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A New Type of NtrC Transcriptional Activator

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The enteric NtrC (NR_I) protein has been the paradigm for a class of bacterial enhancer-binding proteins (EBPs) that activate transcription of RNA polymerase containing the σ^{54} factor. Activators in the NtrC class are characterized by essentially three properties: (i) they bind to sites distant from the promoters that they activate (>100 bp upstream of the transcriptional start site), (ii) they contain a conserved nucleotide-binding fold and exhibit ATPase activity that is required for activation, and (iii) they activate the σ^{54} RNA polymerase. We have characterized the NtrC protein from a photosynthetic bacterium, *Rhodobacter capsulatus*, which represents a metabolically versatile group of bacteria found in aquatic environments. We have shown that the *R. capsulatus* NtrC protein (RcNtrC) binds to two tandem sites that are distant from promoters that it activates, *nifA1* and *nifA2*. These tandem binding sites are shown to be important for RcNtrC-dependent nitrogen regulation in vivo. Moreover, the conserved nucleotide-binding fold of RcNtrC is required to activate *nifA1* and *nifA2* but is not required for DNA binding of RcNtrC to upstream activation sequences. However, *nifA1* and *nifA2* genes do not require the σ^{54} for activation and do not contain the highly conserved nucleotides that are present in all σ^{54}-type, EBP-activated promoters. Thus, the NtrC from this photosynthetic bacterium represents a novel member of the class of bacterial EBPs. It is probable that this class of EBPs is more versatile in prokaryotes than previously envisioned.

Enhancer-binding proteins (EBPs) are present in a wide range of bacteria and activate the expression of genes required for diverse cellular processes, including nitrogen assimilation, the development of cell polarity, pathogenicity, and biological nitrogen fixation (46). Members of the EBP family characteristically activate transcription from promoters recognized by the alternative sigma factor σ^{54} (36, 42), and the binding sites for EBPs are located distal to the promoter, usually at sites greater than 100 bp upstream from the transcription start (13, 35). Like many eukaryotic activators, prokaryotic EBPs are composed of several domains which are modular in structure, since in some cases the activation and DNA-binding functions may be separated (see reference 49 for a review). All members of the EBP family share a highly conserved central domain which includes a nucleotide-binding fold (46) and a C-terminal domain which includes a helix-turn-helix motif required for DNA recognition (14, 44). Several activators are members of two-component regulatory systems (e.g., NtrC, DctD, FlbD, and HoxA) and possess an amino-terminal regulatory domain which is phosphorylated at a conserved aspartate in response to an environmental stimulus (32, 47; for reviews, see references 50, 62, and 66). This phosphorylation induces ATPase activity (2, 70) and probably oligomerization (43, 53, 71), which are considered prerequisites in the activation process.

The enteric NtrC was the first prokaryotic EBP to be extensively characterized in vivo and in vitro (38, 72, 73). Many aspects of its activation mechanism appear to be conserved among EBP members but distinct from activation by proteins which act at promoters requiring the housekeeping sigma factor σ^{70}. The promoters recognized by σ^{54} RNA polymerase (RNAP) contain highly conserved sequences at -12 and -24 (6, 24, 36, 45), unlike the -10 and -35 promoters recognized by σ^{70} RNAP. In contrast to σ^{70}, σ^{54} can specifically recognize some promoters in the absence of the core RNAP (8). The σ^{54} RNAP closed complex cannot isomerize to form an open complex in the absence of the activator and ATP (2, 52), and thus regulation of transcription occurs through activation rather than repression. Although EBPs typically bind to sites located around -100, in some cases the recognition site can be moved to sites greater than 1 kb from the transcriptional start and still retain activation function (7, 9, 48, 57). The ability of NtrC-like activators to contact bound σ^{54} RNAP from distal sites (64, 69) and to catalyze transcription in an ATP-dependent fashion are qualities shared with the activation of eukaryotic RNA polymerase II transcription (23).

In the photosynthetic bacterium *Rhodobacter capsulatus*, synthesis of nitrogenase in response to low nitrogen requires *rpoN*, the σ^{54} gene (33), and the product of one of two functional copies of *nifA*, *nifA1* (copy I), or *nifA2* (copy II) (40). The expression of each copy of *nifA* in turn requires the product of *R. capsulatus ntrC*, RcNtrC (20, 29, 34). According to genetic experiments, σ^{54} is not required either for the expression of *nifA1*- and *nifA2-lacZ* fusions or for the synthesis of *nifA1*/*nifA2* transcripts as detected by primer extension analysis (20). Importantly, the *nifA1* and *nifA2* promoters share no resemblance to the highly conserved σ^{54} -12, -24 consensus promoter (reviewed in reference 42). However, it remained possible that RcNtrC does not directly activate the *nifA1* and *nifA2* promoters but instead functions as an intermediate regulator; such is the case for the enteric intermediate regulatory protein called Nac (5). The *nac* gene in *Klebsiella aerogenes* is activated by NtrC at a σ^{54} promoter, and subsequently Nac activates transcription of σ^{70}-dependent genes like *hut* (histidine utilization) and *put* (proline utilization). In this report, we provide evidence that purified RcNtrC directly contacts the promoters of *nifA1* and *nifA2* at tandem distal binding sites greater than 100 bp upstream of the transcriptional start site. These sites are shown to be important for RcNtrC-dependent nitrogen control in vivo. It is also shown

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that the ATP-binding site of RcNtrC is required for activation of *nifA1* and *nifA2* but not for its DNA-binding properties. Accordingly, RcNtrC represents the first member of the NtrC-type of EBP family which requires an ATP-binding site but does not activate σ^{54} promoters.

MATERIALS AND METHODS

Media and growth conditions. *R. capsulatus* basal medium (RCV) has been described previously (4). The sources of fixed nitrogen was either $(NH_4)_2SO_4$ (7.5 mM) or glutamate (10 mM); tetracycline was added to a final concentration of 0.1 μg/ml for liquid cultures. Cells grown for β-galactosidase assays were typically inoculated into 5 to 10 ml of RCV (NH_4^+) and grown at 34°C in illuminated, unshaken 20-ml test tubes. Inductions under anaerobic conditions were carried out by adding a small inoculum of cells to 12.5-ml screw-cap tubes and filling the tubes completely with induction medium. When cells were induced in different media, they were first washed in RCV (without NH_4^+), then resuspended in induction medium, and incubated in the light at 34°C for 12 to 18 h.

Strains and plasmids. The wild-type *R. capsulatus* strain used in this study was SB1003 (76). NtrC$^-$ strains were 102-C4 (4) and J61 (68), kindly provided by Judy Wall, University of Missouri, Columbia. The J61::*nifA2-lacZ* integration strain, BMMA2c, has been described previously (19). *Escherichia coli* NtrBC$^-$ strain SN24 and plasmid pBLS8 were kindly provided by Lawrence Reitzer, University of Texas, Dallas.

Figure 1 shows the *nifA1*- and *nifA2-lacZ* fusions used in this study. pPA1Bc1, pPA2TB, pDFH100P, pDFH100Y, pDFH 200H, and pDFH200T have been described previously (20). All *nifA1* and *nifA2* upstream fragments were first cloned into pUC118 (65), sequenced to verify the insert, and then subcloned into the broad-host-range vector described below. pPA1P16, pPA1P15, and pPAP12 are 0.36- to 0.37-kb *Xba*I-*Bam*HI *nifA1* PCR products cloned into the *Xba*I-*Bam*HI sites of pUC118. The template in each case was pPA1Bcl. The 5′ primers (shown 5′ to 3′) were: TATCTAGAGTCTGCCAG ATTTTCCGGCCC (pPA1P16), TATCTAGATTTTCCGGC CCCCGGGGCCGGT (pPA1P15), and TATCTAGAGTCTG CCAGATTTGGTCCCCG (pPA1P12) the 3′ primer in each case was the reverse primer ACCATGATTACGAATTCGA GCTCGGTACCC. pPA2P1, pPA2P8, pPA2P9, and pPA2P11 are 0.38- to 0.42-kb *Xba*I-*Bam*HI *nifA2* PCR products cloned into the *Xba*I-*Bam*HI sites of pUC118. The 5′ primers were TATCTAGAACCTCTGCCAGACCGGAAGCGC (pPA2P1), TATCTAGACCGGAAGCGCCATTTTTTTCGG (pPA2P8), TATCTAGATCCGGCCATTCCCGCCTCA (pPA1P9), and TATCTAGATCTTAACGAAGCCTCAATCA (pPA1P11); the 3′ primer was the reverse primer described above. The template in each case was pPA2TB.

Fragments containing insertions in the *nifA1* upstream region were constructed from pPA1Bcl. pPA1M4 was produced by digesting pPA1Bc1 with *Mlu*I, filling the ends with Klenow enzyme, and religating the plasmid to generate a 4-bp insertion which included a new *Bss*HII site. pPA1M4 was then digested with *Bss*HII, and the ends were filled with Klenow enzyme and ligated in the presence of 12-bp *Xho*I linkers (CCGCTC GAGCGG; New England Biolabs). pPA1M20 contains a 20-bp insert including one *Xho*I linker; pPA1M36 contains a 36-bp insert including two *Xho*I linkers and 4 bp of unknown origin. Construction of pPA1M58 required two steps. First, pPA1Bc1 was digested with *Mlu*I (the ends filled with Klenow enzyme) and ligated in the presence of the 1.4-kb *Bam*HI (ends filled with Klenow enzyme) Kan^r cassette from pUC4 KIXX (Pharmacia). The resulting plasmid, pPA1MKm, was then digested with *Xho*I to release the cassette and religated, leaving a 58-bp insert. pPA1B5 was constructed by digesting pPA1Bcl with *Bst*EII, filling the ends with Klenow enzyme, and religating the plasmid. pPA1B17 and pPA1B29 were constructed in an identical fashion except the ligation mixture included *Xho*I linkers (described above). pPA1B17 contains 17 bp inserted at the *Bst*EII site including one *Xho*I linker; pPA1B29 contains two linkers. pPA1B59 was constructed by a strategy identical to that used for pPA1M58 except that the initial insertion of the Kan^r cassette was at the *Bst*EII site (pPA1BKm). To construct pPA1BstEIIa, pPA1BKm was digested with *Hin*dIII and religated, excising both the Kan^r cassette and the *nifA1* region upstream of the *Bst*EII site. pPA1B130 was constructed by a strategy identical to that used for pPA1B59, except in an earlier experiment. The insert in this case included the same sequence as pPA1B59 plus an additional 71 bp (±2 bp) of unknown origin.

nifA1- and *nifA2-lacZ* fusions containing various upstream fragments were generated in the following manner. Fusions constructed in the a direction with respect to the vector were made by excising 0.36- to 0.45-kb *Xba*I-*Bam*HI fragments from the pUC118 plasmids described above and ligating each one independently to the 18-kbp *Xba*I-*Bam*HI fragment of pDFH200P1 (20). Fusions constructed in the b direction with respect to the vector were made by excising 0.36- to 0.41-kb *Sph*I (site in polylinker)-*Bam*HI fragments from the pUC118 plasmids described above and ligating each one independently to the 18-kbp *Sph*I-*Bam*HI fragment of pDFH100Y. Thus, pDFH100Bcla contains the 0.38-kb *Xba*I-*Bam*HI fragment from pPA1Bcl, pDFH100P16-1a contains the 0.37-kb *Xba*I-*Bam*HI fragment from pPA1P16-1, pDFH100P15-5a contains the 0.36-kb *Xba*I-*Bam*HI fragment from pPA1P15-5, pDFH 100P12a contains the 0.37-kb *Xba*I-*Bam*HI fragment from pPA1P12, and pDFH100BstEIIa contains the *Xba*I-*Bam*HI fragment from pPA1BstEIIa. pDFH200Ta contains the 0.45-kb *Xba*I-*Bam*HI fragment from pPA2TB, pDFH2009a contains the 0.41-kb *Xba*I-*Bam*HI fragment from pPA1P1, pDFH 200P8a contains the 0.40-kb *Xba*I-*Bam*HI fragment from pPA1 P8, pDFH200P11a contains the 0.38-kb *Xba*I-*Bam*HI fragment from pPA1P11, and pDFH200P9a contains the 0.36-kb *Xba*I-*Bam*HI fragment from pPA1P9. pDFH2009a and pDFH 200P8a have been described previously (20) except that pDFH 200P8a was referred to as pDFH200d9. pDFH100B5a contains the 0.38-kb *Xba*I-*Bam*HI fragment from pPA1B5, pDFH 100B17a contains the 0.40-kb *Xba*I-*Bam*HI fragment from pPA1B17, pDFH100B29a contains the 0.41-kb *Xba*I-*Bam*HI fragment from pPA1B29, pDFH100B59a contains the 0.44-kb *Xba*I-*Bam*HI fragment from pPA1B59, and pDFH100B130a contains the 0.51-kb *Xba*I-*Bam*HI fragment from pPA1B130. pDFH100M4a contains the 0.38-kb *Xba*I-*Bam*HI fragment from pPA1M4, pDFH100M20a contains the 0.40-kb *Xba*I-*Bam*HI fragment from pPA1PM20, pDHF100M36a contains the 0.44-kb *Xba*I-*Bam*HI fragment from pPA1M36, and pDFH 100M57 contains the 0.46-kb *Xba*I-*Bam*HI fragment from pPA1M57. pDFH200Tb contains the 0.45-kb *Sph*I-*Bam*HI fragment from pPA2TB, pDFH2008b contains the 0.39-kb *Sph*I-*Bam*HI fragment from pPA2P8, and pDFH100Bclb contains the 0.38-kb *Sph*I-*Bam*HI fragment from pPA1Bcl (described previously as pDFH100Bcl [20]).

All β-galactosidase values shown in Fig. 1 are taken from assays using *lacZ* fusions constructed in the a orientation with the exception of pDFH200P8, which is in the b orientation. However, cells containing these plasmids in the a orientation gave nearly identical results. In general, orientation with respect to the vector made no difference in the patterns or levels of expression. The numbering system with respect to the

A

ß-gal Assays
-NH3 +NH3

0 300 600 900 1200 1500 1800

nifA1-lacZ

-165 -142 -133 -84 -47 +14

pDFH100 Bcl (-167) TCTGCCAGATTTTCCGGCC
BclI BstEII MluI PstI BamHI

pDFH100P16-1 (-142) TCTGCCAGATTTTCCGGCC

pDFH100P12 (-142) TCTGCCAGATTTGGTC--C

pDFH100P15-5(-133) TTTTCCGGCC

pDFH100BstE2 (-84)

pDFH100P (+11)

nifA2-lacZ

-220 -151 -139 -114 -10

pDFH200T (-220) ACCTCTGCCAGACCGGAAGCGCCATTTTTTTCGGCGCT
TaqI HindIII BamHI

pDFH2009 (-151) ACCTCTGCCAGACCGGAAGCGCCATTTTTTTCGGCGCT

pDFH200P8 (-139) CCGGAAGCGCCATTTTTTTCGGCGCT

pDFH200P11 (-114)

pDFH200P9 (-90)

pDFH200H (-10)

B

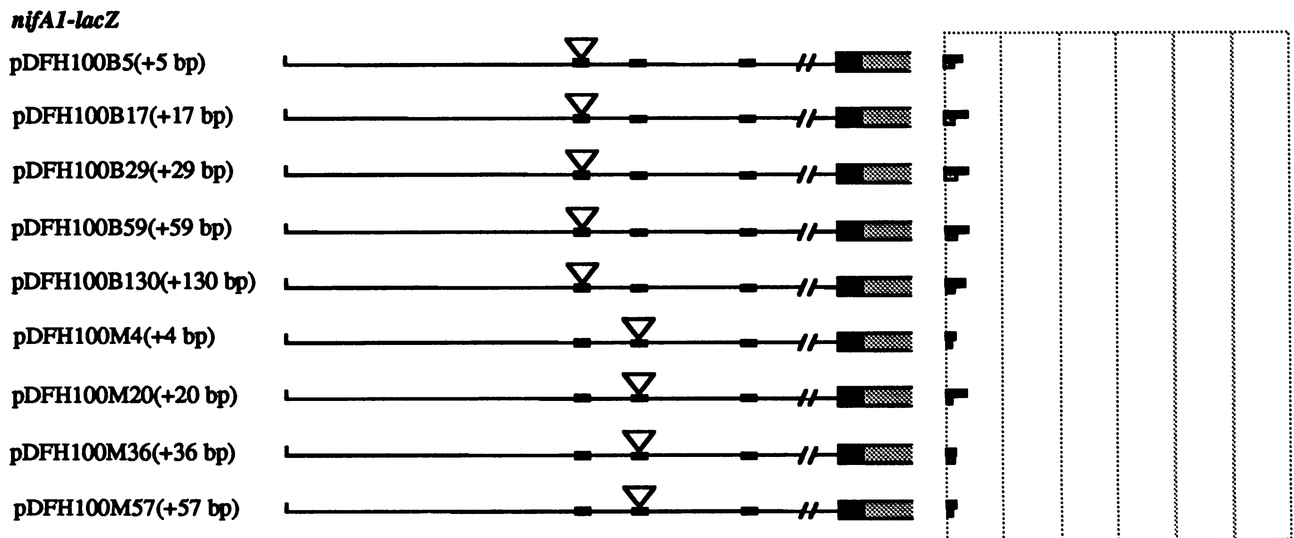


FIG. 1. Deletion and insertion analysis of *nifA1*- and *nifA2-lacZ* fusions. (A) β-Galactosidase (β-gal) activities of *nifA1*- and *nifA2-lacZ* plasmids containing 5′ deletions. The deletion endpoint is shown in parentheses next to the plasmid designation; numbers are distances (in bases) from the most distal transcription start sites; in the case of *nifA2*, two potential start sites were determined (20, 54). Inverted repeats are marked with arrows; the conserved 9-bp region is lightly shaded. (B) β-Galactosidase activities of *nifA1-lacZ* plasmids containing insertions in either the *Bst*EII site or the *Mlu*I site. Sizes of the insertions are shown in parentheses. β-Galactosidase assays are described in Materials and Methods; units of activity are expressed in nanomoles of *o*-nitrophenol per minute per milligram of protein. Values for pDFH100P, pDFH200H, pDFH2009, and pDFH200T have been reported previously (20). All assays were done with the wild-type strain SB1003.

promoter is slightly different for *nifA1* in Fig. 1 than reported by Foster-Hartnett and Kranz (20), reflecting the sequence difference that we observed; the numbering for *nifA2* is also shifted by −3 bp. These changes are described further in Results.

Expression and purification of RcNtrC. The coding region of *R. capsulatus ntrC* was amplified by PCR using CGGGATC CCATATGGATGGCACCGTTCTCG as the 5′ primer, CCC AAGCTTACATCAGTTTGCGCCGCCTTG as the 3′ primer, and pDQ2013 (31) as the template DNA. The 1.4-kb *Bam*HI-*Hin*dIII PCR fragment was first cloned into the *Bam*HI-*Hin*dIII sites of pUC118 to generate pNTRC118. To construct pNTRCPET21b, the 1.4-kb *Nde*I-*Hin*dIII fragment of pNTRC118 was subcloned into the *Nde*I-*Hin*dIII sites of the inducible T7 expression vector pET21b (Novagen) (63). (This *R. capsulatus ntrC* gene was shown to be functional in complementation assays with RcNtrC⁻ strains; that is, it complements an NtrC⁻ strain [Nif⁻] to a Nif phenotype [19].) One-liter cultures of *E. coli* BL21(DE3) transformed with pNTRC PET21b were grown in LB-carbenicillin (50 μg/ml) broth to an optical density (A_{600}) of 0.6 to 0.9 at 37°C before induction with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM, final concentration). After a 3-h induction at 32°C, cells were concentrated by centrifugation and frozen at −80°C. The purification procedure was performed at 4°C. The cell pellets were thawed on ice, resuspended in buffer A (50 mM Tris-HCl [pH 8.0], 0.5 mM EDTA [pH 8.0], 0.5% glycerol, 1 mM dithiothreitol) plus 150 mM NaCl and 0.5 mM phenylmethylsulfonyl fluoride. Resuspended cells were lysed by repeated sonication; unbroken cells were removed by low-speed centrifugation. Streptomycin sulfate was added to the cell lysate (1 mg/ml), and the mixture was centrifuged at 40,000 × *g* for 1 h. $(NH_4)_2SO_4$ (20%) was added to the supernatant, and the mixture was centrifuged at 12,000 × *g*. The $(NH_4)_2SO_4$ saturation of the supernatant was increased to 40%, and the mixture was centrifuged again at 12,000 × *g*. The 40% $(NH_4)_2SO_4$ pellet containing RcNtrC was resuspended in buffer A and dialyzed in the same buffer for 1 h before loading on a heparin-agarose column. Fractions were step eluted at 0.1, 0.3, 0.5, 0.7, and 0.9 M NaCl. The 0.5 M fraction containing RcNtrC was concentrated by precipitation with $(NH_4)_2SO_4$ and then loaded onto a Sephacryl S200HR column (Pharmacia). Fractions containing RcNtrC were stored in 20% glycerol at −20°C. RcNtrC was assayed by DNA footprinting and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) during the purification (Fig. 2). RcNtrC was determined to be at least 95% pure, as determined by visual inspection of a Coomassie blue-stained SDS-polyacrylamide gel (Fig. 2). Protein was quantitated by the bicinchoninic acid (Pierce) method with bovine serum albumin as a standard. The RcNtrC mutant (G-176→N) was purified in the same way except that dialyzed 0.5 M fraction of the heparin-agarose column was used without further purification.

DNA sequencing. DNA sequencing was carried out by the dideoxy method of Sanger et al. (59), using [^{35}S]dATP and [^{32}P]dATP sequencing and both the Sequenase and Taquenase enzymes (U.S. Biochemical Corp.). Single-stranded template was prepared with inserts in pUC118 and pUC119 (65).

Site-directed mutagenesis of *R. capsulatus ntrC*. The 168-bp *Apa*I-*Bcl*I fragment of *R. capsulatus ntrC* was PCR amplified by using the degenerate 5′ primer (shown 5′ to 3′) TGATGAT CATGGGCGAATCCGGCACCANCAA and the 3′ primer AGACGACGAGCCGCCCTTGA. *Apa*I-*Bcl*I PCR fragments were recloned into the *Apa*I-*Bcl*I sites of pBMM5, which is the 2.2-kb *Hinc*II fragment of *ntrC* cloned into the *Sma*I sites of pUC119. The entire PCR-amplified region was sequenced by

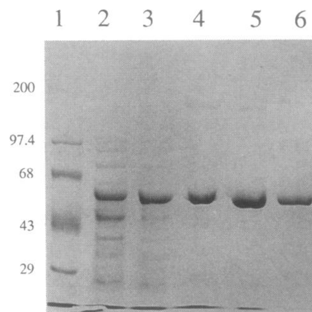


FIG. 2. SDS-PAGE analysis of RcNtrC purification from an *E. coli* overexpression system. Lanes 2 to 6 contain 10 μg each of extracts from *E. coli* BL21(λDE3) transformed with a plasmid containing RcNtrC cloned behind an inducible T7 *lac* promoter. Lanes: 1, molecular weight markers; 2, sonicated cell supernatant; 3, 40% ammonium sulfate precipitate following 40,000 rpm centrifugation; 4, 0.5 M NaCl fraction from heparin-agarose column; 5, 40% ammonium sulfate precipitate of 0.5 M NaCl heparin-agarose fraction; 6, fraction 10 from Sephacryl S200 column. Molecular weight size standards: myosin (H chain), 200 kDa; phosphorylase *b*, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa. The gel was stained with Coomassie brilliant blue.

methods described above. *Hin*dIII-*Eco*RI (vector-derived sites) fragments (2.2 kb) of wild-type and mutant alleles of *ntrC* were subcloned into the *Hin*dIII-*Eco*RI sites of the broad-host-range plasmid pUCA9 (19).

DNase I footprinting. End-labeled fragments were prepared by first digesting plasmids (pPA2TB, pPA1Bcl, pPA1P16, and pPA1P12) with a single restriction enzyme (described in figure legends). The 5′ ends were dephosphorylated with shrimp alkaline phosphatase (U.S. Biochemical) and labeled with γ-[^{32}P]dATP and T4 polynucleotide kinase. Following a second restriction enzyme digest, the labeled fragments were loaded onto 5% native acrylamide gels (50 mM Tris-borate-EDTA). Gel slices containing the appropriate fragments (122 to 506 bp) were excised, and the probes were eluted in Tris-EDTA buffer at 37°C overnight. End-labeled DNA (15,000 to 35,000 cpm) was mixed with various concentrations of NtrC in binding buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 50 mM KCl, 1.0 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) including 100 ng of poly-(dIdC) as a nonspecific competitor. Complexes were allowed to form at 23°C for 20 min in a final volume of 50 μl. The mixtures were then treated with 50 μl of DNase I (1 ng in 10 mM $MgCl_2$–5 mM $CaCl_2$) for 1 min at 23°C, and the reactions were stopped with 90 μl of stop buffer (20 mM EDTA, 1.0% SDS, 200 mM NaCl, 125 μg of yeast tRNA per ml). The DNA was purified by one extraction with phenol-chloroform (1:1), ethanol precipitated, washed with 70% ethanol, and resuspended in formamide dyes. Sequencing gels (6 and 8%) were used to analyze the products. Using the combination of size standards described in the text, we located areas of protection or DNase I hypersensitivity to ±1 bp.

Other methods. Conjugations with triparental matings were carried out as described previously (4). Complementation of *R. capsulatus ntrC* strains, as defined by ability to fix nitrogen, have been described previously (19). β-Galactosidase activities were determined as described previously (20). Protein measurements for β-galactosidase assays were determined by the modified Lowry method (39). Values for pDFH2009, pDFH100P, and pDFH200H have been reported previously (20). Other values shown in Fig. 1 are averages of at least four

independent assays with the same pattern of expression in each experiment. Values shown in Fig. 7B are the average of two to four independent assays with the same result in each case.

RESULTS

Deletion analysis of sequences upstream of the *nifA1* and *nifA2* promoter regions. To define the sequences upstream of *nifA1* and *nifA2* that are essential for RcNtrC-dependent nitrogen control, and therefore a putative RcNtrC-binding site, we constructed *nifA1*- and *nifA2-lacZ* fusions with progressive 5′ deletions generated by PCR. One obvious region of homology between the *nifA1* and *nifA2* promoters is a 9-bp palindrome, TCTGCCAGA, beginning at −142 for *nifA1* and −148 for *nifA2*. Largely on the basis of this homology, Preker et al. (54) proposed that this was the RcNtrC-binding site. However, when this sequence was deleted from the *nifA2-lacZ* promoter, NtrC-dependent activation was not reduced significantly (20). To control for the possibility that the vector or the vector/insert junction provided an activation site for NtrC, we cloned this fragment (deletion to −139) in the opposite orientation with respect to the vector, pDFH200P8 (Fig. 1). The β-galactosidase activity expressed by pDFH200P8 in wild-type *R. capsulatus* is not significantly lower than the β-galactosidase expression of pDFH2009 (deletion to −151). We observed an 8- to 10-fold induction of both pDFH2009 and pDFH200P8 β-galactosidase expression when wild-type SB1003 strains containing these plasmids were induced in media lacking fixed nitrogen (Fig. 1); β-galactosidase activity was at background levels in an *ntrC* strain (not shown). When the same 9-bp sequence was deleted from *nifA1*, however, activity was reduced fivefold (compare pDFH100P16-1 with pDFH100P15-5).

During the construction and sequencing of DNA in these plasmids, we observed a difference from the published sequence (40) in the region immediately downstream of the *nifA1* 9-bp palindrome. We have sequenced the chromosomal DNA amplified by PCR from strains SB1003 and B10 and both strands of the cloned *nifA1*. In each case, the sequence is as indicated in pDFH100P16-1 (Fig. 1). Our sequence differed at five bases (with a net +2 bp) from that published by Masepohl et al. (40) (Fig. 1; compare underlined sequences in pDFH 100P12 and pDFH100P16-1). The presence of the originally published 5 bp in the place of the new sequence decreased expression nearly sevenfold, to nearly background levels. Thus, this region of DNA is essential for NtrC-dependent activation of *nifA1*.

The *nifA1* and *nifA2* promoters show homology in another larger palindromic region located from −139 to −125 for *nifA1* and −134 to −114 for *nifA2*. Removal of upstream sequence to −114 for *nifA2* (pDFH200P11) decreased activation to ammonia-independent background levels (Fig. 1). Removal of upstream sequence to −84 decreased activation of *nifA1* (pDFH 100BstE2) an additional twofold (compared with the −133 deletion). This low level of β-galactosidase activity expressed by SB1003(pDFH100BstE2) may represent a basal level of NtrC-independent activity previously observed (reference 20 and unpublished data). These results define the putative RcNtrC-binding site(s) at −114 to −139 for *nifA2* and −133 to −142 for *nifA1*. Furthermore, these results indicate that the sequence required for RcNtrC activation is not contained solely in the 9-bp palindrome TCTGCCAGA, as suggested by Preker et al. (54), but is present in a larger palindromic region that includes this sequence for *nifA1* but not *nifA2*.

Analysis of *nifA1-lacZ* fusions containing insertions at two sites in the promoter region. Since DNA upstream of the *Bst*EII site (−84) is required for induction of high levels of *nifA1* expression, we wanted to determine if the upstream activation sequence could function as an enhancer. We constructed *nifA1-lacZ* fusions containing DNA insertions of various lengths at both the *Bst*EII site and the *Mlu*I (−47) site. Insertions of 5, 17, 29, 59, and 130 bp in the *Bst*EII site reduced activation approximately 10-fold (Fig. 1B). This is the same level of activity observed when the sequence upstream of the *Bst*EII site is deleted (pDFH100BstE2; Fig. 1A). Similar results were obtained when DNA insertions of 4, 20, 36, or 57 bp were cloned into the *Mlu*I site of *nifA1-lacZ*, although the levels of activity were slightly lower. β-Galactosidase activity in SB1003(pDFH100M20) was 4-fold higher in low-nitrogen media than in high-nitrogen media, but the level of expression measured in low-nitrogen media was still 9- to 10-fold lower than expression from plasmids with the upstream activating sequence in the wild-type position. Thus, even when the position of the upstream region is predicted to be on approximately the same face of the DNA helix (insertions of 20, 59, or 130 bp), activation by RcNtrC is substantially reduced.

Purification of recombinant RcNtrC. We purified the RcNtrC protein to test whether NtrC directly activates the *nifA1* and *nifA2* genes and if so to determine the exact binding sites for RcNtrC. The RcNtrC gene product was overexpressed in *E. coli*, using an inducible T7 expression system (63). Transformed cells containing *R. capsulatus ntrC* cloned behind the T7 *lac* promoter (pNTRCPET21b) expressed a protein of approximately 54 kDa at high levels after the addition of IPTG to the growth media (Fig. 2, lane 2). The apparent molecular mass of 54 kDa is slightly larger than the 49.4 kDa predicted by sequence analysis (31). The induced 54-kDa protein was purified from sonicated extracts in several steps, and the NtrC protein was assayed both by SDS-PAGE (Fig. 2) and by DNase I footprint analysis (discussed below). After streptomycin sulfate and ammonium sulfate precipitations, followed by heparin-agarose affinity and Sephacryl S200 (gel filtration) chromatography, the RcNtrC protein was greater than 95% pure as determined by SDS-PAGE (Fig. 2). By gel filtration chromatography, we observed that the RcNtrC behaves as a dimer.

DNase I footprint analysis of the *nifA1* and *nifA2* promoter regions. Since transcription of *nifA1*- and *nifA2-lacZ* is dependent on the *ntrC* gene, we predicted that RcNtrC might directly contact the *nifA1* and *nifA2* promoters around −130, in regions defined by deletion analysis to be critical for activation of *nifA1*- and *nifA2-lacZ* fusions. We used DNase I footprint analysis (22) to identify DNA sequences involved in contacting RcNtrC. Labeled *nifA1* fragments containing the upstream region −166 to +256 (upper strand) or −166 to −47 (lower strand) (Fig. 3) were incubated with increasing concentrations of purified RcNtrC and then subjected to limited DNase I digestion. The positions of DNase I cleavage sites were localized by comparison with chemical sequence reactions specific for guanines, labeled DNA size standards, and dideoxy DNA sequence ladders generated with a primer originating at the position of the label on the bottom-strand probe. At concentrations starting at 20 nM RcNtrC, sites of enhanced DNase I cleavage could be seen at −137 on the upper strand probe (Fig. 3A, lane 3, large arrowhead) and at −98 on the lower strand probe (Fig. 3B, lane 3, large arrowhead). For both the upper and lower strands, several areas of protection were apparent from 80 to 160 nM RcNtrC, extending from −88 to −143 (upper strand) and −89 to −145 (lower strand). At 160 nM RcNtrC, several other minor DNase I-hypersensitive bands appeared (Fig. 3A and B, lanes 6C, marked by small arrowheads). Two areas that share homology with *nifA2*, region I

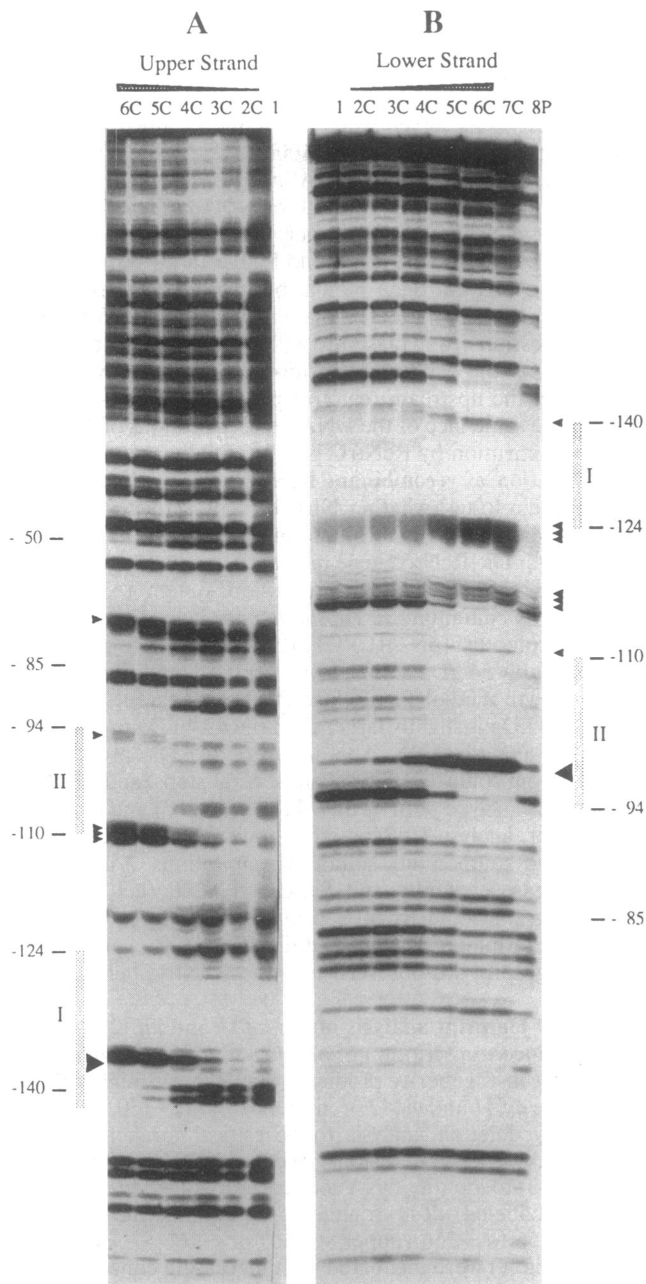


FIG. 3. Protection of the *nifA1* promoter region from DNase I digestion by RcNtrC. (A) Analysis of the upper strand, using the 422-bp *Hin*dIII-*Eco*RI fragment from pPA1Bc1 (approximately 0.8 nM probe per reaction). (B) Analysis of the lower strand, using the 148-bp *Hin*dIII-*Mlu*I fragment from pPA1Bc1 (approximately 0.2 nM per reaction). For locations of fragment endpoints, see text and Fig. 5. RcNtrC concentrations (dimer) were 0 nM (lane 1), 10 nM (lane 2C), 20 nM (lane 3C), 40 nM (lane 4C), 80 nM (lane 5C), and 160 nM (lane 6C). Lanes 7C and 8P contained 1.0 μg of the 0.5 M NaCl heparin-agarose fraction purified from *E. coli* BL21(λDE3) transformed with either pNTRCPET21b (lane 7C) or pET21b (lane 8P). Shaded bars mark potential RcNtrC-binding sites (see text); numbers refer to the distance from the transcription start; large and small arrowheads mark areas of increased DNase I sensitivity.

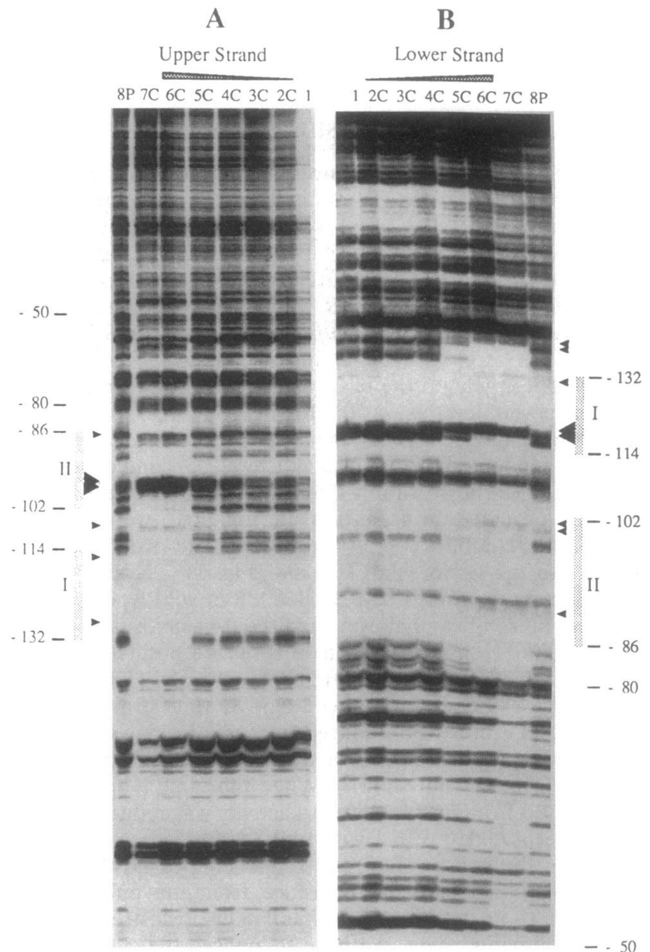


FIG. 4. Protection of the *nifA2* promoter region from DNase I digestion by RcNtrC. (A) Analysis of the upper strand, using the 506-bp *Sal*I-*Eco*RI fragment from pPA2TB. (B) Analysis of the lower strand, using the 217-bp *Xba*I-*Hin*dIII fragment from pPA2TB. Both probes were used at approximately 0.1 nM per reaction. For locations of fragment endpoints, see text and Fig. 5. RcNtrC concentrations were 0 nM (lane 1), 10 nM (lane 2C), 20 nM (lane 3C), 40 nM (lane 4C), 80 nM (lane 5C), and 160 nM (lane 6C). Lanes 7C and 8P contained 2.0 μg of the 0.5 M NaCl heparin-agarose fraction purified from *E. coli* BL21(λDE3) carrying pNTRCPET21b (lane 7C) or pET21b (lane 8P). Shaded bars mark potential RcNtrC-binding sites (see text); numbers refer to the distance from the transcription start; large and small arrowheads mark areas of increased DNase I sensitivity.

and region II (see below), are centered at −140 to −124 and −110 to −94, respectively.

The *nifA2* upstream region was analyzed in the same way (Fig. 4). The upper-strand probe extended from −220 to +260, and the lower-strand probe extended from −220 to −10. Intense hypersensitive sites at −98 and −99 (upper strand) and −119 and −120 (lower strand) and several minor hyperreactive bands were present when binding reaction mixtures contained 160 nM RcNtrC (Fig. 4A and B, lanes 6C). Protection extended from −80 to −135 (upper strand) and −82 to −138 (lower strand) at an RcNtrC concentration of 160 nM. Similar to the analysis with *nifA1*, region I centered at −132 to −115 and region II centered at −102 to −85 were completely protected at 160 nM RcNtrC.

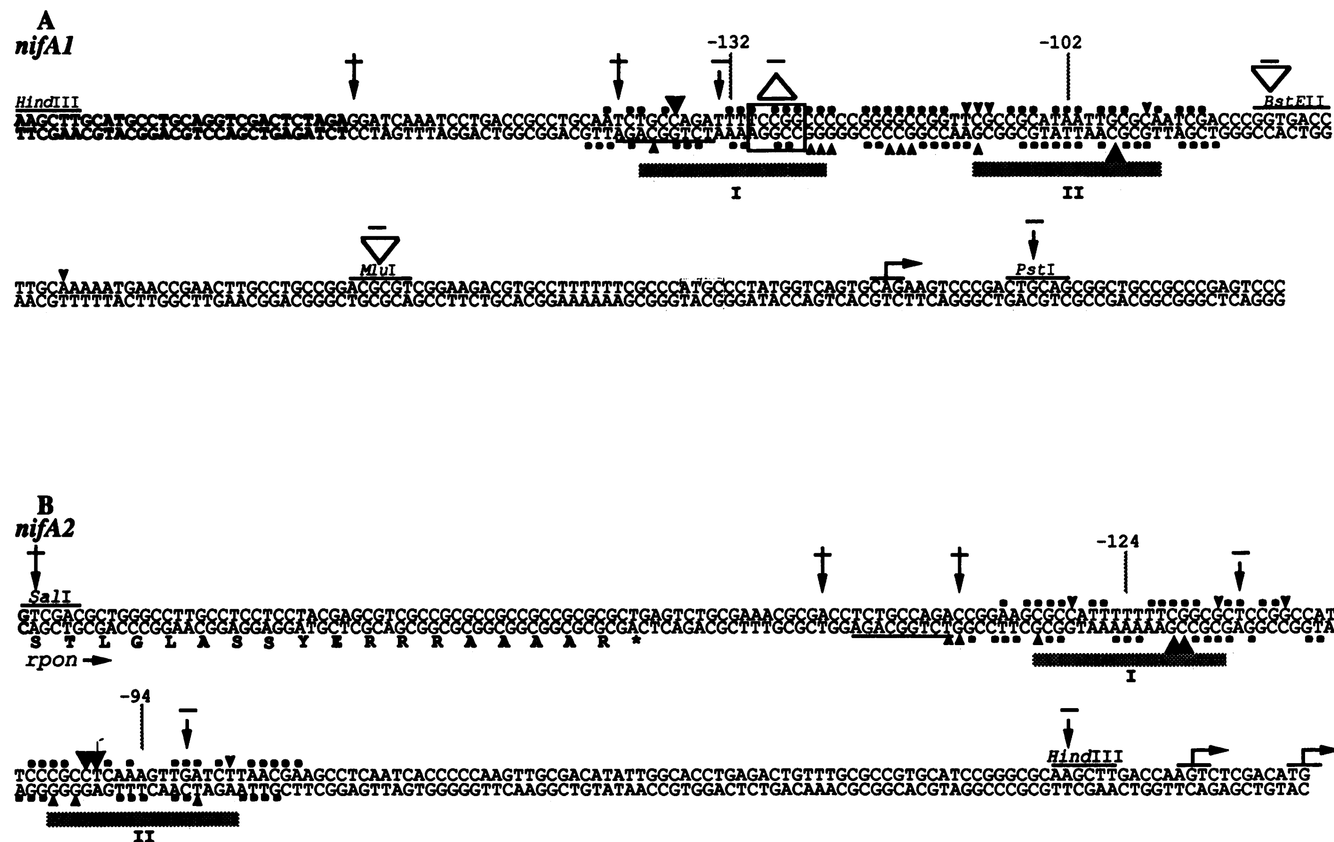


FIG. 5. Summary of DNase I protection data and deletion analysis for the *nifA1* (A) and *nifA2* (B) promoter regions. Boldface letters indicate vector-derived sequence. The C-terminal amino acids of *rpoN* are shown upstream of the *nifA2* sequence. Major and minor DNase I-hypersensitive sites (large and small arrowheads), regions of DNase I protection (large dots), and potential NtrC-binding sites (horizontal shaded bars) are shown (see text). Horizontal arrows mark the transcription start sites (20, 54). Numbers are the distance from the most distal promoter. (In the case of *nifA2*, two potential start sites were identified.) Sites of 5′ deletions (vertical arrows) and DNA insertions (inverted triangles) in *nifA1*- and *nifA2-lacZ* fusions are depicted. Relative β-galactosidase activities are indicated by + (100%) and − (<20%).

As a control, heparin-agarose fractions purified from *E. coli* containing either RcNtrC (Fig. 3B, lane 7C; Fig. 4A and B, lanes 7C) or the expression vector alone (Fig. 3B, lane 7C; Fig. 4A and B, lanes 7P) were added at equivalent concentrations (micrograms of protein) to labeled *nifA1* and *nifA2* fragments and analyzed by DNase I digestion. The patterns of DNase I digestion for reactions containing 1.0 μg (Fig. 3B, lanes 1 and 8P) and 2.0 μg (Fig. 4A and B, lanes 1 and 8P) of the *E. coli* control extract were essentially the same as those containing no protein. Identical results were obtained with the *nifA1* probes when crude sonicated extracts from the RcNtrC (i.e., footprints) and *E. coli* control (i.e., no footprints) were used (not shown).

For both *nifA1* and *nifA2* promoters, at high concentrations of RcNtrC, we observed areas of weaker protection downstream (closer to the promoter) of region II between approximately −85 to −50 for *nifA1* (Fig. 3A, lane 6C; Fig. 3B, lanes 6C and 7C) and approximately −80 to −50 for *nifA2* (Fig. 4B, lanes 6C and 7C). The extents of DNase I protection in these areas varied slightly from experiment to experiment; however, the degree of protection observed was always weak even at concentrations two- to fourfold greater than that required to completely protect sequences centered at regions I and II (unpublished observations). Since one area of the *nifA1* promoter weakly protected by RcNtrC includes the *Bst*EII site at −84, we also performed DNase I analysis using a 155-bp *Mlu*I-*Hin*dIII probe that contained the same 5-bp insert in the *Bst*EII site as the *nifA1-lacZ* fusion, pDFH100B5. Although the weak protection at −84 was abolished, the pattern of protection with respect to regions I and II was unchanged (not shown).

Locations of DNase I protection and the 5′ deletion endpoints of *nifA1*- and *nifA2-lacZ* fusions. The regions of *nifA1* and *nifA2* protected from DNase I cleavage by RcNtrC are summarized in Fig. 5. For *nifA1* and *nifA2*, the protected areas of both strands are located within the boundaries established by 5′ deletion analysis of *nifA1*- and *nifA2-lacZ* (Fig. 1). Vertical arrows marked with a + indicate deletion endpoints of *nifA1*- or *nifA2-lacZ* which retained high levels of induction, while − indicates a fivefold or greater loss of activity. Regions I and II represent potential binding sites for RcNtrC based on the following criteria: (i) pattern of major hypersensitive sites (marked by large arrowheads) and protected sites, (ii) presence of palindromic sequence (see Fig. 1 for site I), (iii) sequence homology when the four regions are aligned, and (iv) some homology with the consensus sequence identified for the enteric NtrC (1, 25) (see Discussion). We next tested the biological significance of the RcNtrC-*nifA1* promoter interaction at region I and determined whether region I (or region II) could represent an independent recognition site for RcNtrC. To do this, we compared the patterns of DNase I protection for the wild-type sequence present in the *nifA1-lacZ* fusion

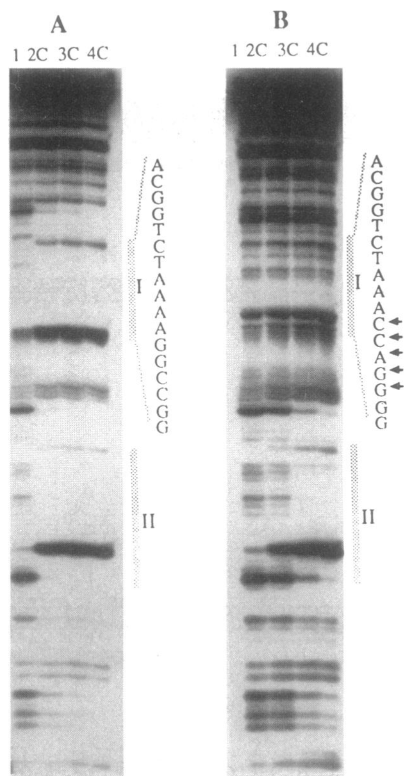
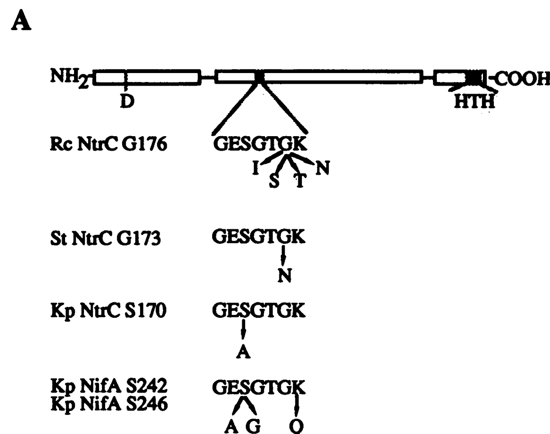


FIG. 6. Comparison of DNase I protection of wild-type and mutated *nifA1* promoter regions. (A) Analysis of the lower strand of the wild-type sequence, using the 124-bp *Hin*dIII-*Mlu*I fragment from pPA1P16. (B) Analysis of the lower strand of a *nifA1* promoter region containing mutations in region I, using the 122-bp *Hin*dIII-*Mlu*I fragment from pPA1P12. The promoter sequences of these probes are the same as those present in Fig. 1 for pDFH100P16-1 and pDFH100P12. RcNtrC concentrations were 0 nM (lane 1), 80 nM (lane 2C), 160 nM (lane 3C), and 320 nM (lane 4C). Arrows indicate bases that were changed in the pPA1P12 sequence. In all lanes, the probe was used at approximately 0.40 nM.



B

Strain	Description	Nif Phenotype
102-C4 (pBMM5)	*wt ntrC*	+++
102-C4 (no plasmid)		
102-C4 (pBMMHc9)	G176N	-
102-C4 (pBMMHc11)	G176T	-
102-C4 (pBMMHc12)	G176S	-
102-C4 (pBMMHc14)	G176I	-

Strain	Description	β-gal Activity -O2/-NH3
J61::*nifA2-lacZ* (pBMM5)	wt *ntrC*	93
J61::*nifA2-lacZ* (no plasmid)		4
J61::*nifA2-lacZ* (pBMMHc9)	G176N	8
J61::*nifA2-lacZ* (pBMMHc11)	G176T	10
J61::*nifA2-lacZ* (pBMMHc12)	G176S	10
J61::*nifA2-lacZ* (pBMMHc14)	G176I	12

FIG. 7. Site-directed mutagenesis of RcNtrC. The ability of NtrC alleles (G176I, -T, -N, or -S) to activate transcription was assayed for Nif phenotype of the 102-C4 ($NtrC^-$) strains (A) and by β-galactosidase (β-gal) expression of chromosomal *nifA2-lacZ* in $NtrC^-$ background (102-C4) (B). β-Galactosidase assays are described in Materials and Methods; units of activity are expressed in nanomoles of *o*-nitrophenol per minute per milligram of protein.

pDFH100P16-1 (Fig. 6A) and pDFH100P12 (Fig. 6B), which is altered by 5 bp in the second half of site I proposed above. The results indicate that the area designated region II plus approximately 5 bp on either side is protected from DNase I digestion using both the wild-type pPA1P16 (Fig. 6A) and the pPA1P12 (Fig. 6B) probes. In contrast, no protection of region I was observed with the pPA1P12 template (Fig. 6B) even at RcNtrC concentrations of 320 nM. Approximately four times as much RcNtrC was required to protect site II of the pPA1P12 template compared with the P16 template, indicating that there may be some cooperativity between RcNtrC bound at these regions (see Discussion).

Site-directed mutagenesis of the ATP-binding loop of RcNtrC. All activators of the σ^{54} RNAP contain the conserved nucleotide-binding motif GX(S)GXGK (67) in the central domain (36, 46). Mutations of this motif that have been characterized in the enteric NtrC and NifA are shown in Fig. 7. In *Salmonella typhimurium*, NtrC G173N has no detectable ATPase activity yet retains DNA-binding specificity (70). Similarly, the mutation S170A in *Klebsiella pneumoniae* NtrC abolishes activation without disruption of specific DNA recognition or formation of closed promoter complexes (3, 18). *K. pneumoniae* mutations of NifA S242A, S242G, and K246Q also demonstrated that the integrity of the ATP-binding motif is necessary for positive control function but is not required for DNA binding (11). We used site-directed mutagenesis to alter the conserved G-176 of RcNtrC to four alternate amino acids (Fig. 7) to determine whether this conserved motif is required for activation function and/or DNA binding of RcNtrC. As shown in Fig. 7B, each of the four mutant alleles of RcNtrC was completely deficient in complementing the *R. capsulatus ntrC* Tn*5* insertion strain 102-C4 (to a Nif^+ phenotype). To test for transcriptional activation of *nifA2* by the mutated RcNtrC proteins, the same alleles were conjugated into the $NtrC^-$ strain J61, which contains a chromosomal *nifA2-lacZ* reporter. In each strain containing a mutant allele of RcNtrC, the expression of chromosomal *nifA2-lacZ* was approximately 10-fold less than expression of the wild-type control (Fig. 7).

To determine whether such point mutations affect the overall structure and stability of RcNtrC and its DNA-binding properties, we partially purified one of the mutant proteins. Extracts containing RcNtrC(G176N) protected both the *nifA1* and *nifA2* promoter regions from DNase I digestion (Fig. 8A and B, lanes 3M), indicating that the activation and DNA-binding activities for RcNtrC may be functionally dissociated.

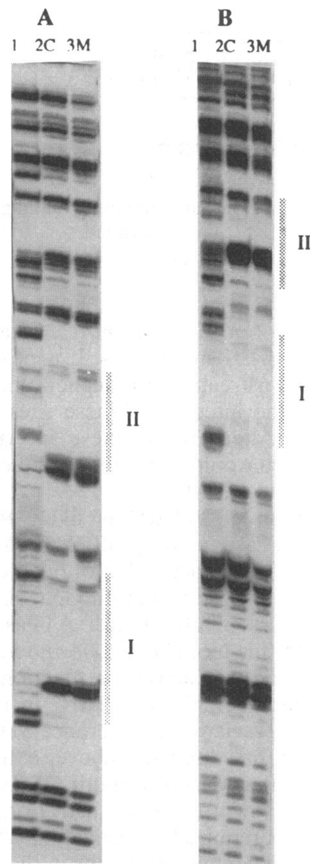


FIG. 8. Protection of the *nifA1* and *nifA2* promoter regions from DNase I digestion by wild-type RcNtrC and RcNtrC(G176N). *nifA1* upper strand (A) and *nifA2* (B) upper strand are the same probes described for Fig. 3 and 4. Lanes: 1, no protein added; 2C, RcNtrC extract; 3 M, RcNtrC(G176N) extract. Extracts of both wild-type and mutant RcNtrC were 0.5 M NaCl heparin-agarose fractions (see text). Approximately equal concentrations (~40 to 50 nM) of RcNtrC and RcNtrC(G176N), as estimated by SDS-PAGE, were used. Shaded regions I and II are potential RcNtrC-binding sites (see text).

DISCUSSION

In other systems studied to date, members of the NtrC class of EBPs characteristically activate transcription from typical −12, −24 promoters recognized by the alternative sigma factor σ^{54}, encoded by *rpoN*. Activators bind to distal sites greater than 100 bp upstream of the transcriptional start and catalyze open complex formation that is dependent on ATP hydrolysis. In our previous work, we determined genetically that the expression of *nifA1* and *nifA2* requires the products of *R. capsulatus ntrC* but not *rpoN*, representing a potentially unique form of transcriptional activation. There are at least three formal explanations of these results. First, it is possible that RcNtrC activates *nifA1* and *nifA2* by activating an intermediate regulator. An example of this type would be the Nac regulon of *K. pneumoniae*, in which NtrC activates the *nac* gene, which then controls the induction of operons like *hut* and *put* in response to nitrogen starvation (5). One immediate difference between the Nac system and RcNtrC is that the expression of Nac-controlled genes still requires *rpoN* because *nac* itself is expressed from a σ^{54} promoter. A second possibility is that another, yet undiscovered copy of *rpoN* directly activates the *nifA1* and *nifA2* promoters or acts indirectly through a Nac-like intermediary. Finally, RcNtrC could directly activate promoters recognized by a sigma factor other than σ^{54}. In this work, we demonstrated that RcNtrC specifically recognizes distal regions of the non-σ^{54} promoters of *nifA1* and *nifA2* and that the activation function of RcNtrC requires the integrity of the highly conserved nucleotide-binding region, similar to other EBPs (3, 11, 18, 70). Specific details of the NtrC system, particularly with respect to this third hypothesis, are discussed below.

Molecular genetic definition of a potential RcNtrC-binding site determined in vivo. Deletion analysis of *nifA1-lacZ* fusions have defined a region of the promoter sequence between −142 and −133 which was essential for low-nitrogen induction and activation by RcNtrC; induction of *nifA2-lacZ* required DNA located between −139 and −114. Alteration of the DNA sequence of *nifA1-lacZ* at −130 to −125 from TCCGG to GGTCC (plus a net −2 bp immediately downstream) reduced activation sevenfold. A large A/T-rich palindromic region located from −139 to −125 for *nifA1* and −134 to −114 for *nifA2* was found to be essential for both *nifA1*- and *nifA2-lacZ* induction (horizontal arrows in Fig. 1). For *nifA2-lacZ*, deletion of this sequence (to −114) had a particularly marked effect, since activation was decreased by approximately 60-fold.

The RcNtrC-binding sites determined by footprint analysis. Purified RcNtrC protected several regions of the *nifA1* and *nifA2* promoters from DNase I digestion. This region extended from −89 to −145 for *nifA1* and from −80 to −138 for *nifA2*. This region of protection agrees almost to the nucleotide with the results of the deletion analysis described above (Fig. 5). The larger A/T-rich palindromic sequence was protected on both strands of the DNA for both the *nifA1* and *nifA2* upstream regions. The specificity of the RcNtrC-promoter interaction was tested with the mutated region of *nifA1* present in pDFH100P12. Even at high concentrations (320 nM), RcNtrC did not protect region I of this probe (Fig. 6). Thus, specific mutations which decreased activation in vivo abolished RcNtrC binding in vitro. The ratio of RcNtrC dimer to labeled DNA required to protect the *nifA1* and *nifA2* promoters was within the range observed for *K. pneumoniae* NtrC binding at the *nifLA* promoter (43) and *E. coli* NtrC at the *glnA* promoter region (48). Although phosphorylation of NtrC increases the cooperativity of oligomerization at the *nifL* (43) and *glnAp2* promoters (53, 71), specific binding site recognition does not require NtrC~P (reviewed in reference 53). RcNtrC is probably phosphorylated in vivo, since the *R. capsulatus ntrB* homolog is required for activation of *nif* promoters and the site of RcNtrC phosphorylation is conserved (31, 33). Very recently, we have shown that RcNtrB, purified as a fusion protein to the maltose-binding protein, phosphorylates RcNtrC in vitro, thus confirming this suggestion (14a).

Within the area of protection, two promoter regions of *nifA1* and *nifA2*, regions I and II, are homologous with respect to sequence, symmetry, and pattern of major hypersensitive sites (Fig. 9). Given that the *R. capsulatus* genome is 65 to 67% G+C, the A/T-rich sequence located in the center of each region is particularly striking. RcNtrC also protects two regions of similar sequence in the NtrC-dependent *glnBp2* promoter of *R. capsulatus* (21). A consensus recognition sequence for RcNtrC was presented in that study (21). We propose that the boxed sequences in Fig. 9 represent potential binding sites for RcNtrC. Each of the four regions contains some homology in sequence and symmetry to the consensus enteric NtrC site (1, 24, 27, 57). For the enteric NtrC, DNase I protection often extends several bases on either side of the recognition site (1, 25, 74), and for the *nifLA* promoter, major DNase I-hypersen-

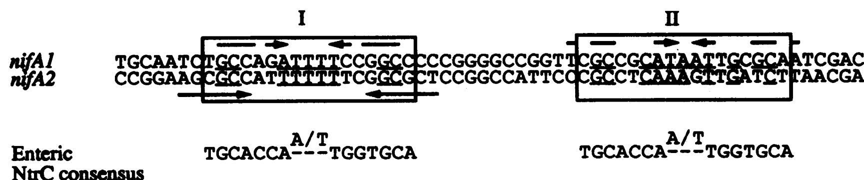


FIG. 9. Comparison of the regions of *nifA1* and *nifA2* protected by RcNtrC. The boxed regions are potential binding sites (see text). Palindromic regions are marked with arrows. Bases homologous to the enteric NtrC consensus (1, 17) are underlined.

sitive sites are present in the same locations as defined here (± 1 bp) (43, 74).

Promoters activated by σ^{54} EBPs typically contain more than one EBP-binding site (reviewed in reference 13). The enteric NtrC is a dimer in solution (25, 56) and binds to each site as a dimer (53). The role of multiple binding sites may be to facilitate the oligomerization of phosphorylated dimers, which is essential for activation (53). Gel filtration analysis and the pattern of migration in native gels suggest that RcNtrC is also a dimer in solution (unpublished observations). Based on the sequence homology of the *nifA1* and *nifA2* upstream DNA and by analogy with enteric NtrC-activated promoters, regions I and II may represent independent sites for interaction with RcNtrC dimers. As for other NtrC-activated promoters, the presence of both potential sites for RcNtrC must be present for optimal activation (43, 57). Disruption or removal of site I for *nifA1* reduced activation by at least 5-fold, while removal of site I for *nifA2-lacZ* reduced activation by 60-fold. For *nifA1*, DNase I protection of the proposed site II is weaker when site I is disrupted (Fig. 6), suggesting possible cooperativity. The results with this template also support the hypothesis that two high-affinity dimer sites are present.

The locations of RcNtrC-binding sites are distal to the promoter. The locations of binding sites for bacterial EBPs is typically outside the range of the RNAP footprint (13). Our DNase protection data show that RcNtrC sites are also located far upstream of the promoter. Proposed sites are centered at −132 and −102 for *nifA1* and −124 and −94 for *nifA2*. For both *nifA1* and *nifA2*, 30 bp (approximately three turns of the helix) separate the two regions, similar to the high-affinity enteric NtrC sites at *glnAp2*. For some promoters, the tandem binding sites of EBPs can be moved greater than 1 kb from the promoter and retain activation properties, similar to eukaryotic enhancer elements (7, 9, 57). Our studies have shown that DNA insertions of various sizes located at −47 or −84 bp upstream of the *nifA1-lacZ* promoter disrupted activation. These results are analogous to those obtained with the DctD-activated promoter, *dctA*, of *Rhizobium leguminosarum* and *Rhizobium meliloti* (37), in which DNA insertions of several lengths reduce expression of *dctA-lacZ* to the same level as when both sites are removed. There are several explanations for the results with the *nifA1* inserts. First, like the promoters activated by DctD, NifA, XylR, and NtrC (at *glnHp2*), RcNtrC may require a coactivator like integration host factor, which presumably facilitates interaction of the activator with RNAP (12, 15, 28, 30, 37, 60). Alternatively, HU or another histone-like protein may assist in correctly positioning bound RcNtrC with respect to the promoter. Insertions could disrupt binding of this coactivator. A second possibility is that phosphorylated RcNtrC dimers form oligomers from the distal sites to the promoter. Although we did not detect any high-affinity sites close to the *nifA1* or *nifA2* promoter, areas of weak DNase I protection could be detected at high concentrations of RcNtrC (160 nM or greater) from approximately −85 to −50 for *nifA1* and −80 to −50 for *nifA2*. For *nifA1*, weaker protection extended over the *Bst*EII site at −84. It is possible that weak RcNtrC sites lie downstream of the higher-affinity sites shown in Fig. 5 and that the integrity of these sites is essential for activation in vivo. Alternatively, these inserts may disrupt RNAP binding, which would reduce the frequency of collision between activator and RNAP.

Activation function of RcNtrC is likely to require ATP hydrolysis. The ATPase activity of NtrC-like activators is absolutely required for the activation of open complex formation. Mutations in the conserved motif GX(S)GXGK of the *S. typhimurium* NtrC essentially abolish ATPase activity (70). Other mutations of this motif in *K. pneumoniae ntrC* (3) and *nifA* (11) impair or destroy activation function. TyrR is a σ^{70} RNAP activator with homology to NtrC in the central and C-terminal domains (reviewed in reference 51). In contrast to the NtrC-like activators discussed above, a mutation of the second G (to D) in the conserved motif GDTGTGK in TyrR does not affect its function as an activator, implying that ATPase activity is not required for activation (75). Alleles of *R. capsulatus ntrC* which contain mutations in the third glycine of the motif (G-176) were unable to significantly activate *nifA2-lacZ* or complement NtrC⁻ strains. This quality of RcNtrC distinguishes it from TyrR and makes it similar to other NtrC-like activators. A second important distinction from the TyrR system is the distal binding properties of RcNtrC; TyrR binds to at least one site less than 80 bp away from the transcriptional start sites (51).

The NtrC family of activators. Two recent reviews discuss the properties of σ^{54} EBPs particularly with respect to the central and C-terminal domains (46, 49). The activation function, located in the central region of EBPs, contains the conserved ATP-binding motif in addition to more extended regions of homology. Interestingly, the RcNtrC protein contains all significantly conserved amino acid residues of the EBP central region with the exception of one short stretch of amino acids (Fig. 10). It has recently been proposed, in part on the basis of this natural deletion in the RcNtrC protein, that this short region may define the contact point to σ^{54} of the σ^{54} RNAP holoenzyme (46). Because of the recently discovered sequence errors at the *R. capsulatus nifR3-ntrB-ntrC* locus (see reference 19 for a description), we resequenced this region of the *R. capsulatus ntrC* gene and confirmed that this natural deletion is present.

Remarkably, Weiss et al. (70) have characterized a single point mutation in this region which changes the *S. typhimurium* NtrC glycine G-219 to a lysine (Fig. 10); NtrC(G219K) was shown to possess significant ATPase activity (54% of the wild-type level) but was unable to activate transcription of the σ^{54}-dependent promoter, *glnAp2*. We transformed several plasmids containing the *R. capsulatus ntrBC* genes into an NtrBC⁻ strain of *E. coli* but did not get complementation of the *ntrC* defect. Plasmids with the *E. coli ntrBC* genes, in contrast, complemented this strain to NtrC⁺ (i.e., growth on

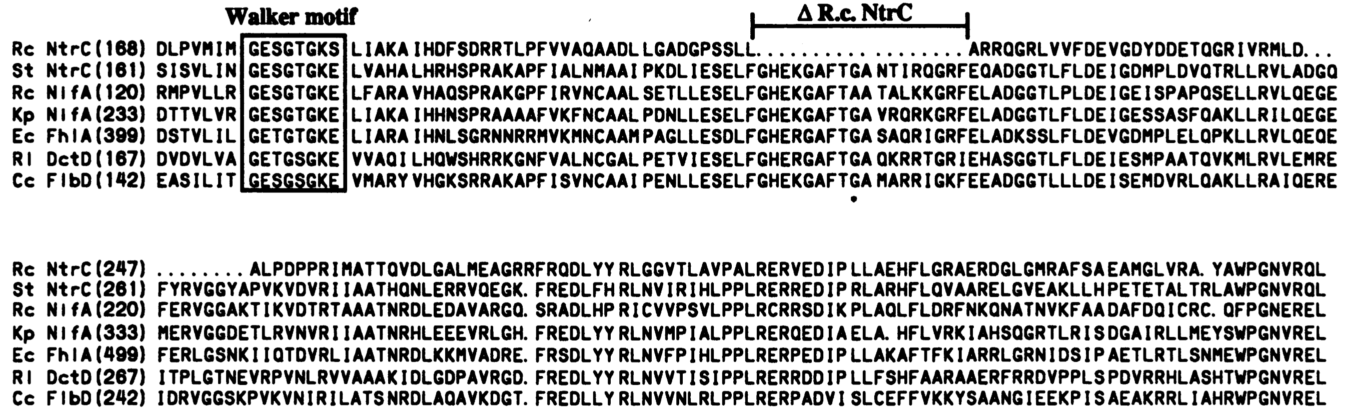


FIG. 10. Comparison of the central domains of RcNtrC protein and several activators of σ^{54} RNAP. The proposed ATP-binding site A (67) is boxed and labeled. The asterisk marks the conserved glycine that was shown to be essential for transcriptional activation in *S. typhimurium* (70). ΔR.c. refers to the natural deletion of this conserved domain in *R. capsulatus*. Amino acid sequences were aligned by the Genetics Computer Group program PILEUP (16); some alignments were performed manually. The first two letters of the designations refer to the organisms, and the last four refer to the protein: RcNtrC, *R. capsulatus* NtrC (31); St NtrC, *S. typhimurium* NtrC (49); Rc NifA, *R. capsulatus* NifA (40); Kp NifA, *K. pneumoniae* NifA (10); Ec FhlA, *E. coli* FhlA (41, 61); Rl DctD, *Rhizobium leguminosarum* DctD (58); and Cc FlbD, *Caulobacter crescentus* FlbD (55).

arginine as the sole nitrogen source). These results are consistent with the idea that the GAFTGA domain (Fig. 10) of the σ^{54} RNAP-dependent EBPs might interact with σ^{54}, as first proposed by Weiss et al. (70).

The *nifA1* and *nifA2* promoters. What RNAP holoenzyme(s) recognizes the *R. capsulatus nifA1* and *nifA2* promoters? The promoter structures of *nifA1* and *nifA2* (and *glnB* [21]) are clearly distinct from the σ^{54} class. The data presented here support the hypothesis that these genes are activated directly by RcNtrC. Could the holoenzyme be the σ^{70} type? Although it was noted previously that little resemblance to the σ^{70} promoters is present at −10 and −35 (20, 54), we should note that the *nifA1* promoter shows some limited homology to the consensus for σ^{70}: TATGGT at −10 (compared with the σ^{70} consensus TATAAT [26]) and GTGCCT at −35 (compared with the σ^{70} TTGACA). However, *nifA2* (and *glnB*) promoters show little resemblance to this consensus. Moreover, *nifA1*- and *nifA2-lacZ* fusions are not expressed in *E. coli* strains that express the RcNtrBC proteins (unpublished data). These results suggest that the σ^{70} RNAP (from *E. coli* at least) is not sufficient for activation in and of itself. Since RNAP holoenzymes other then σ^{54} RNAP are used by the RcNtrC protein, it is clear that the bacterial EBP class of transcriptional activators is more versatile then originally envisioned. Possibly, the evolution of distinct EBPs with distinct RNAP holoenzymes in a single microorganism provides another form of transcriptional control that facilitates remarkable metabolic versatility. Such versatility is characteristic of many photosynthetic bacteria.

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