JOURNAL OF BACTERIOLOGY, Dec. 2004, p. 7980–7987 Vol. 186, No. 23
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.23.7980–7987.2004


# A DNA Region Recognized by the Nitric Oxide-Responsive Transcriptional Activator NorR Is Conserved in β- and γ-Proteobacteria

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Received 3 August 2004/Accepted 27 August 2004

**The $\sigma^{54}$-dependent regulator NorR activates transcription of target genes in response to nitric oxide (NO) or NO-generating agents. In *Ralstonia eutropha* H16, NorR activates transcription of the dicistronic *norAB* operon that encodes NorA, a protein of unknown function, and NorB, a nitric oxide reductase. A constitutively activating NorR derivative (NorR′), in which the N-terminal signaling domain was replaced by MalE, specifically bound to the *norAB* upstream region as revealed by gel retardation analysis. Within a 73-bp DNA segment protected by MalE-NorR′ in a DNase I footprint assay, three conserved inverted repeats, GGT-($N_7$)-ACC (where N is any base), that we consider to be NorR-binding boxes were identified. Mutations altering the spacing or the base sequence of these repeats resulted in an 80 to 90% decrease of transcriptional activation by wild-type NorR. Genome database analyses demonstrate that the GT-($N_7$)-AC core of the inverted repeat is found in several proteobacteria upstream of gene loci encoding proteins of nitric oxide metabolism, including nitric oxide reductase (NorB), flavorubredoxin (NorV), NO dioxygenase (Hmp), and hybrid cluster protein (Hcp).**

Bacteria have to cope with the harmful effects of free radicals that are produced as metabolic by-products or encountered in their environment. The key species superoxide, hydroxyl radical, and nitric oxide can damage DNA, lipids, and proteins. Consequently, bacteria have evolved sophisticated molecular mechanisms to sense free radicals and to activate protective systems. In *Escherichia coli*, OxyR and the SoxRS system regulate a number of oxidative stress proteins in response to hydrogen peroxide and superoxide, respectively (13). Both regulatory systems are also activated by nitric oxide (NO) or derived reactive nitrogen species (RNS) (16, 22). However, specialized NO detoxification systems that appear to be controlled independently of OxyR and SoxRS exist. Several bacteria and fungi contain flavohemoglobins termed Hmp or Fhp that convert NO to nitrate by acting as NO dioxygenases (19, 38). The flavorubredoxin NorV from *E. coli* catalyzes NO reduction under anaerobic and microaerobic conditions (17, 20). Denitrifying bacteria possess nitric oxide reductases (Nor) that convert nitric oxide produced by nitrite reductase (Nir) to nitrous oxide. The ammonia oxidizer *Nitrosomonas europaea* also contains a Nor (5) and induces biofilm formation upon exposure to NO (44). A subfamily of Nor enzymes that oxidize quinols (qNor) is also found in pathogenic bacteria like *Neisseria* species (1, 24), the phototrophic nondenitrifier *Synechocystis* sp. strain PCC6803 (7), and the archaeon *Pyrobaculum aerophilum* (15).

Apparently, RNS-sensing regulatory systems that control expression of NO-detoxifying enzymes in bacteria are diverse. Transcription of the NO dioxygenase gene *hmp* is derepressed upon inactivation of the oxygen sensor Fnr by RNS in *E. coli* (9), whereas transcription of *hmp* is activated by the RNS-responsive ResDE signal transduction system in *Bacillus subtilis* (35). In *Salmonella enterica* serovar Typhimurium, *hmp* appears to be a member of the iron-controlled Fur regulon (12). In the majority of denitrifying bacteria, Nir and Nor are coordinately controlled by Fnr-like transcriptional activators termed NNR (55), NnrR (54), DNR (2), and DnrD (57). In contrast to Fnr, these proteins lack iron-sulfur centers and are activated by RNS (2, 29, 56, 58). It is very likely that further RNS-responsive systems exist since, for example, expression of the qNor of *Neisseria gonorrhoeae* seems to be independent of known RNS-responsive regulators (24).

Recently, a novel transcriptional regulator (NorR) in the denitrifying β-proteobacterium *Ralstonia eutropha* H16 was discovered (37). NorR shows the typical modular structure of $\sigma^{54}$-dependent activators (33, 36, 52) and activates its target genes in response to nitrite and the nitrosating agent sodium nitroprusside. The N-terminal signaling domain carries a GAF module (3) that might be involved in direct interaction with the effector molecule. A NorR ortholog is also present in *E. coli* and controls the expression of the flavorubredoxin NorV and its cognate reductase, NorW (18, 26). In *R. eutropha* H16, NorR controls the *norAB* operon that is located adjacent to *norR* and transcribed divergently. *norB* encodes a single-subunit nitric oxide reductase of the qNor type (11). A physiological function has not yet been ascribed to the *norA* gene product. The *norRAB* genes are part of a 76-kb large denitrification cluster located on the indigenous megaplasmid pHG1 (47). A second gene region, termed *norR2A2B2*, was discovered on chromosome II of *R. eutropha* (37, 46). Either of the two gene regions is sufficient for denitrification (10, 37).

The NO-responsive activation of transcription by NorR raises two questions: how does the protein manage to sense nitric oxide, and what are the recognition sequences of NorR on the target DNA? In this study, the latter question was addressed by using a NorR derivative lacking its signaling domain. Mobility shift assays, footprint analyses, and in vivo ac-

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TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| Strains | | |
| *R. eutropha* | | |
| H16 | Wild type | DSM428, ATCC 17699 |
| HF562 | *norR′* Δ*norR2A2B2* | 37 |
| *E. coli* | | |
| S17-1 | $Tra^+$ *recA pro thi hsdR chr*::RP4-2 | 50 |
| XL1-Blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*$^q$ *lacZ*ΔM15Tn*10*] | Stratagene |
| DH5α | ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*lacZYA-argF*)*U169* | 21 |
| BL21(DE3) | *E. coli* B $F^-$ *dcm onipT hsdS* ($r_B^-$ $m_B^-$) *gal*λ (DE3) | 53 |
| Plasmids | | |
| pBluescript SK(+) | $Ap^r$ *lacZ′* ColE1 *ori*, T7F10 promoter, f1 *ori* | Stratagene |
| pEDY305 | $Tc^r$ RK2 *ori* $Mob^+$, promoterless *lacZ* gene | 46 |
| pLITMUS28 | $Ap^r$ *lacZ′* ColE1 *ori* M13 *ori* | New England Biolabs |
| pMAL-c2X | $Ap^r$ *lacI*$^q$ *malE lacZ*α ColE1 *ori* | New England Biolabs |
| pET22b+ | $Ap^r$ *lacI* f1 *ori* $P_{T7}$ | Novagen |
| pCH510 | 7.0-kb XhoI fragment in pBluescript SK(+) | 10 |
| pCH689 | 1.1-kb SalI-BamHI fragment in Litmus 28 | 37 |
| pCH694 | 3.3-kb EcoRI-BamHI in Litmus 28 | 37 |
| pCH724 | 0.4-kb AgeI fragment containing a 438-bp in-frame deletion in *norR* | 37 |
| pCH791 | 1.5-kb SpeI-PstI fragment of pCH694 in pCH724 | 37 |
| pCH916 | 1.6-kb NdeI-HindIII fragment containing *norR′* in pET22b+ | This study |
| pCH994 | 1.2-kb fragment from pCH916 in pMAL-c2X (*malE-norR′*) | This study |
| pCH996 | 312-bp PstI-AscI fragment of pCH510 in pBluescript SK(+) | This study |
| pCH997 | 4.3-kb inverse-PCR fragment from pCH689 | This study |
| pCH998 | 430-bp PstI-blunt PCR fragment | This study |
| pCH999 | 430-bp PstI-blunt PCR fragment | This study |
| pCH1000 | 430-bp PstI-blunt PCR fragment | This study |
| pCH1001 | 429-bp PstI-blunt PCR fragment | This study |
| pCH1002 | 430-bp PstI-blunt PCR fragment | This study |
| pCH1017 | 322-bp BstEII-AscI fragment from pCH997 in pCH694 | This study |
| pCH1054 | 430-bp PstI-blunt PCR fragment | This study |
| pGE428 | 463-bp AscI-BamHI fragment of pCH689 in pEDY305 | 37 |
| pGE518 | 307-bp PstI-XbaI PCR fragment in pEDY305 | This study |
| pGE520 | 275-bp PstI-XbaI PCR fragment in pEDY305 | This study |
| pGE522 | 250-bp PstI-XbaI PCR fragment in pEDY305 | This study |
| pGE523 | 304-bp PstI-AscI fragment from pCH996 in pEDY305 | This study |
| pGE524 | 345-bp PstI-AscI fragment from pCH997 in pEDY305 | This study |
| pGE525 | 371-bp PstI-AscI fragment from pCH689 in pEDY305 | This study |
| pGE526 | 371-bp PstI-AscI fragment from pCH998 in pEDY305 | This study |
| pGE527 | 371-bp PstI-AscI fragment from pCH999 in pEDY305 | This study |
| pGE528 | 371-bp PstI-AscI fragment from pCH1000 in pEDY305 | This study |
| pGE529 | 370-bp PstI-AscI fragment from pCH1001 in pEDY305 | This study |
| pGE530 | 371-bp PstI-AscI fragment from pCH1002 in pEDY305 | This study |
| pGE548 | 371-bp PstI-AscI fragment from pCH1054 in pEDY305 | This study |

tivities of transcriptional fusions revealed the presence of DNA motifs that are essential for transcriptional activation by NorR.

## MATERIALS AND METHODS

**Strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. *R. eutropha* H16 is the wild-type strain harboring the endogenous megaplasmid pHG1. HF strains are derivatives of strain H16. *E. coli* S17-1 served as the donor in conjugative transfers. *E. coli* XL1-Blue or DH5α was used as a host in standard cloning procedures.

Plasmid pCH510 contains a 7-kb fragment of pHG1 that includes *norB*, *norA*, and most of the *norR* gene (10). Deletion fragments of the *norAB* promoter were generated by PCR with plasmid pCH510 as a template. Vent DNA polymerase (New England Biolabs) was used for DNA amplification. Fragments were amplified by using the synthetic oligonucleotide 5′-CAGCAGTCTAGACTCGAT GGCTGTG-3′ with an insertion of a new XbaI site (underlined bases) and the synthetic oligonucleotides 5′-TGGGCTGCAGGGTGATAAATACCGG-3′ (yielding pGE518), 5′-CAAACTGCAGATGCGAATTTTAAGG-3′ (yielding pGE520), and 5′-AAATCTGCAGGTATGTCTTGTAAGC-3′ (yielding pGE522) with an insertion of a new PstI site (underlined bases). The PCR fragments were cut with PstI and XbaI, and the resulting fragments were ligated into the PstI-XbaI-cut promoter test vector pEDY305 (45). A fragment was amplified from pCH510 by using the synthetic oligonucleotides 5′-GCAGGCGCGCCTCGAT GGCTGTGG-3′ and 5′-CGTCCTGCAGATAAATACCGGTCC-3′ to yield a new a PstI site (pCH996). The PCR fragment was cut with PstI and ligated into the PstI-EcoRV-cut vector pBluescript SK(+). Subsequently, a 303-bp PstI-AscI fragment was ligated into the PstI-AscI-cut promoter test vector pEDY305, yielding pGE523. Plasmid pCH689 contains a 1.1-kb SalI-BamHI fragment that includes 161 bp of *norR* and *norA* and 400 bp of *norB* (37). A deletion of distal inverted repeat 3 was introduced by inverse PCR with pCH689 as a template using the synthetic oligonucleotides 5′-GTATGTCTTGTAAGCCATTG-3′ and 5′-GGTATATTTGACCCGGACCG-3′. The 4.3-kb fragment was phosphorylated and religated, yielding pCH997. A 345-bp PstI-AscI fragment of pCH997 was cloned into pEDY305, yielding pGE524.

Mutations within the inverted repeats were generated by site-directed mutagenesis according to the method of Chen and Przybyla (8). Plasmid pCH510 served as a template and Vent DNA polymerase (New England Biolabs) was used for DNA amplification. Fragments were amplified by using the synthetic oligonucleotide 5′-TACAGTGTTCCAGTCGTTCTCCAC-3′ as the nonmutagenic primer and 5′-CCGGTCCGGGgCAAATATACCATG-3′ (yielding pCH998), 5′-CAAATATcCCATGCGAATTTTAAG-3′ (yielding pCH999), 5′-ttTAAATATGtttGTATGTCTTGTAAGCCATTG-3′ (yielding pCH1000), 5′-caaatatACCGTATGTCTTGTAAGCCATTG-3′ (yielding pCH1001), 5′-AC CATGCGAATTaTAAGGTAAATATGACCG-3′ (yielding pCH1002), and 5′-

ATCGTGATAAATcCCGGTCCGGGTC-3′ (yielding pCH1054) as the mutagenic primers (mutations are indicated in lowercase type). The PCR products and the synthetic oligonucleotide 5′-GCCGGAGAACGCGCCGCGGGCGT G-3′ were used as primers in a second amplification step with pCH791 (*norR′*) as a template. PCR fragments were cut with PstI. The PstI-blunt fragments were cloned into PstI-EcoRV-digested pBluescript SK(+). PstI-AscI fragments of these plasmids were cloned into the PstI-AscI-cut vector pEDY305. All mutations and PCR-amplified fragments were verified by DNA sequencing.

A fusion protein of NorR′ and the maltose-binding protein MalE was constructed by PCR. A 1.2-kb fragment was amplified with *Pfx* polymerase (Invitrogen) and the synthetic oligonucleotides 5′-AACAAGGATCCATACATATGC ACC-3′ and 5′-ATGACAGCAGGATCCGACCCGACG-3′ (underlined bases indicate newly generated restriction sites for BamHI) using plasmid pCH916 (containing *norR′*) as a template and cloned into the BamHI site of the vector pMal-c2X (New England Biolabs), yielding pCH994.

**Media and growth conditions.** *E. coli* strains were grown in Luria-Bertani broth at 37°C. *R. eutropha* strains were cultivated at 30°C in mineral salts medium (43) with 0.4% (wt/vol) fructose as the carbon source (FN medium). Under microaerobic conditions, cells were cultured in 50-ml Erlenmeyer flasks filled with 50 ml of FN medium. Solid media contained 1.5% (wt/vol) agar. The following antibiotics were added: for *R. eutropha*, tetracycline (15 μg $ml^{-1}$); for *E. coli*, tetracycline (10 μg $ml^{-1}$) and ampicillin (50 μg $ml^{-1}$).

**DNA techniques.** Isolation of plasmids, transformation, and cloning were carried out by using standard methods (42). Silica-based matrix (QIAGEN) was used for the preparation of plasmid DNA for sequencing and isolation of DNA fragments from gels. Mobilizable plasmids were transferred from *E. coli* S17-1 (50) to *R. eutropha* by a spot-mating technique. Transconjugants were selected on FN medium plates containing the appropriate antibiotics. Nucleotide sequences were determined by the dideoxynucleotide chain termination method with a Thermosequenase cycle sequencing kit (Amersham Pharmacia Biotech) and fluorescent primers (Metabion). Electrophoresis and band detection were carried out with a LICOR sequencer (MWG Biotech).

**Analytical procedures.** β-Galactosidase was assayed as described previously (60). The activity was calculated according to the method of Miller (32), except that the optical cell density (OD) was measured at 436 nm ($OD_{436}$). Cultures were inoculated to an $OD_{436}$ of 0.4 and incubated at 30°C at 110 rpm and grown under microaerobic conditions for 4 h to reduce the oxygen tension before 2 mM sodium nitroprusside (SNP) (sodium nitrosylpentacyanoferrate [III] dihydrate) was added.

**Protein purification.** The MalE-NorR′ fusion protein was overexpressed in *E. coli* BL21(DE3) with plasmid pCH994. The strain was cultivated in Luria-Bertani medium with 0.2% glucose to an $OD_{600}$ of 0.6 at 30°C. Next, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added, and cells were grown for 3 h. Cells were harvested by low-speed centrifugation. The cell pellet was resuspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 5 mM $MgSO_4$), and cell disruption was carried out by sonification. The extract was centrifuged at 15,000 × *g* for 30 min at 4°C. The crude extract was purified by affinity chromatography using an amylose resin column (New England Biolabs) according to the manufacturer's instructions. Protein was concentrated by ultrafiltration (Centriprep-30; Amicon), and protein concentration was determined according to the protocol of Lowry et al. (30).

**EMSAs.** For electrophoretic mobility shift assays (EMSAs), purified MalE-NorR′ fusion protein and PCR-generated fragments containing the *norAB* promoter were used. The assay was carried out as described previously (4). For binding studies, nonlabeled DNA was used. The standard assay contained TEKMD buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol), 13% glycerol, either two or three competitive DNA fragments, and 0.04 to 2.57 μg of MalE-NorR′. The binding assay mixtures were incubated at 37°C for 30 min and separated on 6% nondenaturing polyacrylamide gels in Tris-glycine buffer (25 mM Tris [pH 8.3], 192 mM glycine) for 30 min at 34 mA. The DNA was stained with ethidium bromide.

**DNase I footprint assay.** DNase I footprint assays were performed according to the method of Ausubel et al. (4). DNA fragments were amplified by PCR with the synthetic oligonucleotides 5′-GCCGGAGAACGCGCCGCGGGCGTG-3′ and 5′-GCCGCCGCAGCAGAAATCCAGCG-3′ using *Pfx* polymerase (Invitrogen). pCH510 was used as a template for a 1,046-bp fragment containing the *norAB* promoter, and pCH1017 was the template for a 1,020-bp fragment harboring the *norAB* promoter with a deletion of the third inverted repeat. The fragments were cut with BglI and end labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-$^{32}$P]dATP. T4 polynucleotide kinase was heat inactivated at 90°C for 3 min. To obtain single, end-labeled DNA, fragments were digested with NcoI, leading to fragments f620 and f594. Unincorporated radioisotopes were removed with a QIAquick nucleotide removal kit (QIAGEN). An assay buffer, TEKMD buffer, was used with 0.1 mM $CaCl_2$ added. Binding reactions contained 200 μl of assay buffer with 1 nM end-labeled DNA, 2 μg of salmon sperm DNA/ml, and MalE-NorR′ fusion protein as indicated in the figure legends. The binding assays were incubated at 37°C for 30 min. Five microliters of DNase I (0.01 mg/ml; Roche Diagnostics GmbH) was then added and incubated at room temperature for 2 min. Reactions were stopped by adding 1 ml of ice-cold PB buffer (QIAGEN). Footprint assay mixtures were purified with a QIAquick PCR purification kit. For the sequence reaction, the dideoxynucleotide chain termination method using a Thermosequenase cycle sequencing kit (Amersham Pharmacia Biotech) and the end-labeled primer 5′-GCCGCCGCAGCAGAAATCCAGCG-3′ was used. The primer was end labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-$^{32}$P]dATP.

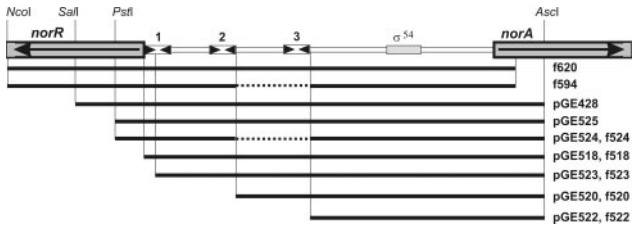


FIG. 1. Physical map of the *norRAB* gene region (not drawn to scale). Grey boxes with arrows represent genes and transcriptional directions. Numbered boxes with arrowheads denote inverted repeats. The $\sigma^{54}$ consensus sequence is depicted as a light grey box. DNA fragments are shown as black bars below the map. Dotted lines show the extent of deletions.

**Genome analysis.** DNA regions containing *norR*-like genes were extracted from the National Center for Biotechnology Information nonredundant database by BLAST analysis and checked for the occurrence of NorR boxes by visual inspection. A consensus pattern was derived from a multiple alignment, matching two consecutive NorR boxes followed by a $\sigma^{54}$ consensus motif, DGT-($N_7$)-ACH-($N_{5\text{-}15}$)-DGT-($N_7$)-ACH-($N_{35\text{-}60}$)-WGGC-($N_8$)-YGC (where W is A or T; D is A, G, or T; H is A, C, or T; Y is C or T; and N is any base). A pattern search with genomic DNA sequences was done with the GeneSOAP analysis workbench program (47). The search algorithm excluded hits that were completely within coding regions and that were not 5′ of genes within a distance of 20 to 100 bp.

## RESULTS

**Location of the NorR binding site.** Previous investigations in which *lacZ* transcriptional fusions were used with DNA fragments of the *R. eutropha* H16 *norRAB* gene region showed that NorR controls expression of both *norA* and *norB* from a promoter upstream of *norA* (37). The upstream regulatory region was located on a 0.5-kb DNA fragment that includes a GG-($N_{10}$)-GC $\sigma^{54}$ promoter consensus motif centered 45 bases upstream of the *norA* start codon. To identify the binding site of NorR, a 620-bp DNA fragment (f620) (Fig. 1), including 366 bp of *norR* and the complete *norR-norA* intergenic region, was used in DNase I footprint experiments. Purification of NorR yielded predominantly insoluble protein, and our attempts to use purified NorR in DNA-binding assays were unsuccessful. Therefore, the footprint assay was carried out by using a purified MalE-NorR′ fusion derivative. NorR′ lacks the N-terminal signaling domain of NorR and was shown previously to activate *norAB* expression in an effector-insensitive manner (37). A 73-bp sequence that overlaps 9 bp of *norR* was protected from cleavage when MalE-NorR′ was added at concentrations of 61 to 158 nM (Fig. 2). Within the protected fragment, we identified three inverted repeats, GGT-($N_7$)-ACC (where N is any base), that were considered putative NorR recognition motifs. Repeat 1 is located directly adjacent to the *norR* translational start, spanning bases 1 to 13 of the *norR-*

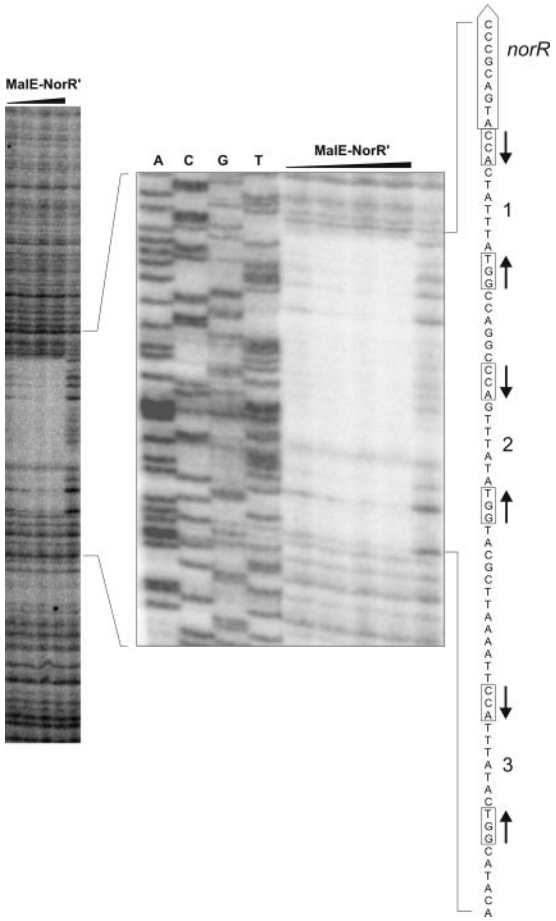


FIG. 2. Footprint analysis. A 620-bp fragment (f620) that includes the *norAB* promoter region was targeted in a DNase I protection assay (left panel). The center panel shows a magnification of the protected region. Lanes containing the DNA sequence reaction are labeled A, C, G, and T. Increasing concentrations of MalE-NorR′ (60, 95, 125, and 160 nM) or no protein (rightmost lane) were added to the target DNA. The DNA sequence of the protected region is shown on the right side. Boxes with arrows indicate inverted repeats.

*norA* intergenic region. The spacing between repeats 1 and 2 and repeats 2 and 3 is 6 and 13 bp, respectively. Repeat 3 is located 40 bp upstream of the $\sigma^{54}$ consensus motif.

To determine the minimal DNA segment required for binding of NorR, nested deletions of the *norRA* gene region (Fig. 1) were used as targets in EMSAs. Fragment f518 (307 bp) spans the *norR-norA* intergenic region and 156 bp of *norA*. Fragment f520 (275 bp) lacks inverted repeats 1 and 2. All three repeats are deleted in fragment f522 (250 bp). As depicted in Fig. 3, the addition of 0.5 μg of MalE-NorR′ to fragment f518 in an EMSA resulted in a retarded band, while a second and a third band became visible upon the addition of 1.5 to 4 μg of MalE-NorR′. The size of the shifting of all bands appears to be rather large, which may indicate that MalE-NorR′ forms multimers. Nonetheless, DNA binding of MalE-NorR′ is specific, since no retardation was observed for fragments f520 and f522, which lack two and all of the inverted repeats, respectively. In contrast, fragment f523 (304 bp), which lacks half of repeat 1, was retarded by MalE-NorR′ (Fig. 4A). Furthermore, a deletion of repeat 3 in fragment f524 (345 bp) did not prevent binding

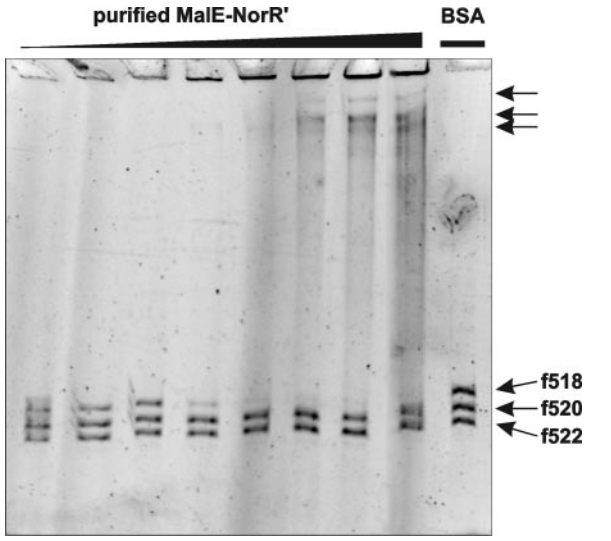


FIG. 3. Electrophoretic mobility shift assay. PCR fragments with nested deletions of the *norA* upstream region were incubated with increasing amounts (0.025, 0.035, 0.05, 0.25, 0.5, 1.5, 2.5, and 4.0 μg) of MalE-NorR′ fusion protein or 5 μg of bovine serum albumin (BSA) and loaded onto a 6% nondenaturing polyacrylamide gel stained with ethidium bromide. Retarded bands are marked by unlabeled arrows.

(Fig. 4B), nor did it change protection of boxes 1 and 2 in a DNase I footprint assay with fragment f594 (Fig. 4C). In conclusion, MalE-NorR′ binds to DNA fragments that include a minimum of two inverted repeats (1 and 2 or 2 and 3 [see Fig. 6A]) that are spaced by 6 and 13 bp, respectively.

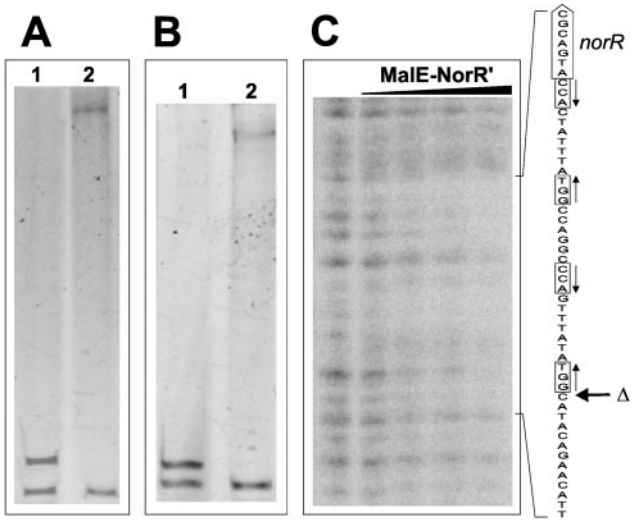


FIG. 4. Binding of MalE-NorR′ to mutated fragments of the *norAB* promoter region. Panels A and B show an electrophoretic mobility shift assay. Fragment f523 (A) lacks one-half of inverted repeat 1. Fragment f524 (B) lacks inverted repeat 3. Fragment f522 (lower band in A and B), lacking all inverted repeats, was used as a control. Lanes labeled with 1 contained 0.05 μg and lanes labeled with 2 contained 2.5 μg of MalE-NorR′ fusion protein. Panel C shows a DNase I protection assay with fragment f594 lacking inverted repeat 3. Lanes containing the DNA sequence reaction are labeled A, C, G, and T. No protein or increasing concentrations of MalE-NorR′ (50, 60, 125, and 300 nM) were added to the target DNA. The DNA sequence of the protected region is shown on the right side. Boxes with arrows indicate inverted repeats. The position of the 26-bp deletion in f524 is indicated with Δ.

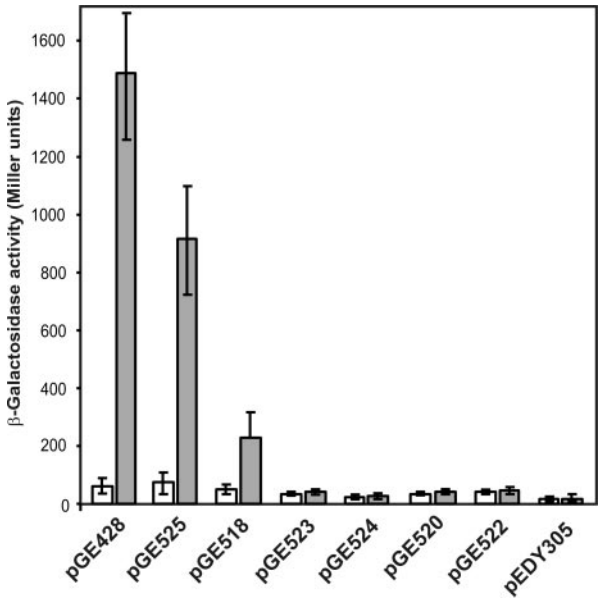


FIG. 5. Transcriptional activation of the *norAB* promoter by NorR. β-Galactosidase activities of transcriptional *lacZ* fusions of fragments with consecutive deletions of the *norA* upstream region were determined in wild-type *R. eutropha* H16 after microaerobic growth in minimal medium. Cells were preincubated for 4 h under oxygen-limiting conditions to reach comparable ODs and to lower the oxygen tension. Promoter activation by NorR was induced by the addition of 2 mM SNP (grey bars) and compared to uninduced cells (white bars). The activities of β-galactosidase are presented in Miller units, with standard deviations (error bars) estimated from three independent samples.

**Minimal NorR binding region for *norAB* promoter activation.** The DNA region required for in vivo activation of the *norAB* promoter by an intact NorR protein was determined by using *norA-lacZ* transcriptional fusions. Fragments generated by nested deletions (Fig. 1) were cloned in the low-copy promoter test vector pEDY305 and assayed for β-galactosidase activity upon transfer into *R. eutropha* H16. Cells were grown microaerobically in the absence of nitrate to prevent the formation of endogenous nitric oxide. Promoter activation by NorR was induced with the nitrosating agent SNP. In a previous study, it was shown that a 463-bp insert of pGE428 that includes 315 bp upstream of *norA* conferred high promoter activity under anaerobic growth conditions and that a longer fragment did not increase that level (37). Under microaerobic conditions, pGE428 produced β-galactosidase activity of about 1,500 Miller units (Fig. 5). The activity of an insert containing only 64 bp of the *norR* gene and the *norR-norA* intergenic region (pGE525, 371-bp insert) was markedly diminished compared to pGE428, yielding about 900 Miller units. An insert lacking these 64 bp (pGE518, 307-bp insert) showed about 25% of the activity of pGE525. Deletions affecting the integrity of the inverted repeats resulted in trace amounts of activity. Removal of one-half of inverted repeat 1 in pGE523 (304-bp insert) yielded approximately 50 Miller units. Plasmid pGE524 (345-bp insert) gave a comparably low activity. This construct lacks inverted repeat 3 but preserves the spacing between the distal inverted repeat (now repeat 2) and the $\sigma^{54}$ consensus site. MalE-NorR′ was able to bind to both of the corresponding DNA fragments f523 and f524 (Fig. 4A and B). On the other hand, the inability of fragments f520 and f522 to bind MalE-NorR′ corresponds to the lack of promoter activation with pGE520 and pGE522.

The role of individual inverted repeats in promoter activation was further investigated by site-directed mutagenesis of the pGE525 insert. The mutations are summarized in Fig. 6A. β-Galactosidase activity of *R. eutropha* H16 transconjugants was determined after the addition of SNP to microaerobically grown cells. A T-to-A exchange in a nonconserved region (pGE530) showed only a slightly reduced β-galactosidase level (Fig. 6B). Mutations altering inverted repeats 1 to 3 yielded promoter activation in the range of about 10 to 14% compared to pGE525. Shortening of the spacing within repeat 3 by 1 bp (pGE529) maintained the highest residual activity, at about 20%. None of the mutations impaired retardation of the corresponding DNA fragments by MalE-NorR′ in mobility shift assays (data not shown). Since all mutated fragments retained at least two intact inverted repeats, this result is in line with our above-mentioned conclusion that two inverted repeats suffice for binding of MalE-NorR′. However, in contrast to the binding behavior of MalE-NorR′, promoter activation by NorR in vivo was very sensitive to base substitutions within all of the inverted repeats. To investigate whether this deviation from the in vitro data is due to the removal of the sensing domain in

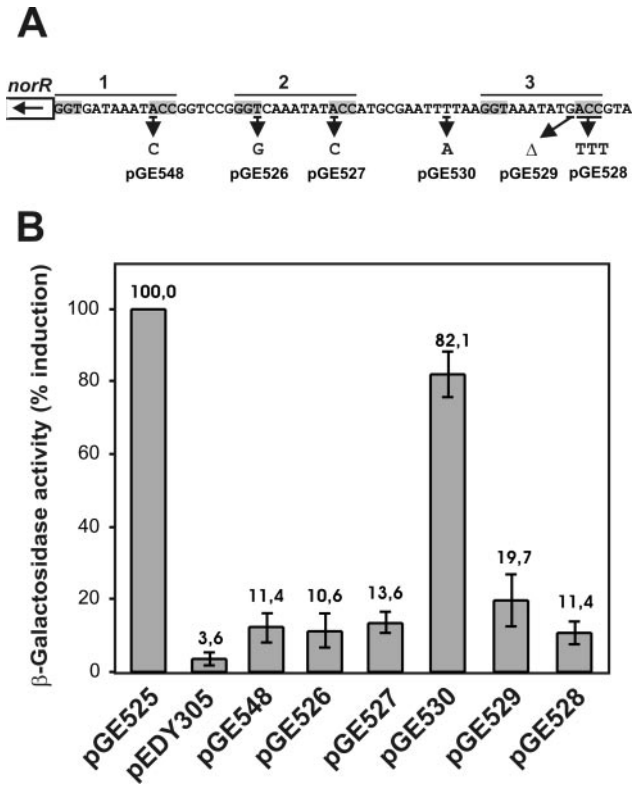


FIG. 6. Mutational analysis of the *norAB* promoter region. A. Shaded boxes labeled with 1, 2, and 3 indicate inverted repeats. Site-directed mutations and designations of the corresponding plasmids with transcriptional *lacZ* fusions are shown. B. β-Galactosidase activity of the reporter constructs was measured in *R. eutropha* H16 wild-type cells in mineral medium under microaerobic conditions after induction with 2 mM sodium nitroprusside. The activity of pGE525 was arbitrarily set to 100%. Standard deviations ($n = 3$) are shown by error bars.

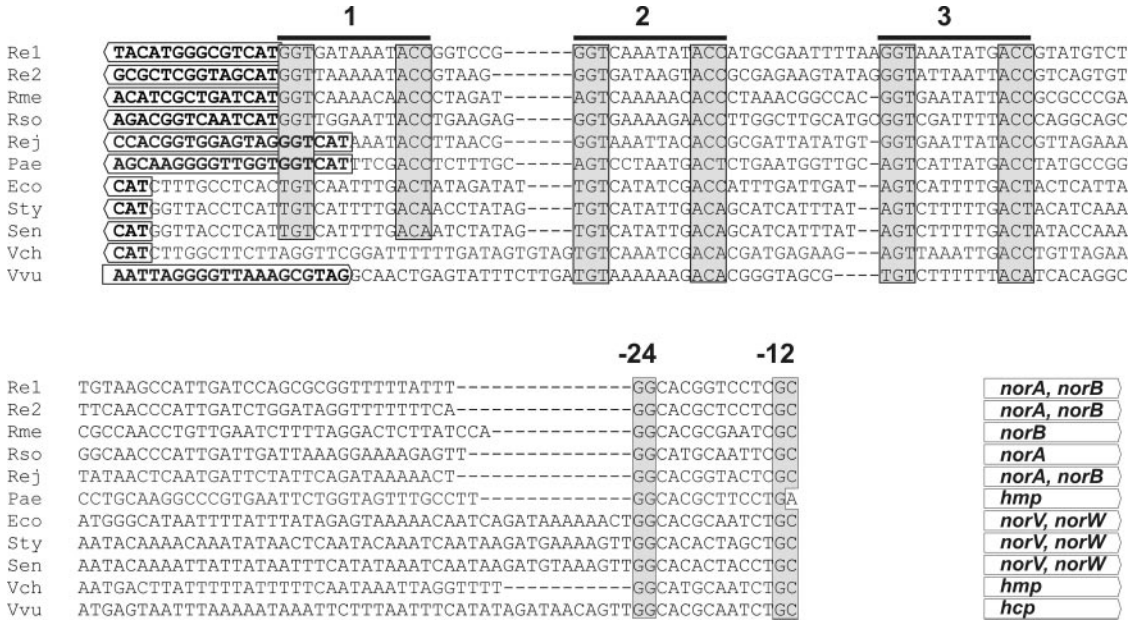


FIG. 7. Comparison of DNA regions adjacent to *norR* genes from various genomes. Shaded boxes labeled with 1, 2, and 3 indicate inverted repeats. Conserved elements of a $\sigma^{54}$ consensus site are also boxed. *norR* and the putative target genes are indicated by arrow boxes. Bases of *norR* coding regions are in boldface type. Re1: *R. eutropha* megaplasmid pHG1; Re2: *R. eutropha* chromosome II; Rme: *Ralstonia metallidurans* unfinished genome (U.S. Department of Energy Joint Genome Institute [http://www.jgi.doe.gov]); Rso: *R. solanacearum* GMI1000 megaplasmid pGMI1000MP (41); Rej: *R. eutropha* JMP134 unfinished genome (U.S. Department of Energy Joint Genome Institute); Pae: *P. aeruginosa* PA01 (51); Eco: *E. coli* K12 (6); Sty: *S. enterica* serovar Typhimurium LT2 (31); Sen: *S. enterica* serovar Typhi Ty2 (14); Vch: *Vibrio cholerae* O1 biovar El Tor (23), Vvu: *V. vulnificus* CMCP6 chromosome I (28).

MalE-NorR′, we checked the β-galactosidase activity of the mutant fragments in strain HF562. HF562 lacks the chromosomal *norR2A2B2* region and expresses NorR′ from a truncated *norR* gene on pHG1, leading to a constitutive activation of *norAB* transcription. The activity pattern in these transconjugants (data not shown) was comparable to that of SNP-induced wild-type cells shown in Fig. 6B.

**Comparison of upstream regulatory regions containing the NorR binding site.** *norA* and *norB* represent the only target genes of NorR that have been identified in *R. eutropha* H16. However, diverse cellular responses to nitric oxide in bacteria that may be subjected to regulation by NorR are known. In fact, NorR orthologs control the expression of flavorubredoxin in *E. coli* (18, 26) and NO dioxygenase in *Pseudomonas aeruginosa* (H. Arai, personal communication). A database survey of DNA regions in the vicinity of *norR*-like genes revealed that in several proteobacteria, gene loci encoding nitric oxide reductase, NO dioxygenase, flavorubredoxin, or hybrid cluster protein are preceded by inverted repeats similar to those identified in *R. eutropha* (Fig. 7). In most cases three consecutive copies of repeats that contain a strictly conserved GT-($N_7$)-AC core sequence are present, whereas the first base (G) and the last base (C) of the *Ralstonia* consensus are frequently replaced by T and A, respectively, in the *Escherichia*, *Vibrio*, and *Salmonella* genomes. To investigate the occurrence of this core sequence in various bacterial genomes, we derived a consensus pattern from the alignment shown in Fig. 7 that matches two or more GT-($N_7$)-AC motifs in noncoding regions upstream of genes. The pattern was used in a computer search with genomic DNA of selected bacteria that contain a putative NorR ortholog. Only one or two matching upstream regions per organism were retrieved by the search, and all but one of these organisms contain a *norR* gene in the immediate neighborhood of the NorR boxes. The only exception was a DNA segment in *Ralstonia solanacearum* upstream of gene *RSc0798*. It appears that very few genes are controlled by NorR in a given organism and that these genes usually are located adjacent to *norR*.

## DISCUSSION

In bacteria, a number of regulators have been shown to modulate transcription in response to RNS. Under anaerobic conditions, the dominant RNS-sensing activity in *E. coli* was attributed to NorR (34) that activates the NO-reducing NorV enzyme (18, 26). Likewise, NorR controls the critical step of NO reduction under anaerobic denitrifying conditions in *R. eutropha*. Thus, NorR acts as a functional equivalent of the Nnr(R)/Dnr(D) proteins known from other denitrifiers, and both types of regulators appear to be specifically designed for sensing RNS in order to induce expression of NO-metabolizing or -detoxifying enzymes.

In this study, we have defined an upstream activator sequence for NorR in *R. eutropha*. The consensus sequence, namely GGT-($N_7$)-ACC, is termed the NorR box. DNase I footprint analysis revealed that the binding of MalE-NorR′ protected a 73-bp segment upstream of *norA* that harbors three NorR boxes. This finding is in agreement with EMSA studies demonstrating a binding site for NorR in that region. The EMSA clearly shows that binding of MalE-NorR′ to its target sequence requires a minimum of two NorR boxes. However, a deletion of half of NorR box 1, a deletion of NorR box 3, and base exchanges within NorR boxes 1 to 3 led to a strong

decrease in β-galactosidase activity, suggesting that the activation of the *norAB* promoter by NorR in vivo depends on all three NorR boxes. Promoter activities in strain HF562 suggest that this discrepancy is not due to the lack of the signaling domain in NorR′. The MalE-NorR′-to-operator ratio that is needed to observe shifting in vitro may be much higher than the NorR-to-operator ratio in vivo, and two NorR boxes may suffice for binding when the protein is present in high concentrations. Another possibility is that three NorR boxes favor the formation of specific NorR multimers that may be needed to activate expression. Furthermore, the DNA-binding assays may not completely reflect the situation in vivo. In fact, a deletion of 93 bp located within the *norR* gene (pGE428 versus pGE525) resulted in an unexpected loss of promoter activity, although no protection by MalE-NorR′ of the additional region on pGE428 was apparent by DNase I footprint analysis (Fig. 2). Promoter activation on both plasmids depends strictly on induction by SNP (Fig. 5) and is thus presumably mediated by NorR. However, we cannot exclude the possibility that full activation of the *norAB* promoter may rely on another SNP-responsive factor that remains to be elucidated.

The comparative analysis of upstream regulatory regions presented in this study is in line with observations by Hutchings et al. (26), who suggested a NorR-dependent *cis*-acting sequence within the *norR* coding region upstream of *norV* in *E. coli*. The 135-bp region defined by those authors includes three conserved NorR boxes (Fig. 7). The NorR boxes are unusually close to the $\sigma^{54}$ consensus site. Typically, $\sigma^{54}$-dependent regulators bind 100 to 200 bp upstream of the target promoter (33). The distance between the distal NorR box and the $\sigma^{54}$ consensus ranges from 40 bp in *R. eutropha* to up to 55 bp in *Salmonella* strains and *Vibrio vulnificus* CMCP6. Most of the spacing regions contain sequence motifs that resemble the binding site WATCAA-($N_4$)-TTR (where W is A or T, R is purine, and N is any base) of the integration host factor (IHF) (40), which is involved in enhanced transcription from several $\sigma^{54}$-dependent promoters (48). The proximity of NorR box 3 to the −24 region implies that NorR may contact RNA polymerase without bending; therefore, a putative role for IHF may be that of a restrictor of cross-activation by other regulators or to help recruit RNA polymerase-$\sigma^{54}$ to the promoter (49). However, to date, there is no experimental evidence for the involvement of IHF in promoter activation by NorR.

Our data suggest that the *norR* gene is mostly, if not always, associated with NorR-dependent promoters. In fact, Mukhopadhyay et al. (34) demonstrated by microarray analyses that only *norVW* and a few other genes were not induced by NO in a NorR mutant of *E. coli*. Thus, it is tempting to postulate that *norR* and its target genes form a gene cassette that is easily interchangeable by horizontal gene transfer and conveys an RNS-detoxifying capability to the host. In most cases, *norR* is transcribed divergently from its target genes. Since binding of NorR near the start codon of *norR* can exert autoregulation by negative feedback inhibition, as shown for *R. eutropha* (37), this arrangement may reflect a mechanistic advantage. On the other hand, *norR* is transcribed in the same direction as *vv12864* encoding a hybrid cluster protein (Hcp) in *V. vulnificus* CMCP6, suggesting a role for Hcp in NO metabolism. In *S. enterica* serovar Typhimurium, *hcp* expression is induced by acidified nitrite (27).

The presence of a consensus NorR binding motif in several proteobacteria suggests that the NorR proteins of these organisms share similar residues that are crucial for base recognition at the DNA target sequence. The C terminus of NorR proteins contains the typical A-L-($X_9$)-A-A-($X_2$)-L-G sequence that is conserved in $\sigma^{54}$-dependent regulators such as NifA and NtrC (33). In these proteins, this sequence overlaps with the helix-turn-helix (HTH) motif that is essential for binding of the protein to the cognate upstream activating sequences (25, 59). The second helix of this motif is termed the recognition helix, as it is critical for specificity. NorR proteins also contain a putative HTH motif that aligns well to the HTH motifs of NifA and NtrC proteins. Within the HTH motif of NifA from *Klebsiella pneumoniae*, several residues that are supposed to make direct base contacts have been identified by nuclear magnetic resonance spectroscopy (39). By analogy, we assume that the corresponding residues in NorR proteins are likely candidates for recognition of conserved DNA sequences. Namely, these residues include a conserved tryptophan residue (W490 in NorR1 of *R. eutropha*) and a K-L-A-K-R-L motif that is conserved in most NorR proteins at the end of the putative recognition helix.

## ACKNOWLEDGMENTS

We thank Thomas Eitinger for critical reading of the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

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