR did not affect the modification of these bases in vitro, and *E. coli* may provide some signal under nitrogen excess conditions that allows GlnR to interact with bases as far upstream as -73 . The significance of these interactions is not understood, and we cannot address their relevance to control in *B. subtilis* since methylation of T residues was not detectable by the protocol used with that organism.

The interaction of GlnR with either *glnRAo*₁ or *glnRAo*₂ may be important in determining how the protein is recruited to interfere with the binding of RNA polymerase. GlnR may have a different affinity for each site, in a manner analogous to systems containing two or more operators (e.g., *lac* [30]), and the binding of GlnR to one or both sites would depend on

the level of GlnR available for repression. Because the left arm of the *glnRAo*₂ dyad has little homology to the half-site consensus, 5'-TCNTNACATC-3', binding of GlnR to this site may not be as efficient as it is for the upstream site. Electrophoretic mobility shift assays have shown that a DNA fragment extending from positions -51 to $+83$, which contains all of *glnRAo*₂ and half of *glnRAo*₁, was a poor substrate for GlnR compared with when upstream sequences were present (38). While the present study did not examine the specific properties of GlnR binding with each of the operator sites, our in vivo results suggested that, qualitatively, GlnR interacts with both sites in approximately the same manner. Using a DNA fragment containing both operators, Brown and Sonenshein have found that GlnR appears to bind to either site without preference (4). One scenario for GlnR's modus operandi involves utilization of one GlnR dimer bound at *glnRAo*₁ as an anchor for immediate binding of a second dimer to *glnRAo*₂.

Our results do not allow us to address the significance of the *glnRAo*₂ operator. By itself, *glnRAo*₂ does not play a role in controlling expression since constructions deleted for *glnRAo*₁ were not regulated (38). Furthermore, single base substitutions at position -20 were not found to alter GS regulation (37). A *glnRAo*₂-like sequence is not found within the *B. cereus* promoter region, which contains sequences homologous to the *B. subtilis* *glnRAo*₁ site (20). One possibility is that the site has an additional role in regulating *glnRA* expression under physiological conditions other than those examined in our studies. We did find evidence for one or more factors other than GlnR that interact at *glnRAo*₂ under nitrogen-limiting conditions (see below).

The positioning of dimers at *glnRAo*₁ and *glnRAo*₂ may result in distortion or bending of the DNA within the binding regions. Upon GlnR binding, several bases within the top and bottom strands of the left arm of *glnRAo*₂ were found to be exposed to potassium permanganate modification (Fig. 5). Such modifications have been described by Sasse-Dwight and Gralla (32) for *lac* transcription complexes and suggest that the binding area possesses single-stranded properties. Dimer-dimer associations may be responsible for these characteristics, and helix distortions as a consequence of stresses resulting from dimer crowding cannot be discounted. While we have no evidence for dimer-dimer interaction in vitro, studies examining such associations are being conducted. Alternatively, binding of a GlnR dimer to *glnRAo*₂ may, by itself, be the cause of tension in the DNA helix. It is noted that modifications were observed in the portion of *glnRAo*₂ having little homology to the *glnRAo*₁ consensus and deformations may be a result of improper protein-operator interactions.

The results obtained from our potassium permanganate and DMS modification studies provided an in vivo perspective to transcription and repressor activities, respectively. Repression was revealed by detection of GlnR-dependent modifications, as well as decreased open complex formation when *glnR*⁺ *glnA*⁺ cells were grown under excess-nitrogen conditions. The opposite was found to occur under nitrogen-limiting conditions. In a *glnA* mutant background [strain YMC11(pJCG10)], repression by GlnR was observed under both limiting- and excess-nitrogen conditions. These results are consistent with a role for GS in providing some nitrogen signal. The degree of repression was surprising, however, since in a *glnA* mutant strain of *B. subtilis* repression was totally alleviated under nitrogen excess conditions (39). On the other hand, when regulation was examined by using *E. coli* as the host, *glnRA* expression was approximately two-

fold lower in a *glnA* mutant strain than in one that was a *glnR* mutant (35), indicating the presence of remaining GlnR activity. The discrepancy may be attributed to a combination of factors, including the host's physiology as well as gene dosage effects. Regulation in *E. coli* was studied by using low-copy-number *glnRAop-lacZ* fusion plasmids to monitor the effect, in *trans*, of mutations in *glnRA*-containing high-copy-number plasmids (35). The levels of GlnR and GS in relationship to the number of operators, as well as other components, may be critical in controlling GlnR activity. The internal environment and the presence of *glnR* and *glnRAO₁-glnRAO₂* in high copy numbers in strain YMC11 (pJCG10) may, together, be responsible for perturbing the signals that control GlnR activity. Thus, in strain YCM11(pJCG10), GlnR represses transcription in the absence of GS, even under nitrogen-limiting conditions. A thorough explanation requires an understanding of the signals involved in controlling GlnR activity, as well as the role GS plays in regulating *glnRA* expression.

The model for *glnRA* control in *B. subtilis* involves interaction of GlnR with the *glnRA* regulatory region. That GS does not directly bind to promoter and operator sequences was apparent from the *E. coli* DMS studies, since modification patterns from *glnR glnA⁺* strains were generally similar to those observed in vitro in the absence of added proteins. The *B. subtilis* modification studies, however, implied the involvement of proteins other than GlnR in controlling *glnRA* expression. Under nitrogen-limiting conditions, strains SMY(pIDG1) (*glnR⁺*) and HJS31(pIDG1) (Δ *glnR57*) demonstrated nearly identical modification patterns at positions -20 and -32 in the bottom strand. Since contributions due to RNA polymerase were not specifically addressed by the DMS studies, involvement of RNA polymerase in the modifications found in this region cannot be ruled out. However, there is evidence suggesting that other genes unlinked to *glnRA* affect *glnRA* control (1, 7). Albertini and Galizzi (1) have shown that a mutation in *outB* affects derepression of a *glnR'-lacZ* fusion, causing 10- to 44-fold lower β -galactosidase expression under nitrogen-limiting conditions. While the association of *outB* with the *glnRA* regulatory region is not known, it may be significant that extracts prepared from *B. subtilis* contain more than one protein with binding activity for the *glnRA* regulatory region (23). Whether these proteins are specific to *glnRA* or more general in their function is being investigated.

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REFERENCES

- Albertini, A. M., and A. Galizzi. 1990. The *Bacillus subtilis outB* gene is highly homologous to an *Escherichia coli ntr*-like gene. *J. Bacteriol.* **172**:5482-5485.
- Backman, K., Y.-M. Chen, and B. Magasanik. 1981. Physical and genetic characterization of the *glnA-glnG* region of the *Escherichia coli* chromosome. *Proc. Natl. Acad. Sci. USA* **78**:3743-3747.
- Borowiec, J. A., L. Zhang, S. Sasse-Dwight, and J. D. Gralla. 1987. DNA supercoiling promotes formation of a bent repression loop in *lac* DNA. *J. Mol. Biol.* **195**:101-111.
- Brown, S. B., and A. L. Sonenshein. 1991. Submitted for publication.
- Claverie-Martin, F., and B. Magasanik. 1991. Role of integration host factor in the regulation of the *glnHp2* promoter of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **88**:1631-1635.
- Cohen, S. N., A. C. Y. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *E. coli* by R-factor DNA. *Proc. Natl. Acad. Sci. USA* **69**:2110-2114.
- Dean, D. R., and A. I. Aronson. 1980. Selection of *Bacillus subtilis* mutants impaired in ammonia assimilation. *J. Bacteriol.* **141**:985-988.
- Dean, D. R., J. A. Hoch, and A. I. Aronson. 1977. Alteration of the *Bacillus subtilis* glutamine synthetase results in overproduction of the enzyme. *J. Bacteriol.* **131**:981-987.
- Deuel, T. F., A. Ginsberg, J. Yeh, E. Shelton, and E. R. Stadtman. 1970. *Bacillus subtilis* glutamine synthetase. *J. Biol. Chem.* **245**:5195-5205.
- Ferrari, F. A., A. Nguyen, D. Lang, and J. A. Hoch. 1983. Construction and properties of an integratable plasmid for *Bacillus subtilis*. *J. Bacteriol.* **154**:1513-1515.
- Fisher, S. H., M. S. Rosenkrantz, and A. L. Sonenshein. 1984. Glutamine synthetase gene of *Bacillus subtilis*. *Gene* **32**:427-438.
- Fisher, S. H., and A. L. Sonenshein. 1984. *Bacillus subtilis* glutamine synthetase mutants pleiotropically altered in glucose catabolite repression. *J. Bacteriol.* **157**:612-621.
- Grossman, A. D. 1991. Integration of developmental signals and the initiation of sporulation in *B. subtilis*. *Cell* **65**:5-8.
- Haima, P., S. Bron, and G. Venema. 1987. The effect of restriction on shotgun cloning in *Bacillus subtilis* Marburg. *Mol. Gen. Genet.* **209**:335-342.
- Hirschman, J., P.-K. Wong, K. Sei, J. Keener, and S. Kustu. 1985. Products of nitrogen regulatory genes *ntrA* and *ntrC* of enteric bacteria activate *glnA* transcription *in vitro*: evidence that the *ntrA* product is a σ factor. *Proc. Natl. Acad. Sci. USA* **82**:7525-7529.
- Holmes, D. S., and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114**:193-197.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
- Magasanik, B. 1988. Reversible phosphorylation of an enhancer binding protein regulates the transcription of bacterial nitrogen utilization genes. *Trends Biochem. Sci.* **13**:475-479.
- Magasanik, B., and F. C. Neidhardt. 1987. Regulation of carbon and nitrogen utilization, p. 1321-1325. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
- Nakano, Y., K. Chiaki, E. Tanaka, K. Kimura, and K. Horikoshi. 1989. Nucleotide sequence of the glutamine synthetase gene (*glnA*) and its upstream region from *Bacillus cereus*. *J. Biochem.* **106**:209-215.
- Nakano, Y., and K. Kimura. 1991. Purification and characterization of a repressor for the *Bacillus cereus glnRA* operon. *J. Biochem.* **109**:223-228.
- Ninfa, A. J., L. J. Reitzer, and B. Magasanik. 1987. Initiation of transcription at the bacterial *glnAp2* promoter by purified *E. coli* components is facilitated by enhancers. *Cell* **50**:1039-1046.
- Nomellini, J. F., and H. J. Schreier. Unpublished data.
- Pan, F. L., and J. G. Coote. 1979. Glutamine synthetase and glutamate synthase activities during growth and sporulation of *Bacillus subtilis*. *J. Gen. Microbiol.* **112**:373-377.
- Popham, D. L., D. Szeto, J. Keener, and S. Kustu. 1989. Function of a bacterial activator protein that binds to transcriptional enhancers. *Science* **243**:629-635.
- Rebello, J. L., and N. Strauss. 1969. Regulation of glutamine synthase in *Bacillus subtilis*. *J. Bacteriol.* **148**:653-658.
- Reitzer, L. J., and B. Magasanik. 1987. Ammonia assimilation and the biosynthesis of glutamine, glutamate, aspartate, aspar-

- agine, L-alanine, and D-alanine, p. 302–320. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
28. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 29. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 30. Sasse-Dwight, S., and J. D. Gralla. 1988. Probing co-operative DNA-binding *in vivo*: the lac O₁:O₃ interaction. *J. Mol. Biol.* **202**:107–119.
 31. Sasse-Dwight, S., and J. D. Gralla. 1988. Probing the *glnALG* upstream activation mechanism *in vivo*. *Proc. Natl. Acad. Sci. USA* **85**:8934–8938.
 32. Sasse-Dwight, S., and J. D. Gralla. 1989. KMnO₄ as a probe for *lac* promoter DNA melting and mechanism *in vivo*. *J. Biol. Chem.* **264**:8074–8081.
 33. Sasse-Dwight, S., and J. D. Gralla. 1990. Role of eukaryotic-type functional domains found in the prokaryotic enhancer receptor factor σ^{54} . *Cell* **190**:945–954.
 34. Schreier, H. J. Unpublished data.
 35. Schreier, H. J., S. W. Brown, K. D. Hirschi, J. F. Nomellini, and A. L. Sonenshein. 1989. Regulation of *Bacillus subtilis* glutamine synthetase gene expression by the product of the *glnR* gene. *J. Mol. Biol.* **210**:51–63.
 36. Schreier, H. J., S. H. Fisher, and A. L. Sonenshein. 1985. Regulation of expression from the *glnA* promoter of *Bacillus subtilis* requires the *glnA* gene product. *Proc. Natl. Acad. Sci. USA* **82**:3375–3379.
 37. Schreier, H. J., and C. A. Rostkowski. Unpublished data.
 38. Schreier, H. J., C. A. Rostkowski, J. F. Nomellini, and K. D. Hirschi. 1991. Identification of DNA sequences involved in regulating *Bacillus subtilis glnRA* expression by the nitrogen source. *J. Mol. Biol.* **220**:241–253.
 39. Schreier, H. J., and A. L. Sonenshein. 1986. Altered regulation of the *glnA* gene in glutamine synthetase mutants of *Bacillus subtilis*. *J. Bacteriol.* **167**:35–43.
 40. Smith, I. 1989. Initiation of sporulation, p. 185–210. In I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development: structural and functional analysis of bacterial sporulation and germination. American Society for Microbiology, Washington, D.C.
 41. Sonenshein, A. L. 1989. Metabolic regulation of sporulation and other stationary-phase phenomena, p. 109–130. In I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development: structural and functional analysis of bacterial sporulation and germination. American Society for Microbiology, Washington, D.C.
 42. Sonenshein, A. L., B. Cami, J. Brevet, and R. Cote. 1974. Isolation and characterization of rifampin-resistant mutants of *Bacillus subtilis*. *J. Bacteriol.* **120**:253–265.
 43. Strauch, M. A., A. I. Aronson, S. W. Brown, H. J. Schreier, and A. L. Sonenshein. 1988. Sequence of the *Bacillus subtilis* glutamine synthetase gene region. *Gene* **71**:257–265.
 44. Wedel, A., D. S. Weiss, D. Popham, P. Droge, and S. Kustu. 1990. A bacterial enhancer functions to tether a transcriptional activator near a promoter. *Science* **248**:486–490.
 45. Wu, S., S. Porter, S. Kustu, and H. Echols. 1990. DNA-looping and enhancer activity: association between DNA-bound NtrC activator and RNA polymerase at the bacterial *glnA* promoter. *Proc. Natl. Acad. Sci. USA* **87**:5504–5508.
 46. Zhang, J., M. Strauch, and A. I. Aronson. 1989. Glutamine auxotrophs of *Bacillus subtilis* that overproduce glutamine synthetase antigen have altered conserved amino acids in or near the active site. *J. Bacteriol.* **171**:3572–3574.