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Regulation and Characterization of Two Nitroreductase Genes, *nprA* and *nprB*, of *Rhodobacter capsulatus*

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Among photosynthetic bacteria, strains B10 and E1F1 of *Rhodobacter capsulatus* photoreduce 2,4-dinitrophenol (DNP), which is stoichiometrically converted into 2-amino-4-nitrophenol by a nitroreductase activity. The reduction of DNP is inhibited in vivo by ammonium, which probably acts at the level of the DNP transport system and/or physiological electron transport to the nitroreductase, since this enzyme is not inhibited by ammonium in vitro. Using the complete genome sequence data for strain SB1003 of *R. capsulatus*, two putative genes coding for possible nitroreductases were isolated from *R. capsulatus* B10 and disrupted. The phenotypes of these mutant strains revealed that both genes are involved in the reduction of DNP and code for two major nitroreductases, NprA and NprB. Both enzymes use NAD(P)H as the main physiological electron donor. The nitroreductase NprA is under ammonium control, whereas the nitroreductase NprB is not. In addition, the expression of the *nprB* gene seems to be constitutive, whereas *nprA* gene expression is inducible by a wide range of nitroaromatic and heterocyclic compounds, including several dinitroaromatics, nitrofuran derivatives, CB1954, 2-aminofluorene, benzo[a]pyrene, salicylic acid, and paraquat. The identification of two putative *mar*/*sox* boxes in the possible promoter region of the *nprA* gene and the induction of *nprA* gene expression by salicylic acid and 2,4-dinitrophenol suggest a role in the control of the *nprA* gene for the two-component MarRA regulatory system, which in *Escherichia coli* controls the response to some antibiotics and environmental contaminants. In addition, upregulation of the *nprA* gene by paraquat indicates that this gene is probably a member of the SoxRS regulon, which is involved in the response to stress conditions in other bacteria.

Nitroaromatics are released into the environment almost exclusively as a consequence of anthropogenic activities related to explosives, paints, dyes, and pharmacology industries (23). Microorganisms have developed many different strategies to remove and degrade these xenobiotic compounds, and oxidative or reductive pathways for the degradation of nitroaromatics have been widely studied (19, 23, 29). Polynitroaromatic compounds are usually degraded through the following two major reductive pathways: (i) reduction of the aromatic ring by the addition of hydride ions to produce hydride-Meisenheimer complexes and (ii) reduction of the nitro group(s) to a hydroxylamino or amino group(s) by nitroreductases (3, 10, 15, 25). The oxygen-sensitive nitroreductases catalyze one electron step reaction to produce a nitro radical anion that can be reoxidized by oxygen with the concomitant production of superoxide. However, the best-studied nitroreductases are oxygen insensitive since they do not produce radicals and they catalyze the sequential addition of pairs of electrons donated by NAD(P)H to convert the nitro groups into hydroxylamines or amines, often through nitroso derivatives (15, 23). These enzymes are usually homodimers of a 25-kDa polypeptide and contain flavin mononucleotide as a cofactor. Among these, the nitroreductases of *Escherichia coli* (16) and *Rhodobacter capsulatus* E1F1 (4) have been purified and characterized. Nitroreductases participate in environmental detoxification processes, although they are also present in the intestinal microflora, reducing more stable compounds to mutagenic nitro derivatives (24). Interestingly, the NfsB nitroreductase of *E. coli* has been used in cancer treatments based on the antibody-directed enzyme prodrug therapy technique (7). Photoreduction of nitroaromatics has been described for phototrophic bacteria, such as strains E1F1 and B10 of *Rhodobacter capsulatus*, which photoreduce 2,4-dinitrophenol (DNP) by use of a nitroreductase. DNP is stoichiometrically reduced to 2-amino-4-nitrophenol (ANP) under anaerobic phototrophic conditions (5). This is clearly a cometabolic process, since DNP is an uncoupler which strongly inhibits nitrogen fixation and since DNP photoreduction enables the bacterium to grow by fixing dinitrogen. However, ANP can be further metabolized by a light-dependent microaerobic pathway that releases nitrite into the medium (28). DNP photoreduction requires the presence of additional carbon and nitrogen sources, and this process is induced by the presence of DNP and repressed by ammonium or glutamine (5, 17, 28). It has been proposed that NtrC, a general regulator in response to ammonium, could regulate DNP reduction in *R. capsulatus* by controlling the expression of the Rnf proteins, which supply electrons for both nitrogenase and nitroreductase enzymes (17). The nitroreductase enzyme of *R. capsulatus* E1F1 is composed of two subunits of 27 kDa each, contains flavin mononucleotide as a prosthetic group, and uses NAD(P)H as a physiological electron donor (4). On the other hand, the

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marRAB operon is a regulatory locus that controls multiple environmental hazard resistances in *E. coli* and therefore is induced by a variety of chemical agents, such as tetracycline, chloramphenicol, menadione, benzoate, salicylate, and 2,4-dinitrophenol (1). The *marRAB* operon consists of an operator-promoter region (*marO*) and the genes *marR*, coding for a repressor of this operon, *marA*, encoding a positive transcriptional regulator of unlinked target genes, and *marB*, of unknown function (1). 2,4-Dinitrophenol and salicylic acid, among others, interact with MarR in vitro, and it is thought that, in vivo, repression is relieved and the activation of *marRAB* results from the direct interaction of these inducers with the repression complex MarR-*marO* (1). The *mar* boxes are often elements of recognition for other regulatory proteins, such as SoxS, which is implicated in the expression of genes related to the response to oxidative stress conditions. The SoxRS regulon in *E. coli* includes at least 15 genes that are upregulated in response to superoxide formed by redox-cycling compounds such as paraquat (14). The SoxR protein acts as a sensor to detect elevated levels of superoxide within the cells and to activate the transcription of *soxS*, which codes for a transcriptional activator. More than 50% of genes upregulated by SoxS are also upregulated by the MarA protein (14).

At present, most nitroreductases characterized at the biochemical and/or molecular level belong to enterobacteria. The aim of this work was the identification, isolation, and characterization of the nitroreductase genes, *nprA* and *nprB*, involved in 2,4-dinitrophenol reduction in the phototrophic bacterium *R. capsulatus* B10. These genes were cloned, and the phenotypes of single and double mutants defective in each of or both the *nprA* and *nprB* genes were analyzed. The control of the expression of both nitroreductase genes by aromatic acids and ammonium was also investigated.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Rhodobacter capsulatus* strains were routinely cultured under diazotrophic conditions at 30°C in RCV medium (26), with 30 mM malate as the carbon source, in the presence of 0.15 mM 2,4-dinitrophenol. Ammonium chloride (5 mM) or 7 mM L-glutamine was used as a nitrogen source when indicated. The *E. coli* strains were cultured in Luria-Bertani (LB) medium or on LB agar plates at 37°C (18). The vectors pGEM-T, pGEM-T Easy (Promega), and pBluescript (Stratagene) were used for routine gene manipulation, and the pSUP202 and pSUP301 plasmids were used as mobilizable vectors (20).

Analytical determinations and enzyme assays. Cell growth was followed turbidimetrically by measuring the absorbance of the cultures at 680 nm. 2,4-Dinitrophenol and 2-amino-4-nitrophenol concentrations were determined with a Gold system (Beckman) high-performance liquid chromatograph with a reverse-phase column as previously described (4). The nitrite concentration was determined colorimetrically (21). The ammonium concentration was estimated with phenol-hypochlorite (22). β-Galactosidase activity was determined at 30°C as previously described (12), and activities are expressed in arbitrary units (11). Nitroreductase activity was determined by following 2-amino-4-nitrophenol formation as previously described (4).

DNA manipulations. DNA manipulations were performed by using standard procedures (18). Insertional mutagenesis of the *nprA* gene was carried out by using total DNA from *R. capsulatus* B10 and amplifying by PCR a 1.3-kb fragment containing this gene. The primers used were synthesized according to the database of the genome sequence of *R. capsulatus* strain SB1003 (6), for which a putative oxygen-insensitive nitroreductase gene has been described (RRCO1791), and their sequences were as follows: primer A, 5′-CGGGATTCG CGAATTCTTTCAGAT-3′; and primer B, 5′-CTGCTTGACTTTCAGCGC GACTTTG-3′. The PCR program was 96°C for 2 min, 96°C for 30 s, 65°C for 30 s, and 69°C for 1.5 min; the last three steps were repeated for a total of 30 cycles, and a final step of 69°C for 10 min was performed. The 1.3-kb PCR fragment was cloned into pGEM-T and subcloned into pBluescript with the restriction enzymes ApaI and SpeI to perform sequence analysis. The kanamycin resistance cassette from the pSUP2021 plasmid (20) was introduced into the *nprA* gene with the restriction enzymes HindIII and SalI, which also deleted 0.5 kb of the *nprA* gene. In order to generate EcoRI restriction sites at both ends of this fragment, a new PCR with the forward and reverse primers from pBluescript was developed, and the PCR fragment was cloned into pGEM-T Easy. Finally, the EcoRI fragment was cloned into the mobilizable vector pSUP202 to produce the plasmid pMO8-AKm (*nprA*Δ::Km), which was further transferred by conjugation from the donor strain *E. coli* S17-1 to the recipient wild-type strain *R. capsulatus* B10 (20). To create the transcriptional *nprA-lacZ* fusion, a 1.3-kb AatII/PstI fragment was isolated from pGEM-T and cloned into pSUP301. The *lacZ* gene was inserted in both orientations into the *nprA* gene with the restriction enzyme SalI to generate the plasmids pMO-AL$^+$ (*nprA-lacZ*$^+$) and pMO-AL$^-$ (*nprA-lacZ*, in reverse orientation). The insertion/deletion *nprB* mutant strain was generated by PCR amplification of two fragments, of 938 bp (primers 1 and 2) and 680 bp (primers 3 and 4), using total DNA from *R. capsulatus* B10. Primers 2 and 3 correspond to the 5′ and 3′ ends of the *nprB* gene, respectively. In the sequence of the *nprB* gene, both primers are separated by 370 bp. The primers were synthesized according to the database of the genome sequence of *R. capsulatus* strain SB1003 (6), for which a second putative nitroreductase gene has been described (RRCO3929). The primer sequences were as follows: primer 1, 5′-CCTCGAGGGCTTCACGTTTTCGCG-3′; primer 2 (with BamHI site underlined), 5′-AGGAAGGTGGGATCCGCGGCGTCG-3′; primer 3 (with BamHI site underlined), 5′-GCGGCGGGGATCCAGCTTCTCAAT-3′; and primer 4, 5′-CGAAGAAAACGACGGGCGCCAGGG-3′. The PCR program was the same as that described above to amplify the *nprA* gene. The 938-bp and 680-bp fragments were cloned into pGEM-T Easy to generate pPCR12 and pPCR34, respectively. The 680-bp BamHI/SpeI fragment was cloned into pPCR12 to generate the pPCR14 plasmid. In this construct, a deletion of 322 bp was created in the *nprB* gene (original coding sequence of 603 bp), and a new BamHI restriction site was created to introduce the 4.3-kb gentamicin/spectinomycin resistance cassette from the Tn*5*B12 plasmid (20). The resulting 5.9-kb NcoI/PstI fragment was cloned into pSUP202 to generate the pMO2-BGmSp (*nprB*Δ::GmSp) plasmid, which was transferred by conjugation from the donor strain *E. coli* S17-1 to either the recipient wild-type strain *R. capsulatus* B10 (to generate the *nprB* mutant strain) or the previously isolated *R. capsulatus nprA* mutant strain (to generate the *nprA nprB* double mutant strain). To create a transcriptional *nprB-lacZ* fusion, the 1.6-kb EcoRI fragment with the *nprB* gene was isolated from pPCR14 and cloned into pSUP202. The *lacZ* gene was inserted in both orientations into the BamHI site of the *nprB* gene to generate the plasmids pMO-BL$^+$ (*npr*B-*lacZ*$^+$) and pMO-BL$^-$ (*nprB-lacZ*, in reverse orientation). The plasmids with the *nprA-lacZ*$^+$, *nprA-lacZ* (in reverse orientation), *nprB-lacZ*$^+$, and *nprB-lacZ* (in reverse orientation) fusions were transferred by conjugation from *E. coli* S17-1 to the wild-type strain *R. capsulatus* B10.

RESULTS AND DISCUSSION

Effect of mutations of the *nprA* and *nprB* genes on DNP reduction in *R. capsulatus*. The phototrophic bacterium *Rhodobacter capsulatus* photoreduces DNP to ANP, which is stoichiometrically released into the medium under anaerobic phototrophic conditions, although it is further degraded by light and microaerobiosis (5, 17, 28). The photoreduction of DNP depends on the presence of alternative nitrogen and carbon sources. Thus, the highest rate of this cometabolic process was observed in the presence of malate and dinitrogen gas (not shown).

Two putative genes coding for possible nitroreductases are annotated in the genome sequence of strain SB1003 of *R. capsulatus*: they are a gene for a putative oxygen-insensitive NAD(P)H nitroreductase (RRCO1791) and a gene for a putative protein from the nitroreductase family (RRCO3929) (6). From the DNA sequences of these genes, different primers were synthesized, and PCRs with total DNA isolated from *R. capsulatus* strain B10 were carried out to clone and sequence the corresponding nitroreductase genes. The sequence of the *R. capsulatus* B10 *nprA* gene (RRCO1791 in strain SB1003) showed 27% identity and 50% similarity with the nitroreductase *nfnB* gene of *E. coli* and other classical nitrore-

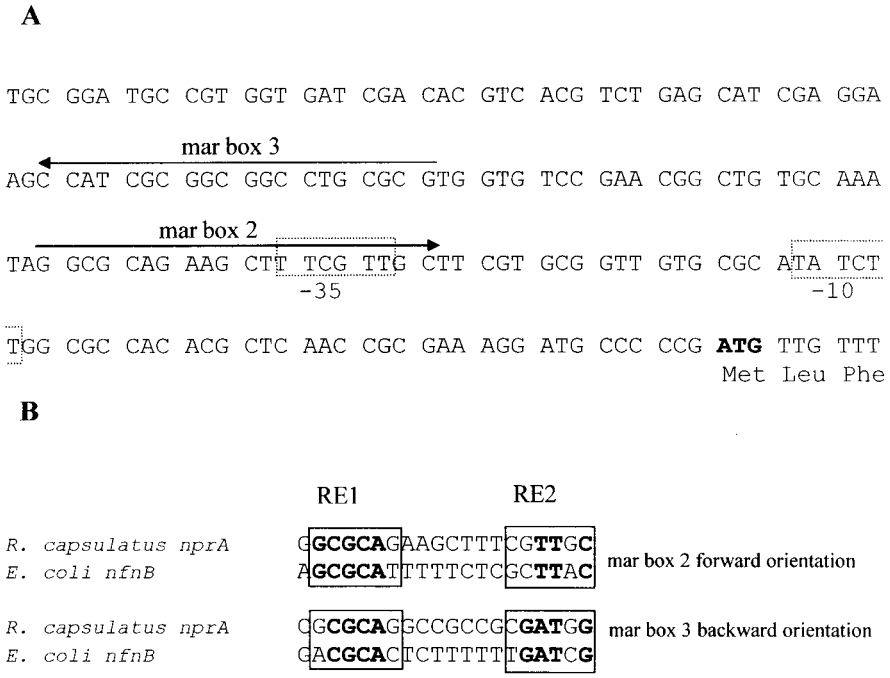

FIG. 1. Locations of hypothetical *mar* boxes in the possible promoter region of the *R. capsulatus nprA* gene. (A) Sequence of the putative promoter region of the *nprA* gene. Arrows indicate the localization and orientation of the two hypothetical *mar* boxes. The putative −10 and −35 heptamers are indicated by dotted lines, and the translation start codon is shown in bold. (B) Sequence alignment of the hypothetical *mar* boxes of *R. capsulatus nprA* and *E. coli nfnB*. The recognition elements RE1 and RE2 for MarA binding are indicated in squares, and the internal conserved nucleotides are shown in bold.

ductase genes of enterobacteria, whereas the *R. capsulatus* B10 *nprB* gene (RRCO3929 in strain SB1003) showed 35% identity and 50% similarity with hypothetical nitroreductase genes from *Caulobacter crescentus* and *Pseudomonas aeruginosa*. The *nprA* and *nprB* genes shared 14% identity with each other (not shown). Activation of the *E. coli* nitroreductase *nfnB* (also named *nfsB*) gene by MarA through a *mar* box in its promoter region has been described (2). *mar* boxes are asymmetric degenerate DNA sequences with two conserved recognition elements, RE1 and RE2, which can be classified into two groups depending on their orientation and location with respect to the −10 and −35 hexamers (2) (Fig. 1). Sequence analysis of the promoter region of the *nfnB* gene of *E. coli* revealed the presence of four putative *mar* boxes. However, studies in vitro have revealed that MarA only binds with a high affinity to *mar* box 2, which belongs to class II and overlaps the −35 hexamer (2). In the possible promoter region of the *R. capsulatus* B10 *nprA* gene, two putative *mar* boxes can be found (Fig. 1A). One of them, *mar* box 2, lies in a forward orientation and overlaps the −35 hexamer, as described for *mar* box 2 of the *E. coli nfnB* gene. Another sequence, *mar* box 3, is located upstream and in the reverse orientation. The putative *mar* box 2 of the *nprA* gene is highly similar to the functional *mar* box 2 described for the *E. coli nfnB* gene, and *mar* box 3 is also very similar to the corresponding *mar* box 3 of the *E. coli nfnB* gene (Fig. 1B). Nevertheless, further studies are needed to determine the transcription start site of the *nprA* gene and the possible interactions between MarA and the two putative *mar* boxes of the *nprA* gene of *R. capsulatus*. On the other hand, the putative promoter region of the *nprB* gene of *R. capsulatus* does not contain *mar* box sequences (not shown).

To determine if the *nprA* and *nprB* genes of *R. capsulatus* B10 code for nitroreductases, three defective strains, the *nprA*, *nprB*, and *nprA nprB* mutants, were constructed by the insertion of antibiotic resistance cassettes into these genes. Bacterial growth and DNP uptake were analyzed in diazotrophic cultures of the wild-type and mutant strains (Fig. 2). The diazotrophic growth rates with DNP of the *nprA* (in which nitroreductase NprB is functional), *nprB* (with nitroreductase NprA being functional), and wild-type (with both NprA and NprB being functional) strains were similar, but the growth of the double *nprA nprB* mutant was considerably affected (Fig. 2A), indicating that simultaneous mutations in both genes impair diazotrophic growth in the presence of DNP, an uncoupler that must be removed to allow dinitrogen fixation. DNP was completely consumed under diazotrophic conditions by the wild-type strain, but in the *nprA* and *nprB* mutant strains this process was delayed for 45 and 60 h, respectively. DNP was even more slowly consumed in the double mutant strain (Fig. 2B). The low rate of DNP reduction observed in the *nprA nprB* mutant strain of *R. capsulatus* can be explained by the presence of other nonspecific nitroreductases that may be present in this bacterium. The presence of several nitroreductases in a single organism is not unusual; for example, in *E. coli* there are at least two oxygen-insensitive (type I) nitroreductases (NfsB and NfsA) in addition to oxygen-sensitive (type II) nitroreductases (27). The *E. coli* NfsA and NfsB proteins have similar enzymatic properties, although they share only 7% identity (8). In

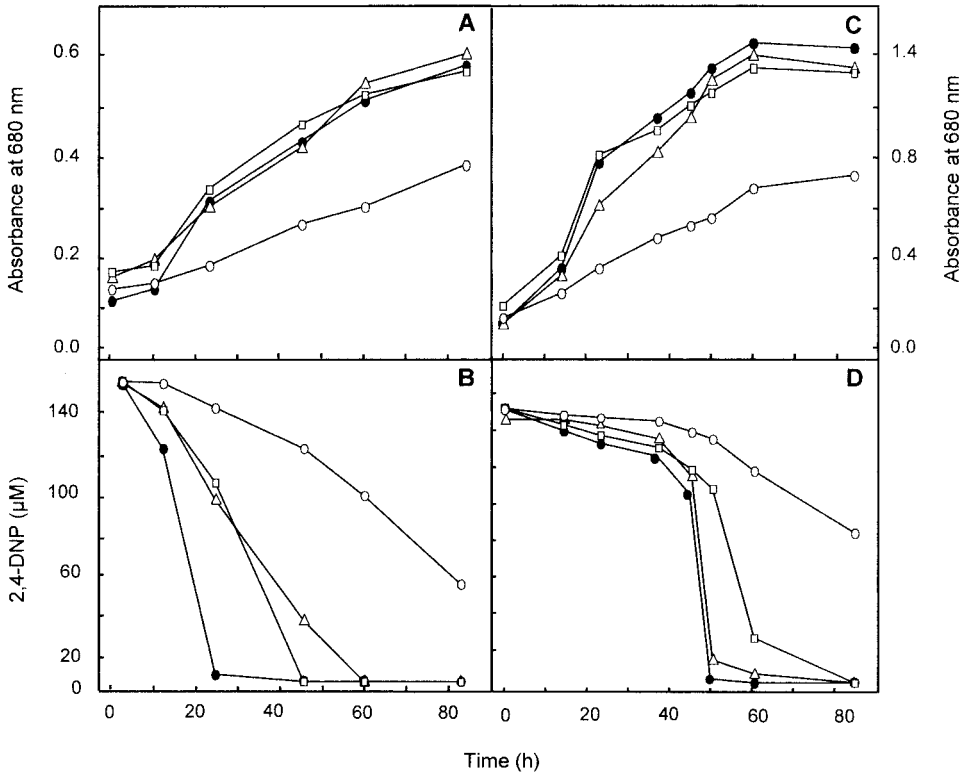

FIG. 2. Bacterial growth (A and C) and 2,4-dinitrophenol uptake (B and D) in wild-type and mutant strains of *R. capsulatus* B10 growing under diazotrophic conditions (A and B) or with 5 mM ammonium chloride (C and D) in the presence of 150 μM 2,4-dinitrophenol. Bacterial growth and 2,4-dinitrophenol uptake were determined as indicated in Materials and Methods at the times indicated in the figure. ●, wild-type strain; △, *nprA* mutant strain; □, *nprB* mutant strain; ○, *nprA nprB* mutant strain. Data correspond to a representative experiment which was repeated three times with variations of <10%.

summary, sequence and mutational analyses indicate that the *nprA* and *nprB* genes of *R. capsulatus* B10 code for the major oxygen-insensitive nitroreductases involved in DNP reduction.

In the presence of ammonium and DNP, cell growth was stimulated in all of the strains, but as observed in medium without ammonium, the *nprA nprB* double mutant showed a lower growth rate than the single mutants and the wild-type strain (Fig. 2C and D). With regard to DNP uptake in the presence of ammonium, the process showed two phases. The initial DNP uptake took place very slowly, corresponding to the presence in the medium of high ammonium concentrations (between 5 and 1.5 mM). This result agrees with that previously reported about the inhibition of DNP uptake by ammonium in *R. capsulatus* E1F1 (5, 17, 28). The second phase took place when the remaining ammonium concentration was lower than 1.5 mM. In this second phase, strong differences in DNP uptake were observed for the wild-type and mutant strains. As shown in Fig. 2D, DNP uptake in the wild-type strain paralleled that in the *nprA* strain (where NprB is functional), whereas the *nprB* strain (where NprA is present) consumed DNP at a lower rate. This result suggests that NprA could be negatively regulated by ammonium. Thus, if the NprA enzyme is not functional in the wild-type strain in the presence of ammonium, the phenotypes of the wild-type strain and the *nprA* mutant strain should be similar. In both strains, only the nitroreductase NprB is active, since this protein seems to not be affected by the presence of ammonium.

Characterization of in vitro nitroreductase activities of *R. capsulatus* B10. Nitroreductase activities were assayed with NADPH or NADH as the electron donor and with DNP as the substrate in crude extracts from wild-type and mutant strains grown under diazotrophic conditions with DNP. In all cases, NADH-dependent nitroreductase activities were lower than NADPH-dependent nitroreductase activities (not shown), thus indicating that *R. capsulatus* nitroreductases preferably use NADPH as the physiological electron donor. It is worth nothing that NprA and NprB nitroreductase activities can be determined in crude extracts in the presence of oxygen, which indicates that both nitroreductases belong to the oxygen-insensitive type of enzymes and that their activity was unaffected in vitro by the addition of ammonium, as previously described for *R. capsulatus* E1F1 (4). A higher level of nitroreductase activity was found in the wild-type strain, corresponding to the presence of both the NprA and NprB nitroreductases (Fig. 3A). The nitroreductase activity of the *nprB* mutant strain was lower than that found in the wild-type strain but higher than the activity showed by the *nprA* defective strain (Fig. 3A). This result indicates that NprA is more effective than NprB under diazotrophic conditions. Finally, the nitroreductase activity of the *nprA nprB* mutant strain was very low (Fig. 3A), confirming that NprA and NprB are the major 2,4-dinitrophenol nitroreductases of *R. capsulatus* B10.

When nitroreductase activities were assayed in crude extracts from cells grown with 5 mM ammonium chloride and in the presence of DNP, the nitroreductase activities were

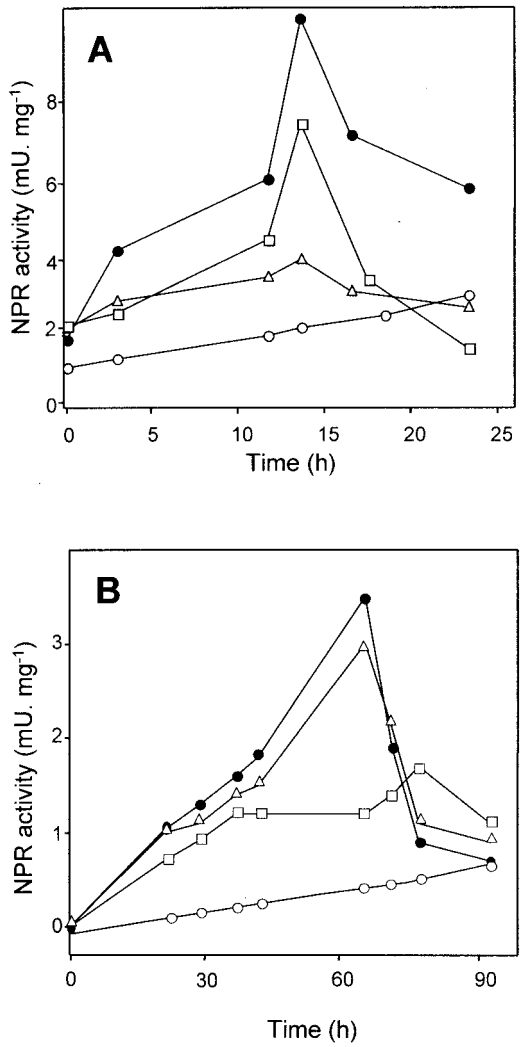

FIG. 3. Nitroreductase (NPR) activities in crude extracts from wild-type and mutant strains of *R. capsulatus* B10 grown diazotrophically (A) or with 5 mM ammonium chloride (B) in the presence of 150 μM 2,4-dinitrophenol. Samples were collected at the indicated times, and crude extracts were obtained as described in Materials and Methods. ●, wild-type strain; △, *nprA* mutant strain; □, *nprB* mutant strain; ○, *nprA nprB* mutant strain. Data correspond to a representative experiment which was repeated three times with variations of <12%.

lower than those in the absence of ammonium (Fig. 3B). In all cases, the highest nitroreductase activities were observed when the remaining ammonium concentration in the medium was in the range of 0.3 to 0.6 mM (not shown). Nitroreductase activities were similar in the wild-type and *nprA* strains, suggesting that in the presence of ammonium, NprA is inactive or absent in the wild-type strain (Fig. 3B). On the other hand, the NprA activity of the *nprB* mutant strain was very low in cells grown in the presence of ammonium. This result, together with the lack of inhibition of NprA by ammonium in vitro, suggests a negative effect of ammonium on *nprA* gene expression.

Analysis of *nprA* and *nprB* gene expression. To study the transcriptional control of and ammonium effect on the expression of the *nprA* and *nprB* nitroreductase genes, transcriptional fusions of the *nprA* or *nprB* gene with the β-galactosidase (*lacZ*) reporter gene were performed. Whereas the expression of the *nprB* gene was constitutive, the expression of the *nprA* gene was induced by a wide range of nitroaromatic and heterocyclic compounds (Table 1). The highest β-galactosidase activity was obtained for cells grown under diazotrophic conditions with 2,4-dinitrophenol, and β-galactosidase activity was not detected in the absence of the inducer (Table 1). When cells of *R. capsulatus* B10 carrying the *nprA-lacZ* fusion were grown in the presence of DNP with ammonium or glutamine, only a 30% β-galactosidase activity was observed. In contrast, other nitrogen sources, such as nitrate, did not show an effect on the expression of the *nprA* gene (not shown). The low expression of the *nprA* gene in the presence of ammonium or glutamine could be explained if these compounds act as repressors and/or if they block the transport of the inducer (DNP) inside the cell. Ammonium or glutamine probably does not affect *nprA* gene expression directly because the putative promoter region of the *nprA* gene does not show sequences typical for binding of the σ^{54} factor or the NtrC regulator, as in most Ntr-controlled promoters. More likely, high concentrations of these two compounds could inhibit DNP uptake, as previously described for *R. capsulatus* E1F1 (5, 17, 28). In agreement with this, in cultures with an initial ammonium concentration of 10 mM, β-galactosidase activity was only detected when the ammonium concentration decreased below 3 mM, showing that a sufficient amount of DNP could be transported inside the cells to induce *nprA* gene expression (not shown). In addition to DNP, expression of the *R. capsulatus nprA* gene was induced in the presence of several nitrofuran derivatives (Table 1). *E. coli* oxygen-insensitive NfsA and NfsB nitroreductase activities are responsible for most of the nitrofuran reduction activity under aerobic conditions (27). The *nprA* gene was also induced by the NfsB substrate 5-[azaridin-

TABLE 1. β-Galactosidase activities of a transcriptional *nprA-lacZ* gene fusion in *R. capsulatus* B10[a]

Compound	β-Galactosidase activity (Miller units)[b]	
	0.1 mM	0.2 mM
None	1	1
Nitrofurazone	10	30
Nitrofurantoine	18	ND
Furazolidone	9	48
CB1954	21	75
2-Aminofluorene	19	116
Benzo[*a*]pyrene	18	25
2,4-Dinitrotoluene	54	90
3,5-Dinitrobenzoate	52	62
2,4-Dinitrophenol	105	144
Salicylate	30	33
Paraquat	ND	25

[a] Cells were harvested at an A_{680} of about 0.5, and β-galactosidase activity was assayed as described in Materials and Methods. Cells were cultured under diazotrophic conditions with 30 mM malate as the carbon source and in the presence of different compounds at a final concentration of 0.1 mM or 0.2 mM. The compounds used were nitrofuran derivatives that are substrates of NfsA and NfsB of *E. coli* (27); the prodrug CB1954, which is a substrate of NfsB of *E. coli* (7); 2-aminofluorene and benzo[a]pyrene, substrates of SnrA of *Salmonella enterica* (13); several dinitroaromatics, substrates of the nitroreductase of *R. capsulatus* E1F1 (4); salicylate, a MarR repressor, as 2,4-dinitrophenol (2); and paraquat, a SoxR activator (9).

[b] ND, not determined. The β-galactosidase activity data are means of the values obtained in three independent experiments with variations of <15%.

1-yl]-2,4-dinitrobenzamide (CB1954), a prodrug used in cancer therapy. This result suggests that CB1954 could be a substrate for the *R. capsulatus* NprA nitroreductase. In addition, two substrates of the Cnr nitroreductase of *Salmonella enterica* serovar Typhimurium used in the Ames test for potential carcinogens (13), 2-aminofluorene and benzo[a]pyrene, are also inducers of the expression of the *R. capsulatus nprA* gene (Table 1). β-Galactosidase activity was not detected when mononitroaromatic compounds were tested (not shown), but expression of the *nprA* gene was induced in the presence of several dinitroaromatic compounds, as well as by salicylic acid and paraquat. Salicylic acid induced expression of the *nprA* gene to the same extent within the range of 0.1 to 3 mM. The induction of *nprA* gene expression with salicylic acid and 2,4-dinitrophenol and the presence of two putative *mar* boxes in its possible promoter region (Fig. 1) suggest that the regulatory MarRA system could be implicated in controlling *nprA* expression in response to environmental hazards, as occurs with the *nfsB* gene of *E. coli* (2).

Effect of cellular redox status on *nprA* gene expression. Expression of the *nprA* gene was induced in the presence of the superoxide-generating compound paraquat (Table 1). The oxygen-insensitive nitroreductases of *E. coli* and *Salmonella*, which are under the control of the MarRA system, are also induced in the presence of paraquat via the *soxRS* system (13, 14). However, it has been postulated that nitroreductases can regulate the accumulation of electron donors in the bacterial cytosol (9). Therefore, many oxidants could trigger induction of the *soxRS* regulon simply by draining the cellular electron source. Thus, the presence of nitroreductases in *E. coli* is not required for regulation of the *soxRS* response, although a depletion of the electron source could contribute to SoxR activation (9). To investigate the possible effect of the cellular redox status on *nprA* gene expression, cells of *R. capsulatus* B10 carrying the *nprA-lacZ* fusion were cultured with different malate concentrations or with different oxidized or reduced carbon sources. The expression of the *nprA* gene was upregulated under carbon-limiting conditions (Fig. 4A). This upregulation was more evident in cells cultured under diazotrophic conditions than in cultures grown in the presence of glutamine since this compound inhibits the transport of the inducer (DNP) inside the cells (Fig. 4A). In addition, electron-rich carbon sources, such as butyrate or caproate, downregulated *nprA* gene expression (Fig. 4B), suggesting that a role of the SoxRS system in the control of this nitroreductase could not be discarded. Thus, in the presence of highly reduced carbon sources or high carbon source concentrations, the redox status of the cells becomes more reduced through NAD(P)H accumulation, and under these conditions, SoxR could probably be reduced in its inactive form.

In conclusion, this work shows that *R. capsulatus* B10 presents two major nitroreductases involved in 2,4-dinitrophenol reduction. NprA is the main oxygen-insensitive NAD(P)H-dependent nitroreductase, whereas NprB is a second oxygen-insensitive NAD(P)H-dependent nitroreductase involved in DNP reduction. The nitroreductases behave in different manners; whereas the expression of the *nprB* gene is constitutive, *nprA* gene expression is induced by DNP and a variety of nitroaromatic and nitroheterocyclic compounds, including the prodrug CB1954 used in cancer therapy. Ammonium and glutamine have a negative effect, probably due to an inhibition of DNP uptake and consequently of *nprA* gene induction. The presence of putative *mar*/*sox* boxes in the possible promoter region of the *nprA* gene and the induction of *nprA* gene expression by 2,4-dinitrophenol and salicylic acid suggest the involvement of the two-component regulatory MarRA system in controlling *nprA* gene expression. In addition, induction of the *nprA* gene by paraquat and by an oxidized redox status in the cell suggests that the SoxRS system could also be implicated in controlling *nprA* gene expression.

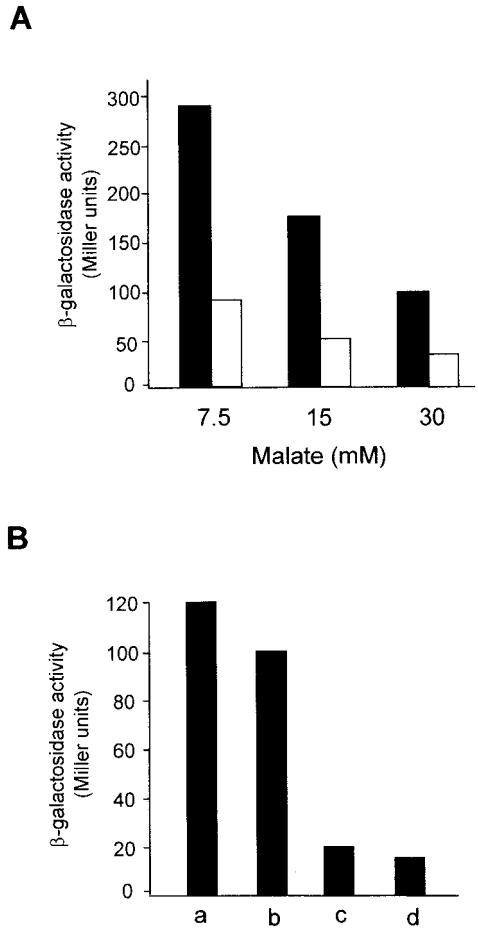

FIG. 4. Effect of carbon source on *nprA* gene expression. (A) *R. capsulatus* cells harboring the transcriptional fusion *nprA-lacZ*$^+$ were cultured with 150 μM 2,4-dinitrophenol either under diazotrophic conditions (black bars) or with 7 mM L-glutamine (white bars) in the presence of several concentrations of malate. (B) *R. capsulatus* cells harboring the transcriptional fusion *nprA-lacZ*$^+$ were cultured with 150 μM 2,4-dinitrophenol under diazotrophic conditions in the presence of the following carbon sources: a, 50 mM acetate; b, 30 mM malate; c, 10 mM butyrate; and d, 5 mM caproate. When cultures reached an absorbance at 680 nm of 0.5, cells were harvested, and the β-galactosidase activity was determined as indicated in Materials and Methods. Data are means of the values obtained in three independent experiments with variations of <15%.

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