JOURNAL OF BACTERIOLOGY, Aug. 2002, p. 4640–4643 Vol. 184, No. 16
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.16.4640–4643.2002

The NorR Protein of *Escherichia coli* Activates Expression of the Flavorubredoxin Gene *norV* in Response to Reactive Nitrogen Species

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Received 28 March 2002/Accepted 9 May 2002

The *Escherichia coli norVW* genes encode a flavorubredoxin and NADH:(flavo)rubredoxin reductase, respectively, which are involved in nitric oxide detoxification under anaerobic growth conditions. Here it is shown that the *norVW* genes also have a role in protection against reactive nitrogen intermediates generated from nitroprusside. Transcription from the *norV* promoter is activated by the presence of nitroprusside in the growth medium; activation requires the product of a divergently transcribed regulatory gene, *norR*.

Genes previously designated *ygaK* and *ygbD* in the *Escherichia coli* genome were recently shown to encode a flavorubredoxin and an NADH:(flavo)rubredoxin oxidoreductase, respectively, which together metabolize nitric oxide (NO) in cells grown anaerobically and preexposed to NO (4, 5). Because of the activity of the proteins in reducing NO, the genes were redesignated *norV* and *norW*. The transcriptional organization of the *norVW* region has not been defined, but the fact that the two coding regions overlap suggests that they are in a single transcription unit and that they are translationally coupled. A *norV* mutant showed a clear defect in the ability to metabolize NO under anaerobic conditions (5). A *norW* mutant showed a partial phenotype, indicating that NorW plays an ancillary role in flavorubredoxin-catalyzed NO reduction, or that it can be replaced by another protein (5). Divergently transcribed from *norVW* there is a gene (previously designated *ygaA*) that is predicted to encode a σ^{54}-dependent transcriptional activator (5, 11, 14). As has been previously noted (11), the product of *ygaA* is ≈42% identical in sequence to the NorR protein of *Ralstonia eutropha*, which activates expression of a nitric oxide reductase in response to NO and to reactive nitrogen intermediates (RNIs) generated from sodium nitroprusside (11). The NorR protein is organized into three domains: an N-terminal GAF domain that is potentially a site for an interaction with a small molecule ligand (8), a central domain that is predicted to interact with σ^{54}-containing RNA polymerase and to hydrolyze ATP, and a C-terminal DNA binding domain (11, 14). On the basis of this sequence similarity and the observation that *ygaA* and *norV* mutants have similar phenotypes, the *ygaA* gene of *E. coli* was redesignated *norR*, on the assumption that the *norR* gene product regulates expression of *norV* (5). There is a predicted σ^{54} promoter in the 111-bp *norR-norV* intergenic region (17) (Fig. 1), which is consistent with the proposal that NorR activates σ^{54}-RNA polymerase-directed transcription of *norV* (and, probably, *norW*). Data presented in this paper demonstrate that the NorR protein is indeed required for transcription of *norV* and that expression of the structural genes is activated in both aerobic and anaerobic cultures by RNIs. It was previously reported that, while the NorVW system efficiently reduces NO in the absence of oxygen, mutants deficient in *norV* or *norW* had no growth defect in anaerobic cultures grown in rich medium in the presence of NO. A growth defect was noted in cultures grown in defined media formulated such that growth was dependent on NO-sensitive enzymes (5). Here it is demonstrated that *nor* mutants are sensitive to RNIs (generated from nitroprusside) and, further, that they show a defect in growth in rich medium in the presence of NO, at least under some conditions.

Phenotypes of *nor* mutants. The *norR* gene was disrupted in *E. coli* strain DH10B [*mcrA* Δ(*mrr hsdRMS mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lac*X74 *deoR recA endA araD* Δ(*ara leu*) *galU galK rpsL nupG*] by a chloramphenicol resistance cartridge, using the one-step inactivation method (3). A single deletion-insertion removing most of the *norV* and *norW* reading frames was constructed by the same method. To test the sensitivity of strains to RNIs, the two mutants and the parent were grown in anaerobic cultures in a rich medium (L broth supplemented with 0.5% glucose) to which increasing concentrations of the NO^+ donor sodium nitroprusside (13) were added. The results revealed that the *norR* and *norVW* mutants are significantly more sensitive to nitroprusside than the wild type. Growth of the *norR* mutant was completely inhibited by 0.2 mM nitroprusside, and growth of the *norVW* mutant was inhibited by 0.075 mM nitroprusside (Fig. 2a). In contrast, the parent strain showed significant growth at nitroprusside concentrations up to 1 mM (Fig. 2a) and was not further inhibited by concentrations as high as 5 mM. Determinations of viable counts after a 2-h exposure to nitroprusside (data not shown) confirmed that nitroprusside is cytotoxic (rather than cytostatic) towards the mutant strains. In aerobic cultures, *nor* mutants were no more sensitive to nitroprusside than the parent strain (data not shown). This can probably be explained by the fact that the enzyme encoded by *norV* is sensitive to oxygen (4). However, a role for the *nor* genes during aerobic growth cannot be excluded, given that the *norV* promoter is activated by nitroprus-

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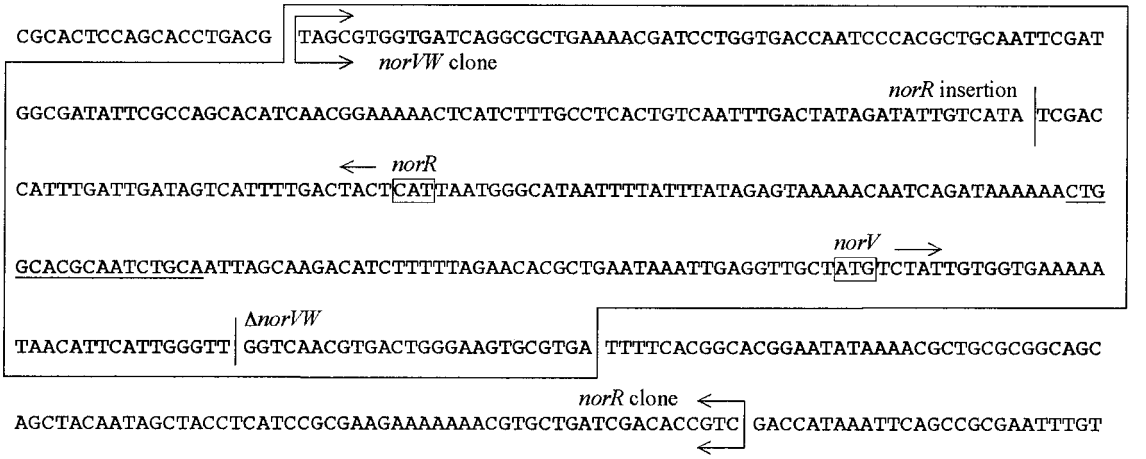

FIG. 1. Organization of the *norR-norVW* intergenic region. The nucleotide sequences of the 5′ ends of *norR* and *norV* and of the intergenic region are shown. The start codons of the two genes are boxed. The 344-bp sequence enclosed within a box is the fragment that was cloned in pRS551 (15) in both orientations to construct transcriptional fusions. The underlined sequence is the previously predicted (17) σ^{54}-type promoter. The site of the *norR* insertion mutation and the 5′ end point of the *norVW* deletion-insertion mutation are denoted by vertical lines, and the square brackets with arrowheads indicate the 5′ ends of the clones used in complementation tests.

side under aerobic growth conditions (see below), and so it will be of interest to investigate further the role of the *nor* genes in aerobic cultures.

The *norVW* disruption mutant could be partially complemented by a clone containing the *norVW* genes and the 111-bp *norR-norV* intergenic region (Fig. 1). The complemented strain grew to final culture densities of about 50% of that of the parent strain across all of the nitroprusside concentrations shown in Fig. 2. However, the *norR* mutant could not be complemented by a plasmid clone (Fig. 1) containing the *norR* gene and the *norR-norV* intergenic region. The most likely explanation for this failure of *norR* to complement the *norR* mutation in *trans* is that the mutation disrupts a *cis*-acting sequence (perhaps a NorR binding site) within the *norR* coding region that is required for *norVW* expression. If this idea is correct, the *cis*-acting sequence can be localized to the 135-bp region defined by the 5′ end of the clone used to construct the *norV-lacZ* reporter fusion (see below) and the site of the *norR* insertion mutation (Fig. 1). For both complementation tests, it is also possible that multiple-copy clones (containing the *norV* regulatory region) titrate out NorR and so contribute to the lack of complete complementation.

In an assay for sensitivity to NO, the three strains were inoculated into rich medium solidified with soft agar in gas-tight glass tubes with rubber septa in the caps. The headspace was flushed with nitrogen and then 1 ml of NO gas was injected. In experiments such as these, a zone of clearing appears during growth, which is interpreted to reflect the sensitivity of the organism to NO (2). In this case, both mutant strains showed a zone of clearing extending into the agar to a depth about twice that found in the wild-type parent (Fig. 2b). The *norVW* mutant consistently showed a sharp boundary between the zones of growth and nongrowth that was not seen in the *norR* mutant (Fig. 2b). The reason for this difference in behavior is not clear, but it may reflect slight differences in the sensitivities of the two strains (Fig. 2). Nevertheless, this experiment shows that the *norR* and *norVW* strains are significantly more sensitive to NO than the parent strain under the growth conditions used in this experiment. This is in contrast to the previous observation that *nor* mutants showed no growth defect in liquid cultures grown in rich medium in the presence of NO (5).

Regulation of the *nor* promoters. To explore the expression pattern of the *nor* genes and the potential role of the NorR regulator, *lacZ* reporter fusions to the *norR* and *norV* promoters were constructed in pRS551, crossed onto phage λRS45 by homologous recombination, and introduced onto the chromosome as single-copy lysogens at the λ attachment site (15). The DNA used for the construction of the *norV* reporter fusion extended into the *norR* coding region (Fig. 1), since the complementation tests indicated the presence of a *cis*-acting regulatory sequence in this region and because σ^{54}-dependent activators typically bind to an upstream activating sequence located 100 to 200 bp away from the target promoter (14). Nitroprusside was used as the source of RNIs in these experiments, since the homologous NorR protein of *R. eutropha* can be activated by nitroprusside in vivo (11). In anaerobic cultures, the *norV* promoter was activated by nitroprusside in both rich and minimal media, though the effect of nitroprusside was greater in rich medium (Table 1). Activation is completely dependent on the product of the *norR* gene, which demonstrates that NorR mediates the activation of the *norV* promoter by RNIs. In minimal medium, nitrate activated the *norV* promoter as effectively as did nitroprusside, which may reflect the fact that nitrate respiration is accompanied by the formation of traces of NO (9). Activation of the *norV* promoter by nitroprusside was virtually abolished in the *norVW* mutant (Table 1). One possible explanation for this observation is that the NorVW proteins act on RNIs to generate a compound that is the true signal recognized by NorR. The *norR* promoter appears to be essentially constitutive, though it did show a small stimulation by the presence of nitrate in the growth medium (Table 1). The *norR* promoter was significantly more active in the *norR* mutant, which is consistent with negative autoregu-

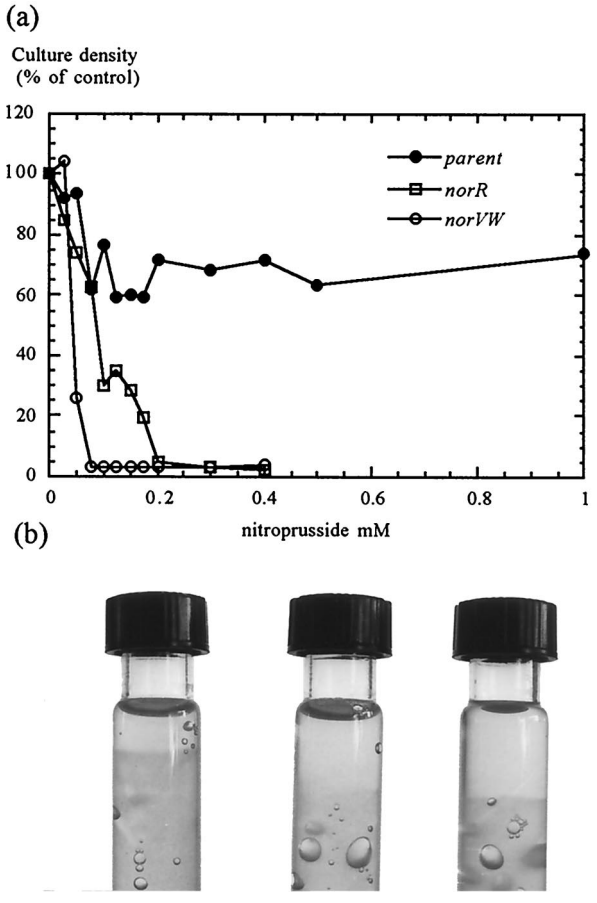

FIG. 2. Sensitivity of the *norR* and *norVW* mutants to nitroprusside and NO. (a) Aerobically grown precultures of DH10B and its *norR* and *norVW* derivatives were inoculated into L broth supplemented with 0.5% (wt/vol) glucose and with the indicated concentrations of sodium nitroprusside. When control cultures grown in the absence of nitroprusside were in late log phase, culture densities were measured at 600 nm and are expressed as a percentage of the optical density of the control culture. There were no significant differences in the optical densities of the three control cultures. (b) Cultures of DH10B (left tube) and its *norR* and *norVW* (right tube) derivatives were grown to mid-log phase, and then 1.5 ml of culture was mixed with 15 ml of 0.3% L agar and poured into a glass tube. The tubes were sealed and sparged with N_2 gas for 10 min. NO gas (1 ml) was then injected into the headspace and the tubes were incubated at 37°C overnight. Controls containing only N_2 in the headspace confirmed that the zone of clearing was due to the presence of NO. Bubbles in the agar are hydrogen formed as a product of glucose fermentation.

lation, as is seen for other members of the σ^{54}-dependent family of regulators, such as XylR (1). The *norR* promoter showed a response to nitrate in the *norR* mutant similar to that seen in the parent strain. The *norR* promoter was inactive in the *norVW* mutant, under all growth conditions, a surprising result that cannot easily be explained at the present time.

In aerobic cultures, the *norV* promoter was strongly activated by nitroprusside, but only in rich medium (Table 2). The reasons for the medium effect and for the different response under aerobic conditions are not known but may reflect the complex interaction of nitroprusside and RNIs with oxygen (13). Nitrate did not cause activation of *norV* in aerobic cultures (Table 2), which supports the idea that activation by nitrate requires nitrate respiration (and the concomitant formation of NO) under anaerobic conditions. Otherwise, *norV* and *norR* promoter activities under aerobic conditions were qualitatively rather similar to those seen under anaerobic conditions. Aerobic expression of *norV* may seem surprising, given that the NO reducing activity of NorVW is sensitive to oxygen (4). On the other hand, the enzyme does seem to have a role under microaerobic conditions (5), and the NorVW proteins have been reported to have oxidase activity (6). Hence, it is possible that the aerobic expression of the *nor* genes under aerobic conditions reflects a physiological role for the enzyme in the presence of oxygen.

Concluding remarks. The NorR protein of *E. coli* appears to be a true orthologue of NorR of *R. eutropha* (11) in that it is activated in vivo by sources of RNIs, specifically nitroprusside. However, the targets for NorR in *E. coli* are different, being two genes, the products of which protect *E. coli* against the harmful effects of NO and RNIs. Bacteria have multiple mechanisms to protect against or reverse the harmful effects of RNIs, including those involving flavohemoglobin (13), cytochrome *c′* (2), peptide methionine sulfoxide reductase (16), *S*-nitrosoglutathione reductase (10), and NO reductase (18). In *E. coli*, the SoxR and OxyR regulatory proteins are activated by NO, and mutants with defects in these systems are more sensitive to RNIs and nitrosative stress (7, 12). The discovery of the role of the *nor* genes increases the diversity of the regulatory and enzymatic systems that have a role in protection against reactive nitrogen species, and it will be of interest to further explore the biochemical mechanisms involved.

TABLE 1. β-Galactosidase activities of *norR*- and *norV-lacZ* fusions in anaerobic cultures of a wild-type strain (DH10B) and its *norR* and *norVW* mutant derivatives[a]

Strain	Promoter	β-Galactosidase activity (Miller units)					
		LG	LG + SNP	M9	M9 + SNP	M9 + nitrite	M9 + nitrate
DH10B	*norV*	3 ± 1	46 ± 2	6 ± 1	22 ± 1	6 ± 1	35 ± 4
norR::*cam*	*norV*	4 ± 1	4 ± 1	14 ± 1	9 ± 1	14 ± 1	14 ± 1
norVW::*cam*	*norV*	1	6 ± 1[b]	10 ± 2	12 ± 2	4 ± 1	94 ± 12
DH10B	*norR*	46 ± 2	46 ± 2	119 ± 2	45 ± 2	86 ± 10	166 ± 11
norR::*cam*	*norR*	215 ± 12	250 ± 62	805 ± 33	344 ± 7	431 ± 56	1,063 ± 11
norVW::*cam*	*norR*	2	7 ± 1[b]	14 ± 2	2 ± 1	11 ± 2	13 ± 1

[a] Activities were determined in duplicate from three independently grown cultures; standard errors are shown. Growth was to log phase in L broth supplemented with glucose (LG) and with 100 μM sodium nitroprusside (SNP) where indicated, or in M9 minimal medium supplemented with SNP (100 μM), nitrite (2 mM), or nitrate (50 mM), as indicated.

[b] Assays were on cultures grown in the presence of 25 μM SNP, since the higher concentration was growth inhibitory to this strain under these conditions.

TABLE 2. β-Galactosidase activities of *norR*- and *norV-lacZ* fusions in aerobic cultures of a wild-type strain (DH10B) and its *norR* and *norVW* mutant derivatives[a]

Strain	Promoter	β-Galactosidase activity (Miller units)					
		LG	LG + SNP	M9	M9 + SNP	M9 + nitrite	M9 + nitrate
DH10B	*norV*	21 ± 4	245 ± 68	2 ± 1	2 ± 1	15 ± 2	1 ± 1
norR::*cam*	*norV*	3 ± 1	2 ± 1	7 ± 1	4 ± 1	7 ± 1	5 ± 1
norVW::*cam*	*norV*	7 ± 1	30 ± 1	1	9 ± 2	70 ± 4	1
DH10B	*norR*	110 ± 19	132 ± 14	77 ± 4	67 ± 1	42 ± 5	60 ± 9
norR::*cam*	*norR*	486 ± 101	969 ± 191	497 ± 29	961 ± 218	537 ± 72	1,426 ± 218
norVW::*cam*	*norR*	9 ± 1	5 ± 1	5 ± 1	3 ± 1	6 ± 1	4 ± 1

[a] Activities were determined in duplicate from three independently grown cultures; standard errors are shown. Growth was to log phase in L broth supplemented with glucose (LG) and with 100 μM sodium nitroprusside (SNP) where indicated, or in M9 minimal medium supplemented with SNP (100 μM), nitrite (2 mM), or nitrate (50 mM), as indicated.

This work was supported by a research grant to S.S. from the Biotechnology and Biological Sciences Research Council and by the Wellcome Trust through provision of a vacation scholarship to N.M.

We are grateful to Tracy Palmer and Barry Wanner for providing strains, plasmids, and phage and to Ray Dixon for helpful discussions.

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