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DNA Binding Activity of the *Escherichia coli* Nitric Oxide Sensor NorR Suggests a Conserved Target Sequence in Diverse Proteobacteria

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The *Escherichia coli* nitric oxide sensor NorR was shown to bind to the promoter region of the *norVW* transcription unit, forming at least two distinct complexes detectable by gel retardation. Three binding sites for NorR and two integration host factor binding sites were identified in the *norR-norV* intergenic region. The derived consensus sequence for NorR binding sites was used to search for novel members of the *E. coli* NorR regulon and to show that NorR binding sites are partially conserved in other members of the proteobacteria.

NorR of *Escherichia coli* is a σ^{54}-dependent transcriptional activator that regulates expression of the *norVW* genes encoding flavorubredoxin and its associated redox partner, respectively (4, 10, 15). The flavorubredoxin has a nitric oxide (NO) reductase activity and submicromolar affinity for NO and appears to have a role in NO tolerance under anoxic and microoxic growth conditions (9, 11, 13, 15). The *norVW* genes are induced ~10-fold in *Salmonella enterica* serovar Typhimurium growing in macrophages (compared to in vitro growth), which is consistent with a role for the flavorubredoxin in protecting against macrophage-derived NO (7). In *E. coli*, NorR activates *norVW* transcription in anaerobic cultures in response to NO, nitrate, and nitrite (which may act as sources of endogenous NO) and nitroprusside and in aerobic cultures in response to *S*-nitrosoglutathione, acidified nitrite, and nitroprusside (4, 10, 15, 19). Transcription of the *norVW* genes can also be activated by NO in aerobic cultures of an *hmp* mutant, which lacks the flavohemoglobin that oxidizes NO to nitrate in the presence of oxygen (10). The fact that NorR can regulate gene expression under oxic conditions (which inactivate the NO reductase activity of the flavorubredoxin) raises the possibility that there are additional genes regulated by NorR, the products of which have roles in aerobically growing cultures. The homologous NorR protein of *Ralstonia eutropha* regulates expression of an NO reductase and can also be activated by nitroprusside and, presumably, NO (20).

A recent transcriptomic analysis using microarrays revealed *norV* and *norW* to be the most highly induced genes in aerobic cultures exposed to either *S*-nitrosoglutathione or acidified nitrite (19). Several *E. coli* regulatory proteins besides NorR have their activities modulated by sources of NO and nitrosative stress, including SoxR, OxyR, Fur, and FNR (3, 5, 6, 16). It has been argued that, of all of these proteins, NorR is perhaps the most important physiological NO sensor and is the only known regulatory protein in enteric bacteria that has evolved exclusively to serve this purpose (19). The array analysis also provided some evidence to suggest that there are other targets for NorR regulation in *E. coli* besides *norVW* (19).

Knowledge of the DNA binding specificity of a regulator is an important complement to array data in the identification of regulon members (23). As part of an effort to understand the mechanism of regulation of the *norVW* promoter and to help identify additional NorR targets, we have set out to determine the DNA sequence(s) recognized by NorR in the *norR-norVW* intergenic region. Taking advantage of the fact that the σ^{54}-dependent transcriptional activators bind to DNA even when they are in their inactive configuration, we have defined three NorR binding sites by using purified protein in DNase I footprinting and methylation protection assays. The characterization of NorR binding sites will facilitate the identification of any novel members of the NorR regulon and leads to the conclusion that NorR DNA recognition sites are partially conserved in several proteobacteria.

Purified NorR binds to the *norR-norVW* intergenic region. The start codon of the *E. coli norR* gene is probably incorrectly annotated in the GenBank database (accession no. NC 000913), because the predicted protein is ~25 residues longer than its homologues at the N terminus. Accordingly, we and others (10) assume that the true start codon is located at coordinate 2830328 (accession no. NC 000913), as was predicted when the gene was first sequenced (22). To confirm that a protein initiating at this position is functional, an NdeI site incorporating the start codon was introduced into the *norR* gene, along with a VspI site adjacent to the stop codon. The NdeI-VspI fragment was cloned into pET21a (Novagen); the recombinant plasmid activated *norV-lacZ* expression in a *norR* mutant (14) when nitroprusside, nitrite, or nitrate was added to anaerobic cultures (data not shown), confirming that the expressed protein can fully complement the *norR* mutation. Complementation presumably requires the low level of expression that can occur from pET clones in a strain lacking the gene for T7 RNA polymerase (17).

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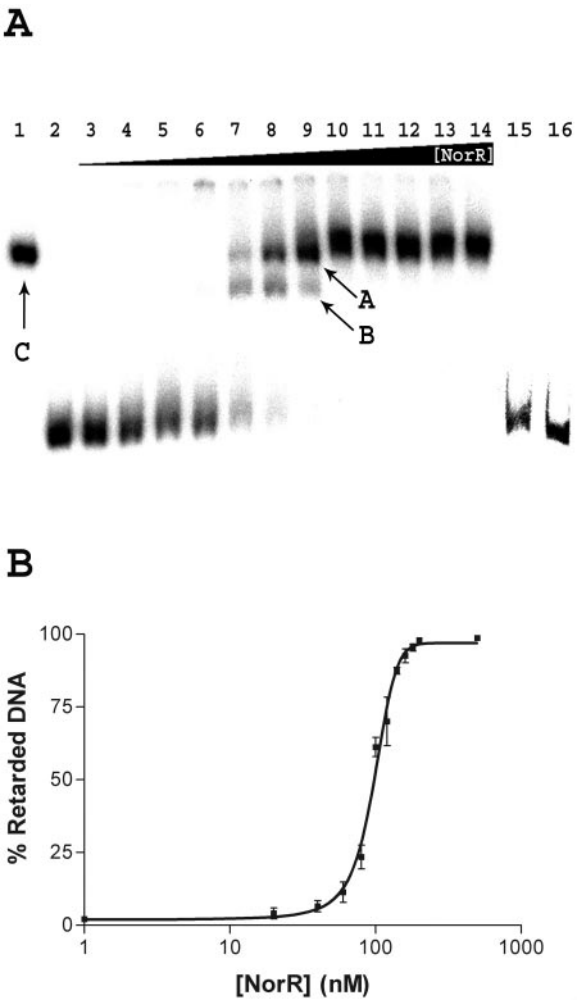

FIG. 1. Binding of NorR to the *norR-norV* intergenic region. (A) Gel mobility shift assays contained a ^{32}P-labeled 362-bp EcoRI-BamHI fragment spanning the *norR-norV* intergenic region. NorR-retarded species are labeled A and B. Concentrations (nanomolar) of NorR were 0 (lane 2), 1 (lane 3), 20 (lane 4), 40 (lane 5), 60 (lane 6), 80 (lane 7), 100 (lane 8), 120 (lane 9), 140 (lane 10), 160 (lane 11), 180 (lane 12), 200 (lane 13), and 500 (lane 14). Lane 1 contained 1 μM IHF; the IHF-bound species is labeled C. A 360-bp fragment containing the *nifH* promoter (lane 15) was not bound by 500 nM NorR (lane 16). (B) Three independent gel mobility shift experiments (including the example shown in panel A) were quantified with a Fujix BAS 1000 phosphorimager. The total amount of retarded DNA is plotted as a percentage of the radioactivity present in each lane.

NorR was overexpressed in strain BL21(DE3) and was purified by heparin agarose chromatography and gel filtration, using procedures similar to those described previously for other σ^{54}-dependent activators, such as NtrC and NifA (1, 8). Gel retardation experiments in 10 mM Tris-HCl (pH 8.0)–5 mM $MgCl_2$–30 mM KCl–0.4 mg of BSA/ml demonstrated that NorR binds to a 362-bp DNA fragment spanning the *norR-norVW* intergenic region (Fig. 1A). At relatively low concentrations of NorR, two distinct retarded species were evident (Fig. 1A, arrows A and B), suggesting the presence of multiple NorR binding sites. At NorR concentrations above 140 nM, only one retarded species (band A) was observed, presumably as a result of the complete occupation of NorR binding sites. No retardation of a labeled *nifH* promoter fragment was observed when incubated with 500 nM NorR, confirming speci-

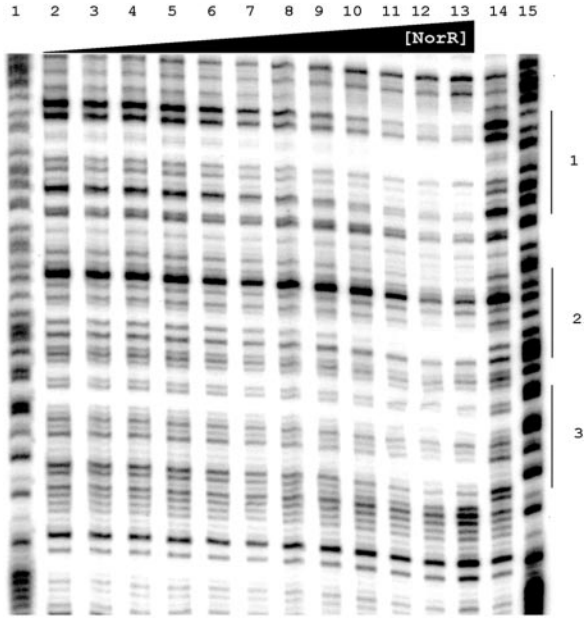

FIG. 2. DNase I footprinting of NorR with the template strand of the *norVW* promoter. The DNA fragment was the 362-bp EcoRI-BamHI fragment, 5′ end-labeled at the EcoRI site. G+A sequencing tracks prepared with the Maxam and Gilbert method are in lanes 1 and 15. Lane 14 contained no NorR. Binding reactions shown in lanes 3 to 14 contained increasing concentrations of NorR identical to those shown in Fig. 1. Regions of NorR protection were deduced from three independent experiments and are denoted by the solid lines to the right of the footprint, labeled 1 for the high-affinity site and 2 and 3 for the low-affinity sites.

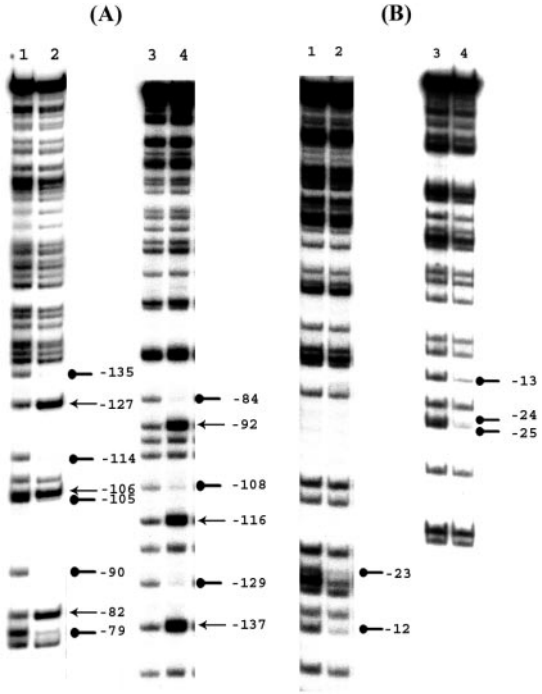

FIG. 3. Methylation protection of the *norR-norV* intergenic region by NorR (A) and σ^{54} RNA polymerase (B). Binding reaction mixtures contained the 362-bp EcoRI-BamHI promoter fragment 5′ end-labeled at either the EcoRI end (lanes 1 and 2) or the BamHI end (lanes 3 and 4) and were treated with dimethyl sulfate. In each case, lanes 1 and 3 contained DNA only and lanes 2 and 4 contained either 500 nM NorR (A) or 1 μM σ^{54} plus 1.5 μM RNA polymerase (B). Residues are numbered with respect to the *norV* transcription start site, designated +1 (see Fig. 4 and 5). Protected G residues are marked with lollipops, and enhanced methylation at G residues is denoted by arrows.

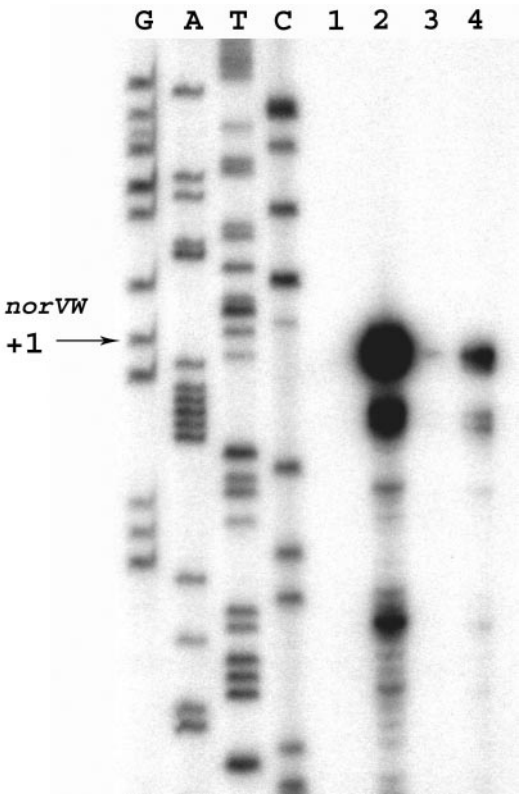

FIG. 4. Localization of the *norVW* mRNA start site by primer extension. Anaerobic cultures of *E. coli* DH10B grown in LB medium supplemented with 0.1% glucose were grown to exponential phase (optical density at 650 nm, 0.45) and were treated with 4 mM $NaNO_2$ (lane 2), 40 mM $NaNO_3$ (lane 3), or 100 μM sodium nitroprusside (lane 4). After 20 min, total RNA was extracted and 20 μg was used for each reaction. Lane 1 contains the untreated control. Dideoxy sequencing reactions (lanes G, A, T, and C) were prepared with the same primer. The arrow marks the major *norVW* transcriptional start site.

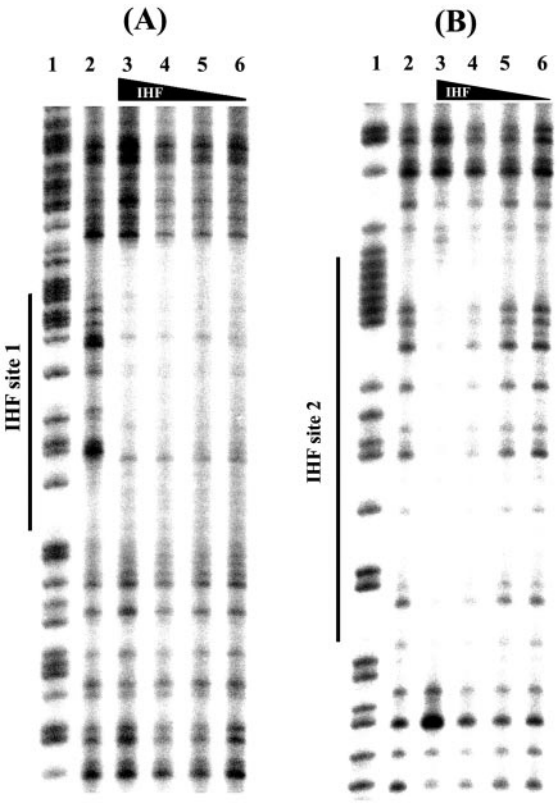

FIG. 5. DNase I footprinting of IHF with the coding strand of the *norVW* promoter. The DNA fragment was the 362-bp EcoRI-BamHI fragment, 5′ end-labeled at the BamHI site. IHF site 1 (A) and IHF site 2 (B) are denoted by solid lines. G+A sequencing tracks prepared with the Maxam and Gilbert method are in lane 1 in each case. IHF concentrations in binding reaction mixtures (micromolar) were 0 (lanes 2), 1 (lanes 3), 0.1 (lanes 4), 0.03 (lanes 5), and 0.02 (lanes 6).

ficity for the *norVW* promoter (Fig. 1A, lanes 15 and 16). Interestingly, retardation of the *norR-norVW* fragment was also observed with integration host factor (IHF) (Fig. 1A, arrow C), which could provide a mechanism for facilitating the interaction between NorR and the σ^{54} RNA polymerase (14, 24). When quantified (Fig. 1B), the NorR binding data give rise to a sigmoidal curve characteristic of cooperative binding, as observed with NtrC (21, 26). This suggests that NorR has a higher affinity for one site, the occupation of which facilitates further binding of NorR to the adjacent site(s).

Footprinting NorR binding sites. DNase I footprinting was performed under the same conditions as the gel retardation assays to identify the location of the NorR binding sites (Fig. 2). Three zones of protection were observed, denoted by bars labeled 1, 2, and 3 in Fig. 2. Similar protection was observed with the opposite strand of the DNA (data not shown). Protection by NorR in region 1 appears to be maintained at lower NorR concentrations (~80 nM) than that observed for regions 2 and 3 (>~140 nM), suggesting a mechanism for the cooperative binding of NorR to the *norVW* promoter that was indicated by the gel retardation experiments. Interestingly, the high-affinity site is ideally positioned to act as an upstream activator sequence for *norVW*, while sites 2 and 3 are located closer to the *norR* coding region. Although the significance of the multiple sites has not been established, it is possible that the high-affinity site is primarily required for NorR-dependent *norVW* regulation, while one or both of the lower affinity sites are involved in *norR* autoregulation (15).

To localize NorR binding sites more precisely and to establish which guanosine residues make close contacts with bound protein, methylation protection patterns were determined on both strands of the DNA fragment, using dimethyl sulfate as a footprinting reagent (Fig. 3A). Clear protection of guanosine residues by NorR was observed at all three of the sites identified by DNase I footprinting (compare Fig. 2 and 3A), thus confirming the locations of the NorR binding sites. There are very similar patterns of methylation protection and enhancement at each site, indicating that NorR interacts with each sequence in a similar manner. Confirmation of the predicted σ^{54} recognition site (25) was obtained from methylation protection experiments with the σ^{54}-RNA polymerase holoenzyme (Fig. 3B). The pattern of protection was very similar to that demonstrated at other σ^{54}-dependent promoters, with protection of G residues corresponding to the consensus GG positions at −25, −24, and −13 on the coding strand and residues corresponding to −23 and −12 on the noncoding strand (2, 18).

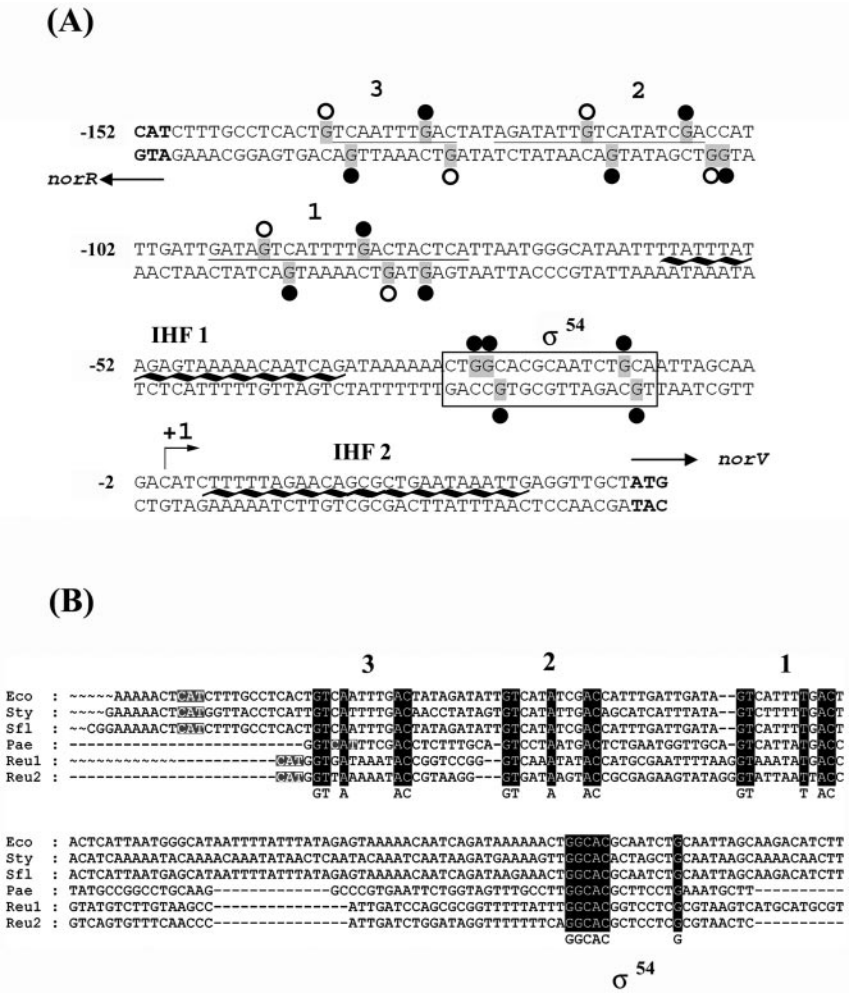

FIG. 6. Protein binding sites in the *norR-norV* intergenic region. (A) The approximate extent of protection by NorR in DNase I footprinting experiments is indicated by underlining. Nucleotides contacted by NorR or σ^{54} RNA polymerase are shaded, those protected from methylation by NorR are marked by filled circles; enhancement of methylation is indicated by open circles. The predicted σ^{54} promoter is boxed. The high-affinity NorR binding site is labeled 1, the low-affinity sites are labeled 2 and 3. The major *norV* mRNA initiates at a cytidine residue indicated by an arrow. Start codons for *norR* and *norV* are shown in bold. IHF binding sites 1 and 2 are labeled and are indicated by wavy lines. (B) Conservation of NorR binding sites among selected bacterial species (Sty, *S. enterica* serovar Typhimurium; Sfl, *S. flexneri*; Pae, *P. aeruginosa*; Reu1, *R. eutropha* megaplasmid genes; Reu2, *R. eutropha* chromosomal genes). The three *E. coli* NorR binding sites are labeled 1, 2, and 3 as in panel A, and *norR* start codons are shaded. Conserved sequence elements in predicted NorR binding sites and σ^{54} promoters are highlighted. The promoter is located upstream of genes for flavorubredoxin (*E. coli*, *S. enterica* serovar Typhimurium, and *S. flexneri*), flavohemoglobin (*P. aeruginosa*), and NO reductase (*R. eutropha*).

To localize the *norVW* transcription start site, RNA was extracted from anaerobically grown cells and was analyzed by primer extension. As observed previously (4, 19), transcripts were not detectable in untreated cultures (Fig. 4, lane 1), but strong activation of the promoter was evident in cells treated with nitrite and nitroprusside (Fig. 4, lanes 2 and 4). Nitrate did not significantly induce transcription under these conditions, possibly because the treatment time (20 min) was too short for the generation of sufficient levels of nitrite and/or NO. As would be predicted, the major *norV* mRNA initiates at a cytidine residue 24 bp downstream of the GG motif of the σ^{54} promoter.

Footprinting IHF binding sites. DNase I footprinting identified two distinct IHF binding sites in the *norVW* promoter region (Fig. 5). Site 1 is fully occupied at IHF concentrations lower than those for site 2, and it is located between the σ^{54} promoter and the promoter-proximal NorR binding site (Fig. 6). The location of this binding site is consistent with a role for IHF in bending DNA to bring NorR into contact with RNA polymerase. The lower affinity IHF site 2 is within the *norV*-transcribed region (Fig. 6). IHF binding sites have been observed in the transcribed regions of other promoters, such as in the *csgD* gene (12).

NorR binding sites are present in several proteobacteria. Alignment of the three *E. coli* NorR binding sites revealed that sites 1 and 3 both contain the inverted repeat GTCA-(N3)-TGAC, while site 2 is GTCA-(N3)-CGAC (Fig. 6). A weight matrix constructed from both strands of the three NorR binding sites was used with PROMSCAN (http://www.promscan.uklinux.net) to search for additional putative targets in the *E.*

coli genome. No other high-scoring multiple NorR sites were found upstream of known or potential σ^{54}-dependent promoters. We cannot exclude the possibility that NorR sites more divergent to those in the *norV* promoter (see below) are functional or that potential NorR sites mediate repression (and are therefore not associated with σ^{54}-dependent promoters). The sequences of the three NorR binding sites that we have identified will facilitate future efforts to identify additional members of the NorR regulon by other techniques. Microarray analysis implicated additional targets for NorR regulation, either direct or indirect, such as *ybiJ* (19). The long noncoding region upstream of *ybiJ* contains neither a predicted σ^{54} promoter nor a strongly predicted site for NorR binding, suggesting that *ybiJ* is not directly activated by NorR.

Alignments of the *norVW* promoters from *E. coli*, *S. enterica* serovar Typhimurium, and *Shigella flexneri* and the chromosomal and megaplasmid copies of the *norAB* promoter from *R. eutropha* (20) indicate that each contains three conserved NorR binding sites with the minimal consensus GT-(N7)-AC (Fig. 5B). The analysis also suggests that NorR activates σ^{54}-dependent promoters upstream of the *hmp* (*fhp*) gene (encoding a putative flavohemoglobin) in *Pseudomonas aeruginosa* (Fig. 5), *Pseudomonas putida*, and *Vibrio cholerae*. Conserved sites are also located upstream of a *norV*-like gene on chromosome II of *Vibrio vulnificus* (data not shown). Thus, there is, apparently, conservation of mechanisms among the proteobacteria for controlling genes encoding diverse enzymes that use NO as a substrate.

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