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A Megaplasmid-Borne Anaerobic Ribonucleotide Reductase in *Alcaligenes eutrophus* H16

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The conjugative 450-kb megaplasmid pHG1 is essential for the anaerobic growth of *Alcaligenes eutrophus* H16 in the presence of nitrate as the terminal electron acceptor. We identified two megaplasmid-borne genes (*nrdD* and *nrdG*) which are indispensable under these conditions. Sequence alignment identified significant similarity of the 76.2-kDa gene product NrdD and the 30.9-kDa gene product NrdG with anaerobic class III ribonucleotide reductases and their corresponding activases. Deletion of *nrdD* and *nrdG* in *A. eutrophus* abolished anaerobic growth and led to the formation of nondividing filamentous cells, a typical feature of bacteria whose DNA synthesis is blocked. Enzyme activity of NrdD-like ribonucleotide reductases is dependent on a stable radical at a glycine residue in a conserved C-terminal motif. A mutant of *A. eutrophus* with a G650A exchange in NrdD showed the DNA-deficient phenotype as the deletion strain, suggesting that G650 forms the glycyl radical. Analysis of transcriptional and translational fusions indicate that *nrdD* and *nrdG* are cotranscribed and that the translation efficiency of *nrdD* is 40-fold higher than that of *nrdG*. Thus, the two proteins NrdD and NrdG are not synthesized at a stoichiometric level.

Reduction of ribonucleotides, mediated by ribonucleotide reductases (RNRs), is an elementary process for all living organisms which provides the four 2′-deoxyribonucleotides for DNA synthesis and repair. Three classes of RNRs are known which use similar radical mechanism for catalysis (reviewed in references 21, 39, and 40). Regulatory feedback mechanisms keep a balanced level of deoxyribonucleotide inside the cell. A major difference between the various classes of RNRs is the nature of the free radical and the way it is formed.

Class I RNRs occur in all higher organisms and certain aerobic bacteria. These enzymes contain a stable tyrosyl radical which is generated by formation of an oxygen-linked diiron center (12). This reaction is strictly dependent on the presence of molecular oxygen (28, 37). The majority of prokaryotes harbor class II RNRs which are active under both aerobic and anaerobic conditions and use adenosylcobalamin as a cofactor for radical production (27, 55). RNRs of the third class function exclusively in the absence of oxygen. These enzymes contain a stable, but oxygen-sensitive glycyl radical which is introduced by an activase (52). In *Escherichia coli*, NADPH and flavodoxin are used to reduce the [4Fe-4S] cluster of the activase, which reductively cleaves *S*-adenosylmethionine to generate the radical (1, 15, 35).

The best-characterized member of class III RNRs is the NrdD protein of *E. coli* (34, 54). Biochemical data are also available for the corresponding protein from phage T4 (61, 62). Evidence for the existence of a class III RNR has also been presented for *Lactococcus lactis* (20) and *Methanobacterium thermoautotrophicum* (17). Furthermore, genome sequences suggest the occurrence of class III RNRs in *Haemophilus influenzae* (10), *Methanococcus jannaschii* (3), and *Pyrococcus horikoshii* (22).

In this report we show that *Alcaligenes eutrophus* H16, a strictly respiratory member of the β-subgroup of proteobacteria, contains an RNR belonging to class III, which is essential for the organism during anaerobic growth with nitrate as the electron acceptor. The enzyme is dispensable in aerobically grown cells. The two genes encoding the class III RNR and its activase are located on a 450-kb megaplasmid which contains genes for denitrification (41), hydrogen metabolism (13), and autotrophic carbon dioxide fixation (19). Sequence comparison suggests that the enzyme from *A. eutrophus* is closer related to class III RNRs from archaebacterial species than to the eubacterial counterparts.

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MATERIALS AND METHODS

Strains, media, and growth conditions. The bacterial strains used here are listed in Table 1. *A. eutrophus* H16 is the wild type, harboring megaplasmid pHG1. Strain HF210 is a megaplasmid-free derivative of strain H16. Strains HF413 and HF456 were derived from the wild type by mutagenesis. *E. coli* XL1-Blue was used as a host in standard cloning procedures. *E. coli* S17-1 served as the donor in conjugative plasmid transfer. *E. coli* strains were grown in Luria-Bertani broth at 37°C. *A. eutrophus* strains were cultivated in mineral salts medium at 30°C (44) with 0.4% (wt/vol) fructose as the carbon source and 0.2% (wt/vol) ammonium chloride as the nitrogen source (FN-medium). For anaerobic growth under denitrifying conditions the cells were cultivated in 150-ml glass flasks sealed with a rubber septum and containing 100 ml of FN-medium supplemented with 0.2% (wt/vol) potassium nitrate. The gas phase consisted of dinitrogen. Solid media contained 1.5% (wt/vol) agar. Antibiotics were added as follows: for *A. eutrophus*, kanamycin (400 μg/ml) and tetracyclin (10 μg/ml), and for *E. coli*, ampicillin (50 μg/ml), kanamycin (30 μg/ml), and tetracyclin (10 μg/ml).

Plasmids. Plasmids used in this study are listed in Table 1. A 6.5-kb *Pst*I fragment from plasmid pPX41 containing *nrdDG* from *E. coli* was subcloned into pGE151, yielding plasmid pGE391. In this plasmid, *nrdD* and *nrdG* of *E. coli* are under control of the *lac* promoter, allowing a constitutive expression in *A. eutrophus* (25). Cosmid pGE26, isolated from a pHG1 DNA library, contains a 30-kb fragment of megaplasmid pHG1. A 6.2-kb *Eco*RI-*Hin*dIII fragment from pGE26 was cloned into the broad-host-range vector pVDZ′2 and into pBluescript KS(+) yielding plasmids pGE291 and pCH447, respectively (Fig. 1A). Exonuclease III treatment of *Eco*RI-*Spe*I-linearized pCH447 resulted in a set of deletion derivatives. Three *Xba*I-*Hin*dIII fragments of 5.0 kb (pCH604), 4.5 kb, and a 4.0 kb were cloned into pVDZ′2, yielding plasmids pGE305, pGE306, and pGE307, respectively (Fig. 1A). Exonuclease III treatment of *Hin*dIII-*Cla*I linearized pCH447 resulted in a second set of deletion derivatives. Four *Eco*RI-*Kpn*I fragments of 4.4, 3.2, 4.7, and 5.2 kb were first cloned into pUC18 and subsequently transferred as *Eco*RI-*Hin*dIII fragments into pVDZ′2, yielding plasmids pGE311, pGE312, pGE343, and pGE344, respectively (Fig. 1A).

Plasmid pGE384 is a derivative of the mobilizable, broad-host-range promoter assay vector pEDY305 carrying the 5′ *nrdD* spanning DNA region inserted upstream of the promoterless *lacZ* gene. This plasmid was generated by inserting a 124-bp *Ecl*136II-*Bam*HI fragment of pCH604 between the *Sca*I and *Bgl*II sites

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
A. eutrophus strains		
H16	Wild type, pHG1	DSM 428, ATCC 17699[c]
HF210	pHG1-free derivative	24
HF413	Δ*nrdDG*	This study
HF456	NrdD G650A	This study
E. coli strains		
S17-1	*pro thi recA hsdS* RP4 *tra* functions	48
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10*)	Stratagene
Plasmids		
pVDZ′2	Tc^r *lacZ′ mob*	6
pBluescript KS(+)	Ap^r, *lacZ′* f1*ori* T7 and T3 promoter	Stratagene
pUC18	Ap^r, *lacPOZ′*	60
pEDY305	Tc^r, *lacZ* RP4 *oriT*	46
pPHU234/pPHU235	Tc^r, *lacZ′*	18
pLO1/pLO2	Km^r, *sacB* RP4 *oriT* ColE1 *ori*	26
pPX41	Ap^r, *E. coli nrdDG*	53
pGE26	*nosZ*, *norB*, *nrdDG*	63
pGE151	Tc^r, *lacZ′* RP4 *oriT*	25
pGE291	6.2-kb *Eco*RI-*Hin*dIII fragment of pGE26 in pVDZ′2 (*nrdDG*)	This study
pGE305	5.0-kb *Xba*I-*Hin*dIII fragment[b] in pVDZ′2	This study
pGE306	4.5-kb *Xba*I-*Hin*dIII fragment[b] in pVDZ′2	This study
pGE307	4.0-kb *Xba*I-*Hin*dIII fragment[b] in pVDZ′2	This study
pGE311	4.4-kb *Eco*RI-*Bam*HI fragment[b] in pVDZ′2	This study
pGE312	3.2-kb *Eco*RI-*Bam*HI fragment[b] in pVDZ′2	This study
pGE343	4.7-kb *Eco*RI-*Bam*HI fragment[b] in pVDZ′2	This study
pGE344	5.2-kb *Eco*RI-*Bam*HI fragment[b] in pVDZ′2	This study
pGE384	124-bp *Ecl*136II-*Bam*HI fragment of pCH604 in pEDY305 (*nrdDP-lacZ*)	This study
pGE385	2.65-kb *Ecl*136II-*Sma*I fragment of pCH604 in pEDY305 ($nrdG_P$-*lacZ*)	This study
pGE386	460-bp *Xba*I-*Hin*cII fragment of pCH604 in pPHU234 [Φ(*nrdD-lacZ*)]	This study
pGE387	2.6-kb *Xba*I-*Sma*I fragment of pCH604 in pPHU235 [Φ(*nrdG-lacZ*)]	This study
pGE388	Derivative of pGE387 containing a 1.7-kb *Xho*I deletion [Φ(Δ*nrdG-lacZ*)]	This study
pGE391	6.5-kb *Pst*I fragment of pPX41 in pGE151 (*E. coli nrdDG*)	This study
pCH447	6.2-kb *Eco*RI-*Hin*dIII fragment of pGE26 in pBluescript KS(+) (*nrdDG*)	This study
pCH604	5.0-kb *Xba*I-*Hin*dIII fragment[b] of pCH447 pBluescript KS(+) (*nrdDG*)	This study
pCH605	3.05-kb *Xba*I-*Xho*I fragment in pLO1 (3.2-kb deletion of *nrdDG*)	This study
pCH606	608-bp *Xho*I-*Msc*I fragment in pLO2 (NrdD G650A)	This study

[a] Ap, ampicillin; Km, kanamycin; Tc, tetracycline; *nrdD*, anaerobic RNR; *nrdG*, activase of NrdD.
[b] Obtained by exonuclease III deletion from pCH447.
[c] DSM, Deutsche Sammlung für Mikroorganismen; ATCC, American Type Culture Collection.

of the pEDY305 polylinker (Fig. 1B). The corresponding plasmid for the *nrdG* promoter region, pGE385, contained a 2.65-kb *Ecl*136II-*Sma*I fragment of pCH604 in the *Sca*I site of the pEDY305 polylinker. Plasmid pGE386 is a derivative of pPHU234 which carries a 460-bp *Xba*I-*Hin*cII fragment of pCH604 inserted between the *Xba*I and *Sca*I sites of pPHU234, resulting in a translational fusion of *nrdD* and *lacZ* (Fig. 1B). A corresponding *nrdG* translational fusion (pGE387) was constructed by insertion of a 2.6-kb *Xba*I-*Sma*I fragment of pCH604 into the *Xba*I and *Sca*I sites of pPHU235. Plasmids pPHU234 and pPHU235 are conjugative broad-host-range vectors with different translational phasing of the polylinker upstream of the *lacZ* gene (18). pGE388 is a derivative of pGE387 carrying a 1.7-kb *Xho*I deletion which removed the 5′ *nrdD* untranslated region and most of the *nrdD* gene. The transcriptional and translational fusions were verified by restriction analysis.

A deletion was introduced into *nrdDG* by digestion of a 6.2-kb *Eco*RI-*Hin*dIII fragment of pCH447 with *Bam*HI (Fig. 1A). The religated fragment was cloned into pBluescript KS(+) and subsequently transferred into pLO1 by digestion with *Xba*I and *Xho*I (pCH605). The G650A exchange in NrdD (Fig. 1A) was obtained by overlap-extension PCR mutagenesis (16) by using pCH604 as the template and the following four primers (mutation sites are underlined): PXHO (5′-CAACCTCGAGGCTACCCCAG-3′), PMSC (5′-CGCTGCATGGCCAGG CAAGG-3′), G650A1 (5′-GCCGGCGAGGTAGTCATGTG-3′), and G650A2 (5′-CCCACACA TGACTACCTCGC-3′) (Fig. 2). The resulting 608-bp *Xho*I-*Msc*I fragment was transferred into the *Sal*I-*Ecl*136II-linearized vector pLO2 to give pCH606. The suicide vectors pLO1 and pLO2 contain the conditionally lethal *sacB* gene from *Bacillus subtilis*, which allows selection of *A. eutrophus* mutants generated by allelic exchange as described previously (26). Mutations were checked by DNA sequencing.

DNA techniques. Standard DNA techniques were used (42). Plasmid DNA was isolated with tip-20 columns (Qiagen) according to the manufacturer's instructions. DNA sequencing was done with an automated DNA sequencer (LI-COR) by using a cycle sequencing kit (Amersham) and fluorescent primers (MWG-Biotech).

Enzyme assays. Anaerobic ribonucleotide reductase activity was determined by using the assay which had been introduced for the anaerobic ribonucleotide reductase of *E. coli* (34). Soluble extracts from anaerobically grown *A. eutrophus* cells were prepared as described previously (59) by sonication under an atmosphere of argon. Protein was determined according to the method of Lowry et al. (30). All components were degassed for 45 min prior to use. The assay mixture contained 1 to 1.5 mg of protein in 50 mM Tris-HCl (pH 7.5), 30 mM KCl, 5 mM dithiothreitol, 1 mM NADPH, 20 μM 5′-deazaflavine, and 0.5 mM *S*-adenosylmethionine. The total volume was 100 μl. The reaction mixture was preincubated for 60 min under illumination. The reductase reaction was started by the addition of 5 mM $MgCl_2$, 5 mM sodium formate, and 1 mM [^{3}H]CTP. After 20 min, the reaction was stopped by the addition of 0.5 ml of $HClO_4$, and the amount of dCTP formed was determined (8). One unit of enzyme activity is expressed as the formation of 1 nmol of dCTP per min. β-Galactosidase was assayed according to the method of Miller (33), except that the optical cell density was measured at 436 nm.

Nucleotide sequence. The nucleotide sequences for the *nrdD* and *nrdG* genes have been deposited in the EMBL database under accession number AJ012479.

RESULTS

Cloning and sequence analysis of a gene locus essential for anaerobic growth of *A. eutrophus*. Curing of megaplasmid pHG1 of *A. eutrophus* H16 led to the loss of anaerobic growth

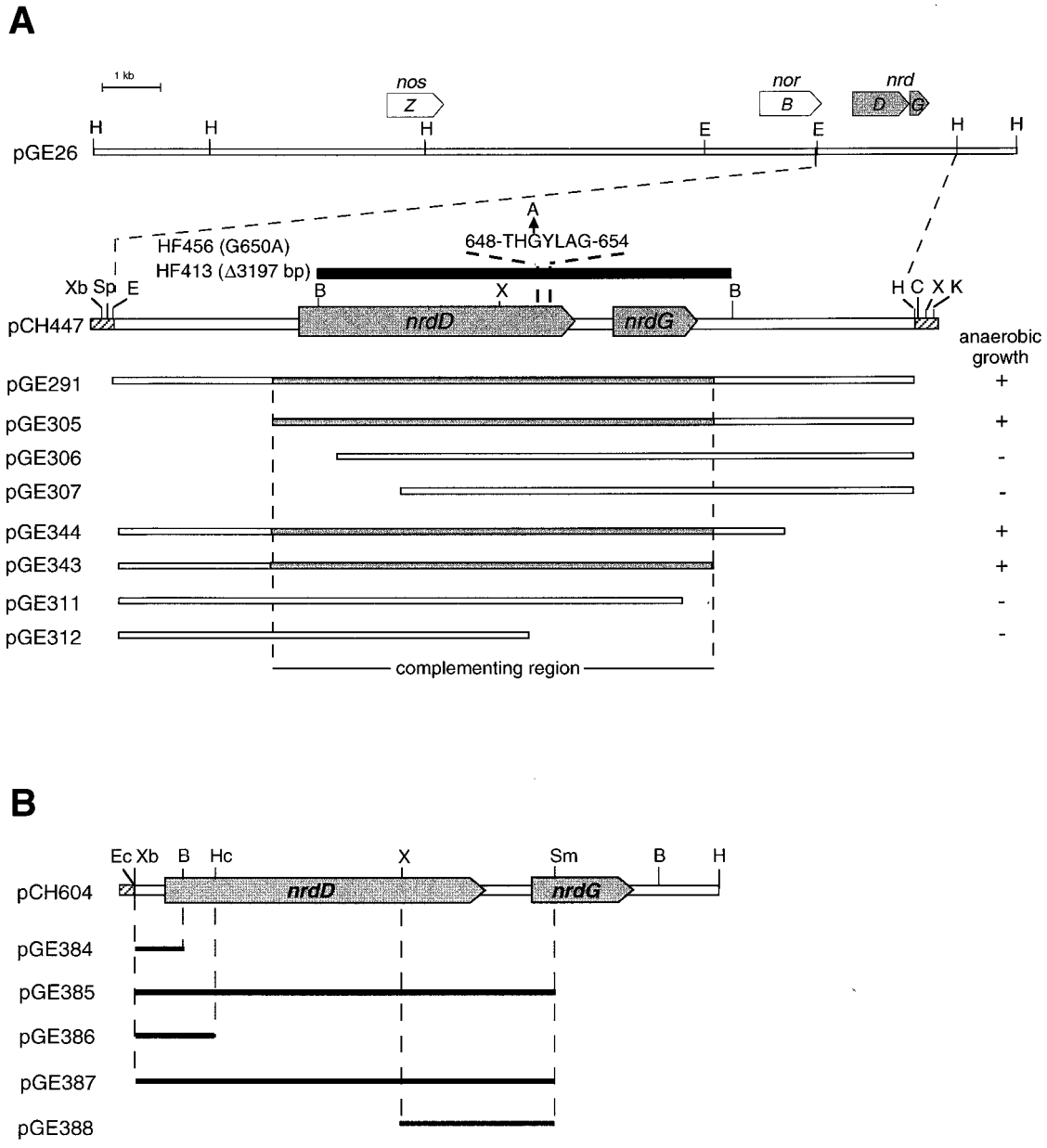

FIG. 1. Subclones of the *nrdDG* region. (A) Three of seven subclones of pGE291, generated by bidirectional deletion, restored anaerobic growth (+) of a megaplasmid-free derivative of *A. eutrophus*. Cloned DNA fragments are indicated by open bars. Highlighted bars depict the region essential for complementation. Identified genes are marked by open arrows: *nosZ*, nitrous oxide reductase; *norB*, megaplasmid-encoded copy of nitric oxide reductase; *nrdD*, anaerobic RNR; *nrdG*, activase. The deleted DNA fragment in HF413 is indicated as solid bar. The G650A exchange in mutant HF456 is marked by an arrow. Relevant restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Sp, *Spe*I; Xb, *Xba*I; X, *Xho*I. (B) DNA fragments used for the construction of transcriptional or translational fusions with *lacZ* as the reporter gene are shown as solid bars. Genes are depicted by open arrows on the restriction map. Relevant restriction sites: B, *Bam*HI; Ec, *Ecl*136II; Hc, *Hin*cII; H, *Hin*dIII; Sm, *Sma*I; Xb, *Xba*I; X, *Xho*I.

ability with nitrate as the terminal electron acceptor (41). This was unexpected since essential enzymes for denitrification, such as the reductases for nitrate, nitrite, and nitric oxide, are encoded on the chromosome of this organism (5, 38, 59). A second functionally equivalent copy of a nitric oxide reductase gene (*norB* [5]) has previously been identified to be closely linked to the gene for nitrous oxide reductase (*nosZ* [63]) on a megaplasmid-borne DNA insert cloned in cosmid pGE26. Subcloning of the 30-kb DNA fragment revealed that the loss of the two denitrification-specific genes did not account for the failure of megaplasmid-free derivatives to grow anaerobically (Fig. 1). However, a third locus (*nrd*), clearly distinct from *nosZ* and *norB*, proved to be essential for anaerobic growth.

***nrdD* and *nrdG* encode an anaerobic ribonucleotide reductase and its activase.** Nucleotide sequence analysis of the complementing DNA segment revealed two open reading frames *nrdD* and *nrdG* within a region of 3.3 kb (Fig. 2). *nrdD* predicts a protein of 676 amino acids (76.2 kDa) and is separated from *nrdG* by 271 bp. *nrdG* has the coding capacity for a protein of 256 amino acids (30.9 kDa). A potential hairpin-like structure

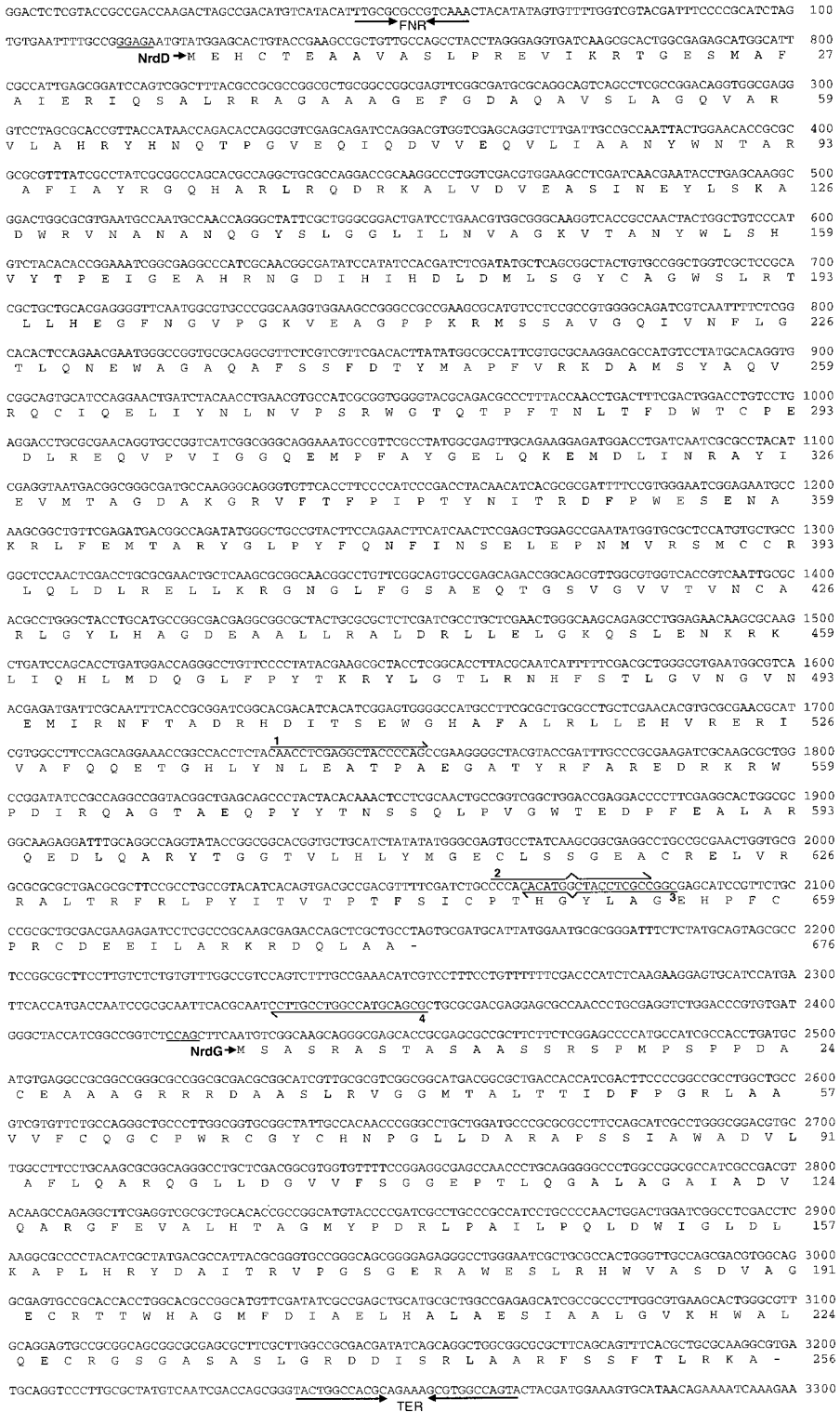

FIG. 2. Nucleotide and derived amino acid sequences of the complementing region. The deduced amino acid sequences of NrdD and NrdG are shown below the nucleotide sequence. A potential Fnr-binding site (FNR) and a possible hairpin structure (TER) are emphasized by inverted arrows. Potential ribosome binding sites are underlined. Primers used for the construction of the G650A mutant are depicted above and below the sequence: 1, PXHO; 2, G650A2; 3, G650A1; 4, PMSC.

($\Delta G_0' = -123.9$ kJ) at bp positions 3236 to 3266 (Fig. 2) points to a transcription termination signal immediately downstream of *nrdG*. The predicted gene product of *nrdD* shows homology to class III ribonucleotide reductases (Fig. 3), with overall identities of 19% (phage T4 [56]), 23% (*E. coli* [53]), 24% (*H. influenzae* [10]), 25% (*M. jannaschii* [3], *M. thermoautotrophicum* [50]), and 27% (*P. horikoshii* [22]). An intein present in NrdD of *M. jannaschii* (36) has been removed from the se-

FIG. 3. Alignment of NrdD from *A. eutrophus* with class III RNRs. The NrdD sequences are from *A. eutrophus* (A_EUT), *M. jannaschii* (M_JAN; National Center for Biotechnology Information [NCB] accession number 1591520), *M. thermoautotrophicum* (M_THE; NCB accession number 2622659), *E. coli* (E_COL; NCB accession number 1790686), *H. influenzae* (H_INF; NCB accession number 1573024), *P. horikoshii* (P_HOR; NCB accession number 3130259), and phage T4 (PH_T4; NCB accession number P07071). Residues conserved in all sequences are marked by asterisks. A consensus motif is underlined. Three conserved cysteine residues are boxed. The potential radical sites are indicated in boldface.

FIG. 4. Alignment of NrdG from *A. eutrophus* with class III RNR activase proteins. The NrdG sequences are from *A. eutrophus* (A_EUT), *M. thermoautotrophicum* (M_THE; NCB accession number 2621339), *M. jannaschii* (M_JAN; NCB accession number 2826326), *P. horikoshii* (P_HOR; NCB accession number 3130260); *E. coli* (E_COL; NCB accession number 1790685), *H. influenzae* (H_INF; NCB accession number 1574712), and phage T4 (PH_T4; NCB accession number P07075). Amino acids conserved in all sequences are marked by asterisks. Cysteine residues which may participate in coordination of an Fe-S-cluster are boxed.

quence to promote alignment. It is interesting to note that the archaebacterial NrdD proteins show the highest similarity to NrdD from *A. eutrophus*. Sequence comparison revealed that three cysteine residues (C186, C392, and C646) are conserved in all NrdD-like proteins available in the database. The highly conserved glycine G650 (marked in boldface in Fig. 3), which corresponds to G681 in the *E. coli* sequence, is the most likely candidate for carrying the stable glycyl radical in the RNR of *A. eutrophus*. An adjacently positioned tyrosine residue was identified in all NrdD proteins. Furthermore, the alignment uncovered a consensus motif AHxxGxIxxH (underlined in Fig. 3).

Sequence comparison of the *A. eutrophus nrdG* showed typical features of class III RNR-associated activases (Fig. 4). The highest identity (26%) was found to NrdG of *M. thermoautotrophicum* (50). A cysteine motif CxxxCxxC, present in all NrdG proteins accessible so far, may participate in the coordination of a [4Fe-4S] cluster. In *E. coli*, a [4Fe-4S] cluster bridges the two NrdG subunits in the homodimer (34, 35). It is interesting to note that the NrdG homolog of *M. jannaschii* has been annotated as a pyruvate formate lyase-specific activase, albeit genome sequence analysis of this archaeon lacks a pyruvate formate lyase but does predict the existence of a class III RNR (3). Hence, we have added this protein to the list of RNR-specific activases (Fig. 4).

RNR mutants. Deletion of a 3.3-kb DNA segment from the *nrd* locus of *A. eutrophus* yielded mutant HF413 (Fig. 1A). HF413 was unimpaired in aerobic growth (data not shown), but under anoxic conditions the cell density increased only slightly (Fig. 5A), and the formation of long cell filaments was observed (Fig. 5B). This morphological change is indicative for inhibited cell division caused by depletion of deoxyribonucleotides under anaerobiosis. Normal growth and cell morphology of HF413 resumed upon introduction of plasmid pGE291, which harbors the *nrdDG* genes of *A. eutrophus* (Fig. 5A). Heterologous complementation of HF413 with the *nrdDG* genes of *E. coli* on plasmid pGE391 was not successful (data not shown). A second NrdD deficient mutant was constructed by replacing the conserved G650 with an Ala residue by using site-directed mutagenesis (Fig. 1A). The resulting mutant HF456 behaved exactly like the deletion strain (Fig. 5A), thus supporting the notion that G650 is essential for the function of NrdD in *A. eutrophus*.

RNR activity. The results of this study point to the existence of two separate RNRs in *A. eutrophus*, one instrumental under anaerobic conditions and a second essential for aerobic growth. Attempts to determine anaerobic RNR activity in crude extracts from anaerobically grown cells of the wild-type H16 by using the protocol designed for *E. coli* (34) yielded an enzymatic activity of 0.01 U per min per mg of protein. This corresponds to 10% of the activity determined in anaerobic extracts of *E. coli* (14). Replacement of the argon atmosphere by air, however, resulted in a significant increase of RNR activity up to 0.8 U per min per mg of protein. This result reflects high level of class I RNR in anaerobically cultivated cells of *A. eutrophus* and differs from the behavior of *E. coli*, which contains only traces of class I RNR during anaerobic growth (4, 14). This interfering activity does not permit a

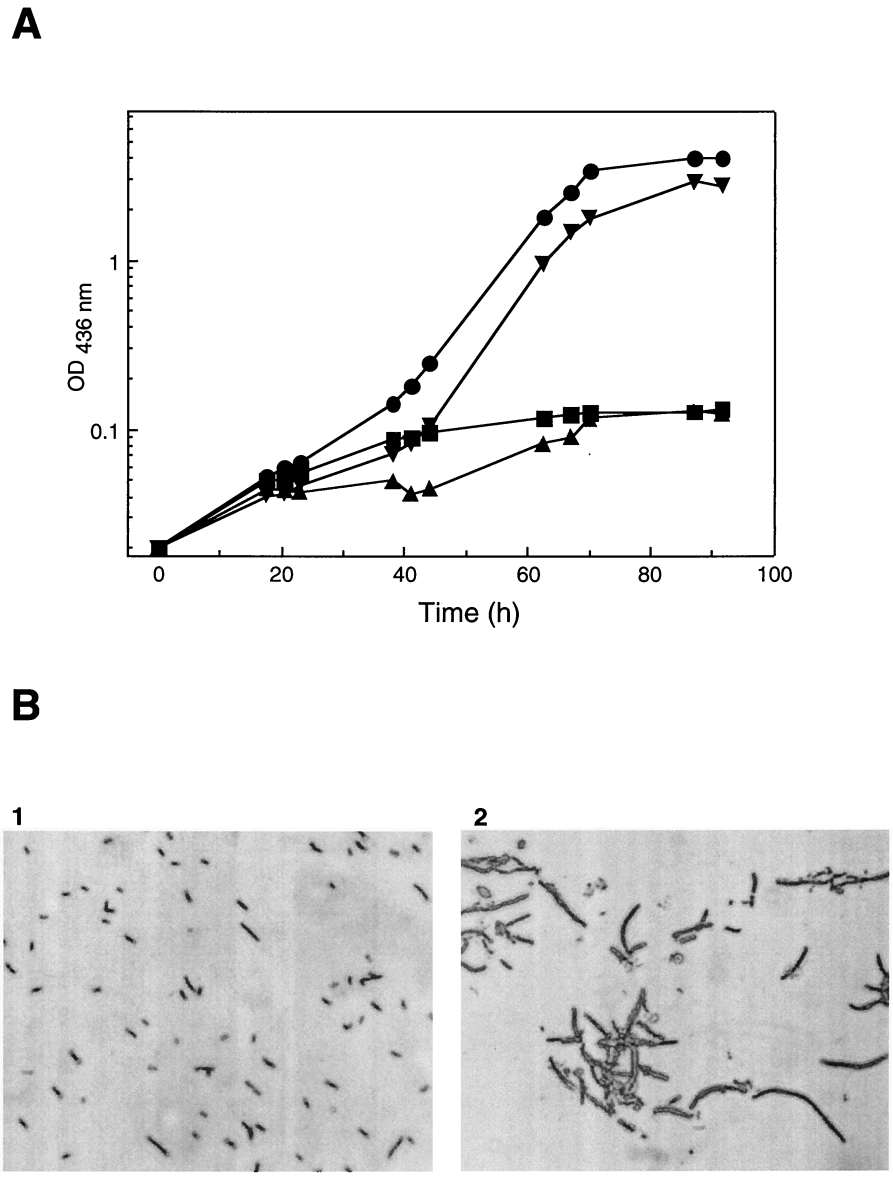

FIG. 5. Phenotype and cell-morphology of RNR mutants. (A) Strains were grown anaerobically in FN-medium supplemented with 0.2% sodium nitrate. Results for *A. eutrophus* H16 (●), *nrdDG* deletion mutant HF413 (▲), complemented mutant HF413(pGE291) (▼), and NrdD G650A exchange mutant HF456 (■) are as indicated. (B) Samples were taken from anoxic cultures after 70 h and examined by light microscopy. Panels: 1, wild-type *A. eutrophus* H16; 2, *nrdD-nrdG* deletion mutant HF413.

reliable assay for class III RNR in crude extracts of *A. eutrophus*.

More evidence for the existence of class I RNR in *A. eutrophus* was obtained by the application of an inhibitor. The addition of 5 mM hydroxyurea to aerobically growing cells led to an increase of the doubling time from 2 to 7 h in both the wild type and the NrdD deficient mutant HF413 (Fig. 6). Hydroxyurea is an efficient radical scavenger and a well-known inhibitor, particularly of class I RNRs (7, 11). The sensitive response of *A. eutrophus* supports the notion that the organism contains a class I RNR in addition to the class III enzyme.

***nrdD* and *nrdG* of *A. eutrophus* form an operon.** A sequence motif 5′-TTGCG N4 GTCAA-3′ was identified 79 bp upstream of the *nrdD*-translational start (Fig. 2) which resembles the binding site of the anaerobic transcriptional activator Fnr from *E. coli* (51). Transcription and translation of *nrdD* and *nrdG* were studied with the aid of reporter gene fusions (Fig. 1B) cloned on a broad-host-range plasmid. The recombinant plasmids were introduced into *A. eutrophus* H16 by conjugation. The level of transcription and translation was monitored by β-galactosidase activity (Table 2). We observed that, under oxic conditions, there was no transcription of *nrdD* and *nrdG*. In the absence of oxygen, β-galactosidase activities of the *nrdD*-transcriptional fusions (pGE384) and the *nrdG*-transcriptional fusions (pGE385) were almost identical, which suggests that *nrdD* and *nrdG* are cotranscribed from a common promoter located upstream of *nrdD*. This assumption is supported by the result obtained with the translational fusion in pGE388 (Table 2), which showed no β-galactosidase activity due to the absence of the *nrdD* promoter region (Fig. 1B). Substantially diverging levels of translation were observed when we compared the activities obtained with pGE386 and

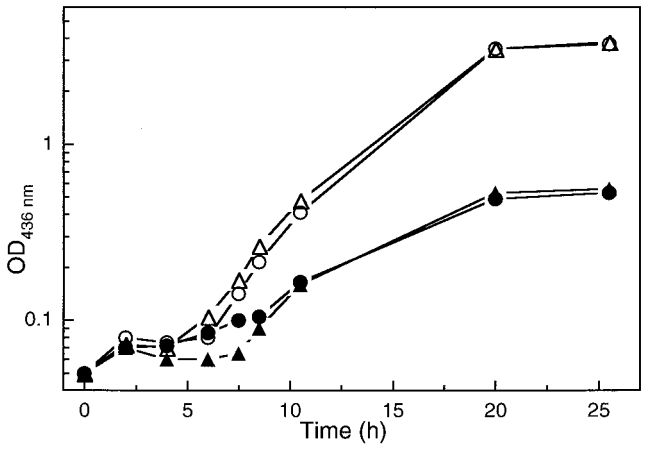

FIG. 6. Aerobic growth of *A. eutrophus* in the presence of hydroxyurea. The wild type (○ and ●) and the *nrdDG* deletion mutant HF413 (△ and ▲) were grown aerobically in FN-medium. Solid symbols indicate growth in the presence of 5 mM hydroxyurea.

pGE387. The expression of the reductase NrdD is 40-fold higher than the expression of the activase NrdG. This result agrees with the observation that the putative ribosome binding site upstream of *nrdD* matches more closely the *E. coli* consensus ribosome binding site than the putative ribosome binding site upstream of *nrdG* (Fig. 2).

DISCUSSION

A. eutrophus H16 harbors a 450-kb megaplasmid pHG1 which carries genetic determinants for the expression of two alternative metabolic pathways: energy generation from the oxidation of molecular hydrogen (13) and anaerobic respiration via denitrification (41). In contrast to hydrogen oxidation, which is encoded entirely on pHG1, genes for denitrification are dispersed on the chromosome and on the megaplasmid of *A. eutrophus*. A megaplasmid-free derivative of the wild type fails to denitrify and forms long filamentous cells. In this report we have shown that this phenotype is due to the absence of the megaplasmid-borne genes *nrdD* and *nrdG*, which encode an anaerobic class III RNR and its corresponding activase. This is the first example of a plasmid-encoded RNR. Introduction of the two genes into a megaplasmid-free recipient restored anaerobic growth and hence denitrification of the cells. It was shown before that transfer of the megaplasmid to taxonomically related bacteria lacking hydrogen oxidation and denitrification capacities yield transconjugants which have gained these metabolic activities (47). In fact, we could now demonstrate that transfer of *nrdD* and *nrdG* into the nondenitrifying strain *Alcaligenes hydrogenophilus* restores anaerobic growth on nitrate (47). This result shows that this host is missing housekeeping functions for anaerobic growth but harbors genes required for denitrification.

Aerobic growth of *A. eutrophus* was very sensitive to hydroxyurea, indicating that an oxygen-dependent class I RNR is instrumental during the aerobic growth of *A. eutrophus*. This enzyme appears to be also formed during anaerobic growth, which strongly interferes with the assay for anaerobic class III RNR activity in crude extracts. Thus, purification of the class III RNR is necessary before reliable statements concerning the enzymatic properties can be made. This result contrasts the situation in *E. coli*, which contains only residual amounts of the class I RNR in extracts from anaerobically cultivated cells (7, 11). Moreover, the assay employed in this study has been specifically designed for the class III enzyme of *E. coli* and may not meet special requirements of the corresponding enzyme from *A. eutrophus*. In particular, it is unknown whether NrdDG of *A. eutrophus* depends also on formate as the electron donor.

A high degree of similarity between NrdD from *E. coli*, *H. influenzae*, and phage T4 made it difficult to identify particular residues of potential structural or functional relevance in class III RNRs. A specific role was assigned to G681 of *E. coli* NrdD and G580 of phage T7 NrdD which carry the stable radical (54, 35). A glycine residue is conserved at the C terminus of all NrdD proteins described so far. Mutational exchange of the corresponding G650 to alanine in NrdD of *A. eutrophus* abolished anaerobic growth of the mutant strain. We therefore conclude that G650 is the site of the radical in NrdD of *A. eutrophus*. Three cysteine residues are conserved at similar positions in all NrdD sequences available so far. These residues may play a role in substrate reduction, as reported for a similar set of cysteines in class I and class II RNRs (2, 31, 49, 58). This assumption is confirmed by the recently published crystal structure of the phage T4 NrdD (29). Cysteine residues C286 and C392 of the *A. eutrophus* NrdD correlate with cysteine residues C79 and C290 in NrdD of phage T4, which reside in the active site of the enzyme (29). A common reaction mechanism, which involves three cysteine residues, has been proposed for all classes of RNRs (9). However, residue N311 in NrdD of phage T4 has been found to reside in place of the third conserved cysteine of class I RNRs (29). N311 is also conserved in all class III RNRs, including NrdD of *A. eutrophus*. In view of the complex reaction and allosteric regulation of class III RNRs, it seems surprising that only a few additional residues are conserved in the NrdD proteins. Particularly worth mentioning are two elements: AHxxGxIxxH and a tyrosine residue adjacent to the postulated radical site. The former motif seems to be involved in binding the phosphate of the substrate (29). Interestingly, two conserved CxxC motifs (residues 543 to 546 and residues 561 to 564 in NrdD of phage T4) are missing in NrdD of *A. eutrophus*. These residues are supposed to be involved in radical generation in NrdD of phage T4 (29).

Comparison of NrdG sequences revealed the presence of a conserved CxxxCxxC motif which may bridge two NrdG monomers via an Fe-S cluster, as has been shown for NrdG of *E. coli* (34, 52). Moreover, a pair of glycines is located at a defined distance to the cysteine cluster within the primary NrdG sequences. Both motifs are also present in pyruvate formate lyase (Pfl) activases and in the PqqE, NifB, and MoaA proteins (57). No specific physiological function has been assigned to PqqE, NifB, and MoaA, which are all involved in cofactor synthesis (32).

TABLE 2. Expression of *nrdD* and *nrdG*

Plasmid	*lacZ* fusion	β-Galactosidase (U)[a]	
		Aerobic	Anaerobic
pGE384	*nrdD*p-*lacZ*	29	4,156
pGE385	*nrdDG*p-*lacZ*	17	4,628
pGE386	Φ(*nrdD-lacZ*)	0	5,986
pGE387	Φ(*nrdG-lacZ*)	0	157
pGE388	Φ(Δ*nrdD-nrdG-lacZ*)	0	0

[a] The activity of β-galactosidase is expressed in arbitrary units. The data represent the means of two independent experiments. Activities were assayed from transconjugant wild-type cells grown to optical densities (measured at 436 nm) of 1.0 to 1.2.

In vivo assays of promoter activity revealed that *nrdD* and *nrdG* are cotranscribed from a promoter upstream of *nrdD*, suggesting an arrangement in an operon. An operon-like structure was also proposed for the *nrdD* and *nrdG* genes of *E. coli* (52). Both gene products assemble into a heterotetramer at $\alpha_2\beta_2$ stoichiometry, which resembles the composition of class I RNRs (34). Translational fusions with *nrdD* and *nrdG* of *A. eutrophus* showed that the expression of NrdD is 40-fold higher than the expression of NrdG. This result suggests that in this organism the two proteins are expressed in nonstoichiometric ratios. Since the glycyl radical is recycled after substrate reduction, a permanently formed reductase-activase complex is not necessarily required for catalysis. This view is supported by the fact that pyruvate formate lyase of *E. coli* is expressed to a significantly higher extent than its activase (43). Both types of activases use a [4Fe-4S] cluster to derive a 5′-deoxyadenosyl-radical from *S*-adenosylmethionine for the activation of their target proteins (23, 35). It is interesting that NrdG of *A. eutrophus* shows a higher degree of similarity to the pyruvate formate lyase-related activase than to NrdG of *E. coli* (data not shown), thus supporting the view of a common, highly related class of proteins which act as a functional module in combination with various enzyme systems.

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