Urea Utilization in the Phototrophic Bacterium \textit{Rhodobacter capsulatus}
Is Regulated by the Transcriptional Activator NtrC

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The phototrophic nonsulfur purple bacterium \textit{Rhodobacter capsulatus} can use urea as a sole source of nitrogen. Three transposon Tn5-induced mutations (Xan-9, Xan-10, and Xan-19), which led to a Ure- phenotype, were mapped to the \textit{ureF} and \textit{ureC} genes, whereas two other Tn5 insertions (Xan-20 and Xan-22) were located within the \textit{ntrC} and \textit{ntrB} genes, respectively. As in \textit{Klebsiella aerogenes} and other bacteria, the genes encoding urease (\textit{ureABC}) and the genes required for assembly of the nickel metallocenter (\textit{ureD} and \textit{ureEFG}) are clustered in \textit{R. capsulatus} (\textit{ureDABC-orf136-ureEFG}). No homologues of Orf136 were found in the databases, and mutational analysis demonstrated that \textit{orf136} is not essential for urease activity or growth on urea. Analysis of a \textit{ureD4-lacZ} fusion showed that maximum expression of the \textit{ure} genes occurred under nitrogen-limiting conditions (e.g., serine or urea as the sole nitrogen source), but \textit{ure} gene expression was not substrate (urea) inducible. Expression of the \textit{ure} genes was strictly dependent on NtrC, whereas \textit{ure} was not essential for urease activity. Expression of the \textit{ure} genes was lower (by a factor of 3.5) in the presence of ammonium than under nitrogen-limiting conditions, but significant transcription was also observed in the presence of ammonium, approximately 10-fold higher than in an \textit{ntrC} mutant background. Thus, \textit{ure} gene expression in the presence of ammonium also requires NtrC. Footprint analyses demonstrated binding of NtrC to tandem binding sites upstream of the \textit{ureD} promoter. Phosphorylation of NtrC increased DNA binding by at least eightfold. Although urea is effectively used as a nitrogen source in an NtrC-dependent manner, nitrogenase activity was not repressed by urea.

\textit{Rhodobacter capsulatus} is a nonsulfur phototrophic purple bacterium which can grow with a variety of different nitrogen sources, such as ammonium, almost all amino acids, purines (xanthine and hypoxanthine), urea, polyamines (putrescine and spermidine), and molecular nitrogen. Like in many other bacteria, ammonium is a preferred N source, and consequently, the highly energy-demanding nitrogen fixation process is repressed by ammonium.

\textit{R. capsulatus} measures the cellular nitrogen status (e.g., availability of ammonium) by an Ntr system similar to that of enterobacteria involving the two-component regulatory system NtrB-NtrC (10, 15, 18). NtrC is the transcriptional activator of a number of genes directly or indirectly involved in nitrogen fixation in \textit{R. capsulatus} (\textit{glnB-glnA}, \textit{nifA1}, \textit{nifA2}, \textit{anfA}, and \textit{mopA-mopABCD} [3]). These genes code for the signal transduction protein PIII (GlnB) and glutamine synthetase (GlnA), the transcriptional activators of the two nitrogenase genes (\textit{nif}- and \textit{anf}-encoded), a molybdenum repressor of the Anf system, and a high-affinity molybdate uptake system. Despite the similarities of the Ntr systems of \textit{R. capsulatus} and of enterobacteria, mutations in \textit{R. capsulatus ntrC} do not produce the “classical” Ntr phenotype of enterobacteria, since the NtrC protein in \textit{R. capsulatus} is not required for the utilization of amino acids as an N source (11, 13, 25).

Under conditions of nitrogen limitation, the NtrB sensor kinase autophosphorylates and transfers the phosphate to the NtrC response regulator. NtrC→P in turn activates transcription of its target genes. Transcriptional activation of these genes requires binding of NtrC→P to enhancer-binding sites distant from the promoters that are activated. Members of the enhancer-binding protein family characteristically activate transcription from promoters recognized by the alternative sigma factor σ54 (NtrA). The NtrC protein from \textit{R. capsulatus} (RntrC) is a unique enhancer-binding protein that does not require σ54 but instead activates transcription of the genes mentioned above together with RNA polymerase containing the σ70-like housekeeping sigma factor (RNAP-σ70 [3, 8]).

Since an \textit{R. capsulatus ntrC} mutant does not grow with urea as the sole N source (17), genes required for utilization of urea (\textit{ure} genes) were likely targets for NtrC-mediated activation. Degradation of urea is catalyzed by the enzyme urease, which is an inducible enzyme in \textit{R. capsulatus} E1F1 (7). Urease is a nickel-containing enzyme that catalyzes the hydrolysis of urea to form ammonia and carbamate, which spontaneously decomposes to produce carbonic acid and additional ammonia. In \textit{Klebsiella aerogenes} and many other bacteria, the \textit{ureA}, \textit{ureB}, and \textit{ureC} genes encode the catalytically inactive apoenzyme, and the \textit{ureD, ureE, ureF}, and \textit{ureG} gene products are required for assembly of the nickel metallocenter (5, 22).

This work describes genetic analyses of the \textit{R. capsulatus ure} gene region showing that expression of the \textit{ure} genes is activated under nitrogen-limiting conditions in an NtrC-dependent manner. DNA footprinting studies demonstrate direct binding of NtrC to the \textit{ureD} promoter. This is the first example...
Nitrogen activities of whole cells were determined by the acetylene reduction assay as described by Wang et al. (31).

RNA isolation and primer extension analysis. RNA was prepared from R. capsulatus nifHDK deletion strain KS36 harboring plasmid pNIRUB5 (ureDA-lacZ). For this purpose, cells were grown in RCV minimal medium containing 4 mM urea as the sole nitrogen source under photoheterotrophic conditions until the early exponential growth phase. Cells were harvested and RNA was isolated according to the method described by Chomczynski and Sacchi (4). The primer extension procedure was performed with Superscript I reverse transcriptase (Life Technologies), using a 5'-fluorescein-labeled oligonucleotide (5'-TGCCTCCAGATCAAAGCC-3', corresponding to codons 35 to 42 of the ureD gene). Conditions for primer extension were as described by Myöhänen and Wahlfors (26). Analysis of primer extension products next to a sequencing ladder generated with the same oligonucleotide was performed using the A. L. F. DNA sequencer (Amersham Pharmacia Biotech).

DNaSe I footprinting. PCR was used to generate a 286-bp fragment spanning the ureD promoter region with ureD primers (5'-CGGGGTTC GGGCAGATCGAAG-3') and ureD.R (5'-CACAGACCTTCCAAGCGC-3') using pNIRUB42-I as a template. In each reaction, one of the primers was labeled with 5'-fluorescein-labeled oligonucleotide (EPICENTRE Technologies, Madison, Wis.) and [γ-32P]ATP. DNA binding reactions with the phosphorylated ReNtR were carried out by the addition of various concentrations of ReNtR to binding buffer (50 mM Tris-HCl [pH 7.5], 0.1 mM EDTA, 10 mM MgCl2, 50 mM KCl, 10 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) which contained either upper- or lower-strand end-labeled probe (approximately 20,000 cpm), either 0 or 1 mM ATP, and 100 ng of poly(dI-dC) as a nonspecific competitor. Complexes were allowed to form for 10 min at 23°C in a total volume of 50 μl, after which DNaSe I digestion and DNA purification were performed as described previously (8). DNA binding reactions with phosphorylated ReNtR were performed by incubation of maltose-binding protein-ReNtR (MBP-ReNtR, 1 μM) in binding buffer that contained 1 mM ATP as

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. Methods for conjugational plasmid transfer between *Escherichia coli* and *R. capsulatus* and the procedures for selection of mutants, anarobic growth conditions, and antibiotic concentrations have been previously described (14, 19, 23, 24).

**DNA techniques.** DNA isolation, restriction enzyme analysis, and cloning procedures were performed according to standard methods (27). Restriction endonucleases, T4 DNA ligase, and Superscript I reverse transcriptase were performed according to standard methods (27). Restriction enzyme analysis and cloning procedures were performed according to standard methods (27).

**Construction of an ureDA-lacZ fusion plasmid.** A 3.9-kb BamHI-HindIII fragment encompassing the ureD promoter region (Fig. 1A) was inserted into the mobilizable broad-host-range vector pPHU235, resulting in hybrid plasmid pNIRUB35 (Fig. 1C) carrying an in-frame ureDA-lacZ fusion.

**β-Galactosidase and in vivo nitrogenase assays.** To determine the β-galactosidase activities of *R. capsulatus* strains carrying pNIRUB5 (ureDA-lacZ), cultures were grown in RCV minimal medium supplemented with tetracycline (23). For growth under nitrogen-limiting conditions, either serine or urea was added to final concentrations of 9.5 or 4.0 mM, respectively. Nitrogen-sufficient conditions were achieved by addition of 15 mM NH4Cl to the medium. Following growth in the media to late exponential phase, β-galactosidase activities of *R. capsulatus* strains were determined by the sodium dodecyl sulfate-chloroform method (10, 20).

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Reference or source</th>
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<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
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<tr>
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<td>JM83</td>
<td>Host for pUC plasmids</td>
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<td>S17-1</td>
<td>RP4-2 (Te::Mu) (Km::Tn7) integrated in the chromosome</td>
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<td><em>R. capsulatus</em> BI0S</td>
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<tr>
<td>KS36</td>
<td>ΔnifHDK::[Spe]</td>
<td>31</td>
</tr>
<tr>
<td>KS11</td>
<td>ntrC::[Gm]</td>
<td>16</td>
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<tr>
<td>Xan-9, -10, -19, -20, -22</td>
<td>Tn5-induced Ure- mutants</td>
<td>17</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pUC18</td>
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<td>Broad-host-range lacZ fusion vector, Te'</td>
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<tr>
<td>pSUP202</td>
<td>Ap' Cm' Te' mob</td>
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</tr>
<tr>
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<td>Ap' Km' mob</td>
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<td>pSUP401</td>
<td>Cm' Km' mob</td>
<td>29</td>
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<td>pNIRUB4</td>
<td>13.3-kb EcoRI fragment from Xan-10 (ureC::Tn5) in pUC18</td>
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<td>pNIRUB35</td>
<td>ureDA-lacZ fusion in pPHU235, Te'</td>
<td>This work</td>
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<td>1.3-kb EcoRI fragment carrying the ureD promoter in pUC18</td>
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<td>pSUP202 derivative carrying lyr2::[Gm']</td>
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</tr>
<tr>
<td>pBKRU16/17</td>
<td>pUC18 derivative carrying orf323::[Km'], Te' mob</td>
<td>This work</td>
</tr>
</tbody>
</table>

* Abbreviations: Ap', ampicillin resistance; Cm', chloramphenicol resistance; Gm', gentamicin resistance; Km', kanamycin resistance; Spc', spectinomycin resistance; Sm', streptomycin resistance; Te', tetracycline resistance.
RESULTS AND DISCUSSION

Genetic organization of the \textit{R. capsulatus} ure region.

Random transposon Tn5 mutagenesis of an \textit{R. capsulatus} \textit{nifHDK} mutant strain (deleted for the structural genes of the molybdenum nitrogenase) led to the identification of five mutants (Xan-9, Xan-10, Xan-19, Xan-20, and Xan-22) unable to grow with urea as the sole nitrogen source (17). Tn5-containing DNA fragments from these mutants were cloned, and the sites of transposon insertions were determined by nucleotide sequence analyses. Comparison with the \textit{R. capsulatus} genome sequence (http://wit.mcs.anl.gov/WIT2/CGI/org.cgi) showed that the Tn5-induced mutations in Xan-9, Xan-10, and Xan-19 mapped within the \textit{ureF} and \textit{ureC} genes (Fig. 1A). Due to the close proximity of the \textit{ureDABC-orf136-ureEFG} genes, it seems likely that the \textit{ure} genes are cotranscribed in \textit{R. capsulatus} from a promoter upstream of \textit{ureD} (see below). The organization of \textit{ure} genes in \textit{R. capsulatus} is similar to that of \textit{K. aerogenes} and many other bacteria (22), except for the presence of an additional open reading frame (orf136) located between \textit{ureC} and \textit{ureE}. This orf did not show homology to any known \textit{ure} gene or any other gene in the databases, indicating that orf136 might be specific for \textit{Rhodobacter}. The remaining mutants Xan-20 and Xan-22 carried the transposon within the \textit{ntrC} and \textit{ntrB} genes, respectively (data not shown).

Mutational analysis of the \textit{R. capsulatus} urease gene region.

As mentioned above, urease catalyzes the hydrolysis of urea to form ammonia and carbamate, which spontaneously decomposes to produce carbonic acid and additional ammonia. Although this process might produce free ammonium within the cell, urea did not prevent nitrogenase synthesis or activity in \textit{R. capsulatus} at least under the conditions used in this study (Table 2). This is similar to the situation in \textit{Azotobacter vinelandii}, where urea has been used as a nonrepressing nitrogen source (e.g., references 1 and 12). Since urease contains a nickel cofactor, different amounts of NiCl$_2$ (Table 2) were added to the \textit{R. capsulatus} medium to rule out that the nickel concentration was limiting urease activity. In other studies

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Nitrogen source} & \textbf{Nickel added (\textmu M)} & \textbf{Nitrogenase activity} \\
& & (nmol of ethylene produced/ [mg of protein \times h]) \\
\hline
Ammonium & None & 3.6 $\pm$ 1.7 \\
Serine & None & 1,069.4 $\pm$ 19.6 \\
Urea & None & 665.9 $\pm$ 50.2 \\
Urea & 0.4 & 941.9 $\pm$ 82.7 \\
Urea & 6.0 & 995.1 $\pm$ 85.2 \\
Urea & 100.0 & 688.6 $\pm$ 145.7 \\
\hline
\end{tabular}
\caption{In vivo nitrogenase activities of \textit{R. capsulatus} B10S in the presence of urea}
\end{table}

*In vivo nitrogenase activities were analyzed by the acetylene reduction assay as described by Wang et al. (31). The results represent the means and standard deviations of at least three independent measurements.
nicker has been added in the micromolar range (e.g., 200 μM for E. coli carrying the K. aerogenes ure gene cluster [5]).

R. capsulatus exhibited growth rates in media containing urea without nickel supplementation comparable to those in media with ammonium (data not shown), demonstrating that sufficient amounts of nickel were present due to impurities of the other chemicals used for the preparation of the media. However, nickel concentrations of up to 100 μM did not significantly affect nitrogenase activities or increase growth rates of R. capsulatus (Table 2; data not shown). At present it remains speculative whether intracellular ammonium cannot be detected by the Ntr system or if ammonium assimilation (via GlnA) is faster than ammonium production by urease.

To avoid any interference between the activities of urease and nitrogenase, all mutations in the ure gene region (see below) were analyzed in the genetic background of the R. capsulatus strain KS36, which is thought to comprise part of a high-affinity, binding-protein-dependent active-transport system for short-chain amidines and urea (21). To determine the role of these four genes and of orf323 in urea utilization, interposon cassette-generated mutants carrying an in-frame nifHK and a gene essential for urea utilization. The Ure phenotype of orf323 mutant NIRUB46 indicates that orf323 and the ure genes are not cotranscribed, suggesting that the ure promoter is located in the intergenic region between orf323 and ureD (see below).

Since Orf433 is thought to be part of a high-affinity urea transport system, the urea concentrations in the growth medium were lowered. The parental strain KS36 and the orf433 mutant strains did not differ in growth within the range from 4 to 0.25 mM urea (data not shown). All strains were unable to grow with less than 0.25 mM urea. However, this does not necessarily exclude the possibility that there are differences between the parental strain and the orf433 mutant strains in urea uptake in the micromolar range.

**Transcriptional analysis of the R. capsulatus ure operon.** Since expression of the ure operon appears to depend on a promoter located directly upstream of ureD, genomic DNA from R. capsulatus carrying an in-frame ureDA-lacZ fusion was constructed (Fig. 1C; Materials and Methods). This broad-host-range reporter plasmid was introduced into the R. capsulatus wild type and selected mutants, and β-galactosidase activity was determined in cultures grown with different nitrogen sources. The results shown in Table 3 can be summarized as follows. (i) Maximum expression of the ure genes occurred under nitrogen-limiting conditions (serine or urea as the sole nitrogen source). (ii) Transcription of urease genes was not substrate (urea) inducible in R. capsulatus B10S. As mentioned above, urease is an inducible enzyme in R. capsulatus E1F1 (7). However, the two R. capsulatus strains differ in several aspects (e.g., their susceptibilities to several bacteriophages) from each other, including those concerning the general nitrogen metabolism. R. capsulatus E1F1 is able to grow with nitrate as the sole source of nitrogen, whereas R. capsulatus B10S is devoid of any nitrate reductase activity. Therefore, it seems not unlikely that regulatory circuits also differ from each other in these two R. capsulatus strains. (iii) Expression of the ureDA-lacZ fusion was down-regulated (3.5-fold) under nitrogen-sufficient conditions, but significant expression remains in the presence of ammonium. (iv) NtrC is essential for expression of the ure genes under both nitrogen-limiting and nitrogen-sufficient conditions, suggesting that expression of the ure genes in the presence of ammonium somehow requires NtrC. At present it remains a matter of speculation whether ammonium-grown R. capsulatus cells contain low amounts of phosphorylated NtrC sufficient for partial activation of promoters with high affinity to NtrC−P. As shown by footprint analysis (see below), NtrC−P indeed efficiently binds to the ureD promoter. (v) σ54 (NtrA or RpoN) is not essential for urease activity or expres-

<table>
<thead>
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<th>Strain (pNIRUB35)</th>
<th>Relevant genotype</th>
<th>NH₄⁺</th>
<th>Serine</th>
<th>Urea</th>
<th>Urea plus NH₄⁺</th>
<th>Urea plus serine</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10S</td>
<td>Wild type</td>
<td>434.4 ± 47.8</td>
<td>1,556.7 ± 109.1</td>
<td>1,529.1 ± 166.1</td>
<td>448.3 ± 53.3</td>
<td>1,577.9 ± 373.5</td>
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<tr>
<td>TD50 ntrC⁺</td>
<td>49.2 ± 4.7</td>
<td>31.6 ± 4.6</td>
<td>67.7 ± 9.0</td>
<td>62.8 ± 12.3</td>
<td>28.8 ± 6.6</td>
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<tr>
<td>KS111 ntrA</td>
<td>556.5 ± 25.5</td>
<td>3,369.4 ± 138.2</td>
<td>2,638.4 ± 287.9</td>
<td>782.7 ± 124.6</td>
<td>2,412.1 ± 196.3</td>
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<tr>
<td>KS36 ΔnifHDK</td>
<td>256.7 ± 15.9</td>
<td>1,471.0 ± 104.3</td>
<td>1,503.8 ± 59.1</td>
<td>309.5 ± 41.7</td>
<td>1,787.9 ± 111.9</td>
<td></td>
</tr>
</tbody>
</table>

* β-Galactosidase activities (in Miller units) were determined in late-exponential-phase R. capsulatus cultures grown photoheterotrophically with the indicated N sources. Miller units (20) and standard deviations were calculated from three independent assays for each strain.
FIG. 2. Protection of the ureD promoter region by RcNtrC from DNase I digestion. (A) Upper-strand protection by RcNtrC at the indicated concentrations (nM) for each reaction. Numbers refer to the distance from the transcription start site. Arrowheads mark areas of increased sensitivity. Numbers refer to the distance from the transcription start site. Arrowheads mark areas of increased sensitivity.

(B) Lower-strand protection by RcNtrC at the indicated concentrations (nM). The concentration of DNA probe was approximately 0.1 nM for each reaction. Numbers refer to the distance from the transcription start site. Arrowheads mark areas of increased sensitivity.

(C) Upper-strand protection by RcNtrC with 1 mM MBP-RcNtrB and 1 mM ATP. The concentration of DNA probe was approximately 0.1 nM for each reaction. Numbers refer to the distance from the transcription start site. Arrowheads mark areas of increased sensitivity.

(D) Lower-strand protection by RcNtrC with 1 mM MBP-RcNtrB and 1 mM ATP. The concentration of DNA probe was approximately 0.1 nM for each reaction. Numbers refer to the distance from the transcription start site. Arrowheads mark areas of increased sensitivity.
pression of ureD. Expression of ureDA-lacZ was somehow enhanced in an ntrA mutant background, but this was typically less than twofold.

DNase I footprint analysis of the ureD promoter region. Since transcription of ureDA-lacZ was dependent on the ntrC gene, we predicted that RcNtrC might directly contact the ureD promoter upstream of the transcriptional start site. To verify this assumption, DNase I footprinting was performed with unphosphorylated RcNtrC to identify DNA sequences involved in contacting RcNtrC. Labeled DNA fragments containing the ureD upstream region from −227 to +59 were incubated with increasing concentrations of purified RcNtrC and subjected to DNase I digestion. The DNase I protection patterns were localized by comparison with dideoxy DNA sequence ladders generated with the ureD.F primer for the upper strand (Fig. 2A) and the ureD.R primer for the lower strand (Fig. 2B). A DNase I-hypersensitive site could be seen at −148 on the upper strand at an RcNtrC concentration of 10 nM and at −118 and −139 on the lower strand at an RcNtrC concentration of 20 nM. At a higher RcNtrC concentration (160 nM), several minor hypersensitive sites were observed on the upper strand. Full protection of the ureD upstream region was observed between −157 and −115 (upper strand) and between −155 and −110 (lower strand) at concentrations of RcNtrC of 80 nM and higher (Fig. 2A and B). This RcNtrC concentration (80 nM) is similar to that reported for complete protection of the glnB (160 nM), nifA1 (80 nM to 160 nM), and nifA2 (80 nM to 160 nM) promoter regions (6, 8). These regions of DNase I protection by RcNtrC encompass the consensus tandem RcNtrC binding sites described previously (Fig. 2A and B, regions I and II [3]).

To determine whether the phosphorylated RcNtrC protein has enhanced DNA binding activity, as described previously for the glnB promoter (6), a comparison between the DNase I protection patterns of unphosphorylated and phosphorylated RcNtrC was performed. The labeled ureD upstream region was incubated with various concentrations of RcNtrC that was phosphorylated with 1 μM MBP-RcNtrB and 1 mM ATP. Complete protection of both the upper and lower strands of the ureD upstream region by phosphorylated RcNtrC was detected at the 10 nM concentration, as compared to 80 nM for the unphosphorylated RcNtrC (Fig. 2, compare panels A and B [80 nM RcNtrC] with panels C and D [10 nM RcNtrC]). We conclude that at the ureD promoter, phosphorylation of RcNtrC increases DNA binding by at least eightfold. Previously, an increase in binding affinity of at least fourfold at the glnB promoter was observed upon phosphorylation of RcNtrC (6). Increased oligomerization at the enhancer tandem binding sites by phosphorylation of RcNtrC may modulate the response to nitrogen via transcriptional activation of RNAP at this promoter.

NtrC-dependent ure gene expression in K. aerogenes occurs indirectly via the action of the positive regulator NAC (nitrogen assimilation control) (2, 9). Under nitrogen-limiting conditions, K. aerogenes NtrC activates expression of the nac gene in concert with RNAP-σ54, and in turn, NAC activates expression of the ure genes together with RNAP-σ70. Thus NAC acts as a bridge between RNAP-σ70-dependent operons and the RNAP-σ54-dependent Ntr system in K. aerogenes. Since RcNtrC acts directly with RNAP-σ70 (3) and directly on the ure operon,
as shown here, there appears to be no requirement for a NAC-like protein in \textit{R. capsulatus}.

To further confirm that the transcriptional start point of the \textit{ure} operon is located within the intergenic region between \textit{hp2} and \textit{ureD} (Fig. 1A), primer extension analysis was carried out (see Materials and Methods). As shown in Fig. 3, putative “−10” and “−35” regions for a \textit{σ}^70-RNAP were identified at reasonable distances upstream of the transcriptional start point of the \textit{ureD} gene. The “−35” region contains at least four of the optimal nucleotides of a “−35” hexamer identified by mutagenesis of the \textit{R. capsulatus nifA1} promoter, which are also present in the corresponding promoter elements of the \textit{R. capsulatus nifA2} and \textit{glnB} genes (3).

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