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

ANJA SIEDOW, RAINER CRAMM, ROMAN A. SIDDIQUI, AND BÄRBEL FRIEDRICH*

Institut für Biologie der Humboldt-Universität zu Berlin, D-10115 Berlin, Germany

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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.

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 *PstI* 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 pBlue-script 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 *Xba1-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-*KpnI* fragments of 4.4, 3.2, 4.7, and 5.2 kb were first cloned into pUCI8 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

^{*} Corresponding author. Mailing address: Institut für Biologie/Mikrobiologie, Humboldt-Universität zu Berlin, Chausseestr. 117, D-10115 Berlin, Germany. Phone: 49-30-20938100. Fax: 49-30-20938102. E-mail: baerbel.friedrich@rz.hu-berlin.de.

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pUC18 $Ap^{f}, lacPOZ'$ 60pEDY305Tc', lacZ RP4 orT46pPHU234/pPHU235Tc', lacZ'18pL01/pLO2Km', sacB RP4 orT ColE1 ori26pPX41 $Ap', E. coli nrdDG$ 53pGE26nosZ, norB, nrdDG63pGE151Tc', lacZ' RP4 orT25pGE291 6.2 -kb $EcoRI-HindIII fragment' in pVDZ'2 (nrdDG)This studypGE3055.0-kb Xba1-HindIII fragment' in pVDZ'2This studypGE3064.5-kb Xba1-HindIII fragment' in pVDZ'2This studypGE3114.4-kb EcoRI-BamHI fragment' in pVDZ'2This studypGE3434.7-kb EcoRI-BamHI fragment' in pVDZ'2This studypGE3445.2-kb EcoRI-BamHI fragment' in pVDZ'2This studypGE3445.2-kb EcoRI-BamHI fragment' in pVDZ'2This studypGE3852.65-kb Ecl136II-BamHI fragment of pCH604 in pEDY305 (nrdDP-lacZ)This studypGE384124-bp Ecl136II-BamHI fragment of pCH604 in pEDY305 (nrdDP-lacZ)This studypGE3852.65-kb Ecl36II-SmaI fragment of pCH604 in pPHU234 [Φ(nrdD-lacZ)]This studypGE386460-by XbaI-HincII fragment of pCH604 in pPHU235 [Φ(nrdG-lacZ)]This studypGE3872.6-kb PxI fragment of pCH604 in pPHU235 [Φ(nrdG-lacZ)]This studypGE388Derivative of pGE387 containing a 1.7-kb XhoI deletion [Φ(ADR-lacZ)]This studypGE3816.5-kb PxI fragment of pCH604 in pPHU235 [Φ(nrdG-lacZ)]This studypGE3816.5-kb PxI fragment of pCH604 in pPHU235 [Φ(nrdG-lacZ)]This study$	pBluescript KS(+)	Ap ^r , <i>lacZ'</i> flori T7 and T3 promoter	Stratagene
pEDY305 Tc^2 , $lacZ RP4 oriT$ 46pPHU234/pPHU235 Tc^2 , $lacZ'$ 18pLO1/pLO2Km ² , $sacB RP4 oriT ColE1 ori$ 26pPX41Ap^2, E. coli nrdDG53pGE26nosZ, norB, nrdDG63pGE151 Tc^2 , $lacZ' RP4 oriT$ 25pGE291 6.2 -kb EcoRI-HindIII fragment of pGE26 in pVDZ'2 (nrdDG)This studypGE305 5.0 -kb Xba1-HindIII fragment ^b in pVDZ'2This studypGE306 4.5 -kb Xba1-HindIII fragment ^b in pVDZ'2This studypGE311 4.4 -kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE312 3.2 -kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE343 4.7 -kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE344 5.2 -kb EcoRI-BamHI fragment of pCH604 in pEDY305 (nrdDP-lacZ)This studypGE385 2.65 -kb Ecl136II-SmaI fragment of pCH604 in pEDY305 (nrdGp-lacZ)This studypGE386460-bp XbaI-HincII fragment of pCH604 in pPHU234 [Φ(nrdD-lacZ)]This studypGE387 2.6 -kb XbaI-SmaI fragment of pCH604 in pPHU235 [Φ(nrdG-lacZ)]This studypGE388Derivative of pGE387 containing a 1.7 -kb XhoI deletion $[Φ(\DeltaDrdG-lacZ)]$ This studypGE391 6.5 -kb PxI fragment of pPCH261 in dEl51 (E. coli nrdDG)This studypGE391 6.5 -kb PxI fragment of pCH604 in pBluescript KS(+) (nrdDG)This studypGE391 6.5 -kb PxI fragment of pCH261 in Bluescript KS(+) (nrdDG)This studypGE391 6.5 -kb PxI fragment of pCH261 in Bluescript KS(+) (nrdDG)This studypGE391 <td>pUC18</td> <td>Ap^r, <i>lacPOZ</i>'</td> <td>60</td>	pUC18	Ap ^r , <i>lacPOZ</i> '	60
pPHU234/pPHU235Tc', $lacZ'$ 18pLO1/pLO2Km', sacB RP4 oriT ColE1 ori26pPX41Ap', E. coli nrdDG53gGE26nosZ, norB, nrdDG63pGE151Tc', $lacZ'$ RP4 oriT25pGE2916.2-kb EcoRI-HindIII fragment of pGE26 in pVDZ'2 (nrdDG)This studypGE3055.0-kb Xbal-HindIII fragment ^b in pVDZ'2This studypGE3064.5-kb Xbal-HindIII fragment ^b in pVDZ'2This studypGE3114.4 kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3434.7-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3434.7-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3445.2-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3852.6-5-kb Ecl13GII-BamHI fragment ^b in pVDZ'2This studypGE386460-bp Xbal-HincIII fragment of pCH604 in pEDY305 (nrdDP-lacZ)This studypGE3872.6-kb Xbal-HincII fragment of pCH604 in pEDY305 (nrdDP-lacZ)This studypGE388Derivative of pGE387 containing a 1.7-kb XhoI deletion [$\Phi(\Delta DnrdG-lacZ)$]This studypGE388Derivative of pGE387 containing a 1.7-kb XhoI deletion [$\Phi(\Delta DnrdG-lacZ)$]This studypGE3916.5-kb Ps/I fragment of pCH604 in pEDI25 ($E. coli nrdDG$)This studypGE3916.5-kb Ps/I fragment of pCH604 rol pBuescript KS(+) (nrdDG)This studypGE38660-bp XbaI-HincIII fragment of pGE26 in pBluescript KS(+) (nrdDG)This studypGE3916.5-kb Ps/I fragment of pCH604 rol G650A)This studypGE3916.5-kb Ps/I fragmen	pEDY305	Tc ^r , <i>lacZ</i> RP4 <i>oriT</i>	46
pLO1/pLO2Km ^r , sacB RP4 oriT ColE1 ori26pPX41Ap', E. coli mdDG53pGE26nosZ, norB, mdDG63pGE151Tc', lacZ' RP4 oriT25pGE2916.2-kb EcoRI-HindIII fragment of pGE26 in pVDZ'2 (nrdDG)This studypGE3055.0-kb Xba1-HindIII fragment ^b in pVDZ'2This studypGE3074.0-kb Xba1-HindIII fragment ^b in pVDZ'2This studypGE3114.4-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3123.2-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3434.7-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3445.2-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3852.65-kb Ecl136II-SmaHI fragment ^b in pVDZ'2This studypGE384124-bp Ecl136II-SmaI fragment of pCH604 in pEDY305 (nrdDP-lacZ)This studypGE3852.65-kb Ecl136II-SmaI fragment of pCH604 in pEDY305 (nrdG-lacZ)]This studypGE386460-bp XbaI-HincII fragment of pCH604 in pPHU235 [\@(nrdG-lacZ)]]This studypGE3872.6-kb XbaI-SmaI fragment of pCH604 in pPHU235 [\@(nrdG-lacZ)]]This studypGE388Derivative of pGE387 containing a 1.7-kb XhoI deletion [\@(\DeltaDnrdG-lacZ)]]This studypGE3916.5-kb PsrI fragment of pCH604 in pElS1 (E. coli nrdDG)This studypGE3916.5-kb XbaI-HindIII fragment of pCH604 in pBU235 [\@(nrdG-lacZ)]]This studypGE388Derivative of pGE387 containing a 1.7-kb XhoI deletion [\@(\DeltaDnrdG-lacZ)]]This studypGE3916.5-kb PsrI fragment of pCH47 pBluescript KS(+) (nrdDG)	pPHU234/pPHU235	Tc^r , $lacZ'$	18
pPX41Ap ^r , E. coli nrdDG53pGE26nosZ, norB, nrdDG63pGE151Tc ^r , lacZ' RP4 oriT25pGE2916.2-kb EcoRI-HindIII fragment of pGE26 in pVDZ'2 (nrdDG)This studypGE3055.0-kb Xbal-HindIII fragment ^b in pVDZ'2This studypGE3064.5-kb Xbal-HindIII fragment ^b in pVDZ'2This studypGE3114.4-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3123.2-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3434.7-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3445.2-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE384124-bp Ecl136II-BamHI fragment ^b in pVDZ'2This studypGE3852.65-kb Ecl18lmAmHI fragment of pCH604 in pEDY305 (nrdDP-lacZ)This studypGE386460-bp Xbal-HincII fragment of pCH604 in pPHU235 [$\Phi(nrdG-lacZ)$]This studypGE3872.6-kb Xbal-SmaI fragment of pCH604 in pPHU235 [$\Phi(nrdG-lacZ)$]This studypGE388Derivative of pGE387 containing a 1.7-kb XhoI deletion [$\Phi(\Delta DnrdG-lacZ)$]This studypGE3916.5-kb PsrI fragment of pCH604 in pBHU235 [$\Phi(nrdG-lacZ)$]This studypGE388Derivative of pGE387 containing a 1.7-kb XhoI deletion [$\Phi(\Delta DnrdG-lacZ)$]This studypGE3916.5-kb PsrI fragment of pCH404 in pBHU235 [$\Phi(nrdG-lacZ)$]This studypGE386berivative of pGE387 containing a 1.7-kb XhoI deletion [$\Phi(\Delta DnrdG-lacZ)$]This studypGE388Derivative of pGE387 containing a 1.7-kb XhoI deletion [$\Phi(\Delta DnrdG-lacZ)$]This studypGE3891 <t< td=""><td>pLO1/pLO2</td><td>Km^r, sacB RP4 oriT ColE1 ori</td><td>26</td></t<>	pLO1/pLO2	Km ^r , sacB RP4 oriT ColE1 ori	26
pGE26 $nosZ$, $norB$, $nrdDG$ 63 pGE151Tc', $lacZ'$ RP4 orT 25 pGE291 6.2 -kb $EcoRI$ -HindIII fragment of pGE26 in pVDZ'2 ($nrdDG$)This studypGE305 5.0 -kb $XbaI$ -HindIII fragment ^b in pVDZ'2This studypGE306 4.5 -kb $XbaI$ -HindIII fragment ^b in pVDZ'2This studypGE307 4.0 -kb $XbaI$ -HindIII fragment ^b in pVDZ'2This studypGE311 4.4 -kb $EcoRI$ -BamHI fragment ^b in pVDZ'2This studypGE312 3.2 -kb $EcoRI$ -BamHI fragment ^b in pVDZ'2This studypGE343 4.7 -kb $EcoRI$ -BamHI fragment ^b in pVDZ'2This studypGE344 5.2 -kb $EcoRI$ -BamHI fragment ^b in pVDZ'2This studypGE384 124 -bp $Ecl136II$ -BamHI fragment of pCH604 in pEDY305 ($nrdDP$ -lacZ)This studypGE385 2.65 -kb $Ecl136II$ -BamHI fragment of pCH604 in pEDY305 ($nrdDP$ -lacZ)This studypGE386 460 -bp $XbaI$ -HincII fragment of pCH604 in pHU234 [$\Phi(nrdD$ -lacZ)]This studypGE387 2.6 -kb $XbaI$ -HincII fragment of pCH604 in pHU235 [$\Phi(nrdG$ -lacZ)]This studypGE388Derivative of pGE387 containing a 1.7-kb $XhoI$ deletion [$\Phi(\Delta DnrdG$ -lacZ)]This studypGE391 6.5 -kb PsI fragment of pPX41 in pGE151 ($E.$ coli $nrdDG$)This studypGE391 6.5 -kb $EcoRI$ -HindIII fragment of pCH447 pBluescript KS(+) ($nrdDG$)This studypGE391 6.5 -kb $DsAI$ fragment in pLO1 (3.2 -kb deletion of $nrdDG$)This studypCH605 3.05 -kb $XbaI$ -HindIII fragment in pLO1 (3.2 -kb deletion of $nrdDG$)This studypCH606	pPX41	Ap ^r , E. coli nrdDG	53
pGE151Tc', $lacZ'$ RP4 $oriT$ 25pGE291 6.2 -kb Eco RI-HindIII fragment of pGE26 in pVDZ'2 ($nrdDG$)This studypGE305 5.0 -kb $XbaI$ -HindIII fragment ^b in pVDZ'2This studypGE306 4.5 -kb $XbaI$ -HindIII fragment ^b in pVDZ'2This studypGE307 4.0 -kb $XbaI$ -HindIII fragment ^b in pVDZ'2This studypGE311 4.4 -kb Eco RI-BamHI fragment ^b in pVDZ'2This studypGE312 3.2 -kb Eco RI-BamHI fragment ^b in pVDZ'2This studypGE343 4.7 -kb Eco RI-BamHI fragment ^b in pVDZ'2This studypGE344 5.2 -kb Eco RI-BamHI fragment ^b in pVDZ'2This studypGE385 2.6 -kb Eco RI-BamHI fragment of pCH604 in pEDY305 ($nrdDP$ -lacZ)This studypGE386 460 -bp $XbaI$ -HincII fragment of pCH604 in pEDY305 ($nrdG_P$ -lacZ)This studypGE387 2.6 -kb $XbaI$ -BinnI fragment of pCH604 in pPHU234 [$\Phi(nrdD$ -lacZ)]This studypGE388Derivative of pGE387 containing a 1.7 -kb $XhoI$ deletion [$\Phi(\Delta DnrdG$ -lacZ)]This studypGE391 6.5 -kb $PSII$ fragment of pPX41 in pGE151 ($E. coli nrdDG$)This studypGE391 6.5 -kb Eco RI-HindIII fragment of pGE26 in pBluescript KS(+) ($nrdDG$)This studypCH447 6.2 -kb Eco RI-HindIII fragment of pGE151 ($E. coli nrdDG$)This studypCH605 3.05 -kb $XbaI$ -HindIII fragment of pCH447 pBluescript KS(+) ($nrdDG$)This studypCH606 608 -bp $XhoI$ -MindIII fragment in pLO2 (NrdD G650A)This study	pGE26	nosZ, norB, nrdDG	63
pGE291 6.2 -kb Eco RI-HindIII fragment of pGE26 in pVDZ'2 ($nrdDG$)This studypGE305 5.0 -kb $XbaI$ -HindIII fragment ^b in pVDZ'2This studypGE306 4.5 -kb $XbaI$ -HindIII fragment ^b in pVDZ'2This studypGE307 4.0 -kb $XbaI$ -HindIII fragment ^b in pVDZ'2This studypGE311 4.4 -kb Eco RI-BamHI fragment ^b in pVDZ'2This studypGE312 3.2 -kb Eco RI-BamHI fragment ^b in pVDZ'2This studypGE343 4.7 -kb Eco RI-BamHI fragment ^b in pVDZ'2This studypGE344 5.2 -kb Eco RI-BamHI fragment ^b in pVDZ'2This studypGE384 124 -bp $Ecl136II$ -BamHI fragment of pCH604 in pEDY305 ($nrdDP$ -lacZ)This studypGE385 2.65 -kb $Ecl136II$ -SmaI fragment of pCH604 in pEDY305 ($nrdGP$ -lacZ)This studypGE386 460 -bp $XbaI$ -HincII fragment of pCH604 in pPHU235 [$\Phi(nrdD$ -lacZ)]This studypGE387 2.6 -kb $XbaI$ -SmaI fragment of pCH604 in pPHU235 [$\Phi(nrdG$ -lacZ)]This studypGE388Derivative of pGE387 containing a 1.7 -kb $XhoI$ deletion [$\Phi(\Delta DnrdG$ -lacZ)]This studypGE391 6.5 -kb $PstI$ fragment of pCH604 in pBHU235 [$\Phi(nrdG$ -lacZ)]This studypGE391 6.5 -kb $PstI$ fragment of pCH604 in pGE151 ($E.$ coli $nrdDG$)This studypCH447 6.2 -kb $ZoaI$ -HindIII fragment of pGE26 in pBluescript KS(+) ($nrdDG$)This studypCH604 5.0 -kb $XbaI$ -HindIII fragment of pCH604 in pCH447 pBluescript KS(+) ($nrdDG$)This studypCH605 3.05 -kb $XbaI$ -HindIII fragment in pLO1 (3.2 -kb deletion of $nrdDG$)This studypCH606	pGE151	Tc^r , $lacZ'$ RP4 $oriT$	25
pGE3055.0-kb Xba1-HindIII fragment ^b in pVDZ'2This studypGE3064.5-kb Xba1-HindIII fragment ^b in pVDZ'2This studypGE3074.0-kb Xba1-HindIII fragment ^b in pVDZ'2This studypGE3114.4-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3123.2-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3434.7-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3445.2-kb EcoRI-BamHI fragment ^b in pVDZ'2This studypGE3852.65-kb Ecl136II-BamHI fragment of pCH604 in pEDY305 (nrdDP-lacZ)This studypGE386460-bp Xba1-HincII fragment of pCH604 in pEDY305 (nrdGP-lacZ)This studypGE3872.6-kb Xba1-SmaI fragment of pCH604 in pPHU234 [$\Phi(nrdD-lacZ)$]This studypGE388Derivative of pGE387 containing a 1.7-kb XhoI deletion [$\Phi(\Delta DnrdG-lacZ)$]This studypGE3916.5-kb PstI fragment of pCH604 in pEHU235 [$\Phi(nrdG-lacZ)$]This studypGE44776.2-kb EcoRI-HindIII fragment of pGE361 (E. coli nrdDG)This studypCH6045.0-kb Xba1-HindIII fragment of pGE26 in pBluescript KS(+) (nrdDG)This studypCH6053.05-kb Xba1-XhoI fragment in pLO1 (3.2-kb deletion of nrdDG)This studypCH606608-bp XhoI-MscI fragment in pLO2 (NrdD G650A)This study	pGE291	6.2-kb <i>Eco</i> RI- <i>Hind</i> III fragment of pGE26 in pVDZ'2 (<i>nrdDG</i>)	This study
pGE3064.5-kb Xba1-HindIII fragment ^b in pVDZ'2This studypGE3074.0-kb Xba1-HindIII fragment ^b in pVDZ'2This studypGE3114.4-kb $EcoRI$ -BamHI fragment ^b in pVDZ'2This studypGE3123.2-kb $EcoRI$ -BamHI fragment ^b in pVDZ'2This studypGE3434.7-kb $EcoRI$ -BamHI fragment ^b in pVDZ'2This studypGE3445.2-kb $EcoRI$ -BamHI fragment ^b in pVDZ'2This studypGE384124-bp $Ecl136II$ -BamHI fragment of pCH604 in pEDY305 (nrdDP-lacZ)This studypGE3852.65-kb $Ecl136II$ -BamI fragment of pCH604 in pEDY305 (nrdDP-lacZ)This studypGE386460-bp Xba1-HincII fragment of pCH604 in pPHU234 [$\Phi(nrdD-lacZ)$]This studypGE3872.6-kb XbaI-SmaI fragment of pCH604 in pPHU235 [$\Phi(nrdG-lacZ)$]This studypGE388Derivative of pGE387 containing a 1.7-kb XhoI deletion [$\Phi(\Delta DnrdG-lacZ)$]This studypGE3916.5-kb PstI fragment of pPX41 in pGE151 (E. coli nrdDG)This studypCH6045.0-kb Xba1-HindIII fragment ⁶ of pCH447 pBluescript KS(+) (nrdDG)This studypCH6053.05-kb Xba1-XhoI fragment in pLO1 (3.2-kb deletion of nrdDG)This studypCH606608-bp XhoI-MscI fragment in pLO2 (NrdD G650A)This study	pGE305	5.0-kb XbaI-HindIII fragment ^b in pVDZ'2	This study
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pCH606 608-bp XhoI-MscI fragment in pLO2 (NrdD G650A) This study	pCH605	3.05-kb XbaI-XhoI fragment in pLO1 (3.2-kb deletion of nrdDG)	This study
	pCH606	608-bp XhoI-MscI fragment in pLO2 (NrdD G650A)	This study

TABLE 1. Bacterial strains and plasmids used in 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-*Smail* fragment of pCH604 in the *ScaI* site of the pEDY305 polylinker. Plasmid pGE386 is a derivative of pPHU234 which carries a 460-bp *XbaI-Hinc*II fragment of pCH604 inserted between the *XbaI* and *ScaI* 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 *XbaI-Smail* fragment of pCH604 into the *XbaI* and *ScaI* 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 *XhoI* deletion which removed the 5' *nrdD* untranslated region and most of the *nrdD* gene. The transcriptional and translational fusion

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 *XbaI* and *XhoI* (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'-CCCACAA TGACCTCGC-3') (Fig. 2). The resulting 608-bp *XhoI-MscI* fragment was transferred into the *SaII-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

DNA techniques. Standard DNA techniques were used (42). Plasmid DNA was isolated with tip-20 columns (Qiagen) according to the manufacturer's in-

structions. 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 MgCl₂, 5 mM sodium formate, and 1 mM [³H]CTP. After 20 min, the reaction was stopped by the addition of 0.5 ml of HClO₄, 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. $\beta\mbox{-}Galactosidase$ 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, *ClaI*; E, *Eco*RI; H, *Hind*III; K, *KpnI*; Sp, *SpeI*; Xb, *XbaI*; X, *XhoI*. (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; C, *HincIII*; H, *HindIII*; Sm, *SmaI*; Xb, *XbaI*; X, *XhoI*.

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

GGACTCTCGTACCGCCGACCAAGACTAGCCGACATGTCATACATTGCGCGCCGTCAAACTACATATAGTGTTTTGGTCGTACGATTTCCCCCGCATCTAG	100
$\label{eq:construction} TGTSAATTTTGCCGGGAGAATGTAATGGAGGACATTGGAGAGAGGATGAAGGCGACTGGAGAGAGA$	800 27
CGCCATTGAGCGGATCCAGTCGGCTTTACGCCGCGCCGGCGCGGCGCGGCGAGGCGAGGCAGGC	300 59
GTCCTAGCGCACCGTTACCATAACCAGACACCAGGCGTGCAGGCGTGGTGGAGGAGGTGTTGATTGCCGCCAATTACTGGAACACCGCGC V L A H R Y H N Q T P G V E Q I Q D V V E Q V L I A A N Y W N T A R	400 93
GCGCGTTTATCGCCTAGGCCAGGCCAGGCCAGGACCGCAAGGCCTGGTCGACGTGGAAGCCTCGATCAACGAATACCTGAGCAAGGC A F I A Y R G Q H A R L R Q D R K A L V D V E A S I N E Y L S K A	500 126
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GTCTACACACCGGAAATCGGCGAGGCCCATCGCAACGGCGATATCCATATCCACGATCTCGAGATATGCTCAGCGGCTACTGTGCCGGCTGGTCGCTCGC	700 193
CGCTGCTGCACGAGGGGTTCAATGGCGTGCCGGGAAGGGGGAGGCCGGGGGCGCGGGGGCAGGTCGGGGGGAGATCGTCCAGG L L H E G F N G V P G K V E A G P P K R M S S A V G Q I V N F L G	800 226
CACACTECAGAACGAATGGGCCGGTGCGCAGGCGTTCGGCGTTCGGCGACGCCATGGCGCAGGACGCCATGCCCATGCCCATGCACAGGTG T L Q N E W A G A Q A F S S F D T Y M A P F V R K D A M S Y A Q V	900 259
CGGCAGTGCATCCAGGAACTGATCTACAACCTGACCTGA	1000 293
AGGACCTGCGCGAACAGGTGCCGGTCATCGGCGGGGAGAGTGCGGGGAGAGGGGAGTGGACCTGATCGATC	1100 326
CGAGGTAATGACGGGGGGGGGGGGGGGGGGGGGGGGGGG	1200 359
ARGCGGCTGTTCGAGATGACGGCCAGATATGGGGCTGCCGTACTTCCAGAACTTCATAACTCCGAGCTGGAGCCGAATATGGTGCGGCTCCATGTGCTGCC K R L F E M T A R Y G L P Y F Q N F I N S E L E P N M V R S M C C R	1300 393
GGCTCCAACTGGACCTGGCGGGAACTGCTCCAAGCGGGGCAACGGCCGCGGGGGGGG	1400 426
ACGCCTGGGCTACCGGCGACGACGACGACGACGAGCGCCGCTACTGGAGCTGCGCGAACCAGGCGAAGCAGAGCGGGAACAAGCGGCAAG R L G Y L H A G D E A A L L R A L D R L L E L G K Q S L E N K R K	1500 459
CTGATCCAGCACCTGATGGACCAGGGCCTGTTCCCCTATACGAAGCGCTACCTCGGCACCTTAGCGATCATTTTTCGACGCTGGCCGTGAATGGCGTCALLING CONTRACTATION CONTRACTOR CONTRACTOR CONTRACTANCE CONTRACTOR	1600 493
ACGAGATGATTCGCGATTCGCCGGGATCGGCATCGGACATCACATCGGAGTGGGGGCCATGCCTTGCGGCTGCGCCTGCCCTGCGACACGGGAACGCAT E M I R N F T A D R H D I T S E W G H A F A L R L L E H V R E R I	1700 526
CGTOGCCTTCCAGGAAAACCGGCCACCTCTCAACCTCGAGGCTACCCCAAGGCGCTACGTACCGATTTGCCCGCGAAGATCGCAAGGCGCTGG V A F Q Q E T G H L Y N L E A T P A E G A T Y R F A R E D R K R W	1800 559
CCGGATATCCGCCAGGCCGGTACGGCTGAGCAGCCCTACCACACCTCCCCAACTGCCGGTCGGCTGGACCGAGGACCCCTTCGAGGCACTGCCG P D I R Q A G T A E Q P Y Y T N S S Q L P V G W T E D P F E A L A R	1900 593
GGCAAGAGGATTIGCAGGCCAGGTATACCGGCGGGCACGGTGCTGCATCTATATATGGGCGAGTGCCTATCAAGCGGGGAGGCCTGCCGCGAACTGGTGGG Q E D L Q A R Y T G G T V L H L Y M G E C L S S G E A C R E L V R	2000 626
$\begin{array}{c} GCGGCGCTGACGCGCTTCCGCCTGCCGTACATCACAGTGACGCCGACGTTTTCGATCTGCCCCCGCGTTCCGCCGCGGCGAGCATCGCTTCCGCCGCGACGTTCCGCCGCGACGTTCCGCCGCGACGTTCCGCCGCGACGTTCCGCCGCGACGTTCCGCCGCGACGTTCCGCCGCGACGTTCCGCCGCGACGTTCCGACGCGACGTTCGACCGCGACGTTCCGACGCGACGTTCCGACGCGACGTTCGACGCGACGTTCGACGCGACGTTTCGATCTGCCCGACGACGTGCGCGACGTTCGGCCGACGTTCGACGCGACGTTCGACGCGACGTTCGACGCGACGTTCGACGCGACGTTCGACGCGACGTTCGACGCGACGTTCGACGCGACGTTCGACGCGACGTTCGACGCGACGTTCGACGCGACGTTCGACGCGACGTGCGCGACGTTCGACGCGACGTTCGACGCGACGTTGGCGACGTTGGACGCGACGTTGGACGCGACGTTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGTGGCGACGACGACGACGACGACGACGACGACGACGACGACG$	2100 659
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TCCGGCGCTTCCTTGTCTCTGTGTTTGGCCGTCCAGTCTTTGCCGAAACATCGTCCTTTCCTGTTTTTCGACCCATCTCAAGAAGGAGTGCATCCATGA	2300
TTCACCATGACCAATCCGCGCAATTCACGCAATCCTTGCCTGGCCATGCAGCGCTGCGCGAGGAGCGCCAACCCTGCGAGGTCTGGACCCGTGTGAT	2400
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ATGTGAGGCCGCGGGCGCGGGGCGCGGCGCGCGCGCGCGC	2600 57
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	2700 91
TIGGCTTCCTGCAGGGGGGGGGGGGGGGGGGGGGGGGGGG	2800 124
acaagccagaggcttcgaggtcgggctggactggaccggcatgtacccggatggcctggcctggcctggactggactggactggactggaccggcctcgacctg a r g f e v a l h t a g m y p d r l p a l l p q l d W I g l d l	2900 157
AAGGCGCCCCTACACCGCTATGACGCCATTACGCGGGGGGGG	3000 191
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	3100 224
$\begin{array}{llllllllllllllllllllllllllllllllllll$	3200 256
TGCAGGTCCCTTGCGCTATGTCAATCGACCAGCGGGGTACTGGCCACGCAGAAAAGCGTGGCCAGTACTACGATGGAAAGTGCATAACAGAAAATCAAAGAA	3300

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.

 $(\Delta G_0' = -123.9 \text{ kJ})$ 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-

A_EUT M_JAN M_THE E_COL H INF	MEHCTEAAVASLPREVIKRTGESMAFAIERIQSALRRAGAAGEFGDAQAVSLAGQVARVLAH	63 82 90 40 41
A_EUT M_JAN M_THE E_COL H_INF P_HOR PH_T4	RYHNQTFGVEQIQDVVEQVLJAANYWNTARAFIAYRGQHARLRQDRKALVDVEASINEYEYEY	122 171 180 109 112 10 14
A_EUT M_JAN M_THE E_COL H_INF P_HOR PH_T4	-LSKADWRVNANANQGYSLGGLILNVAGKVTANYWLSHVYT-PE-IGE <u>AHRNGDIHIH</u> DLDMLSGYCAGWSLRTLLHEGFNGV VYDITKLIKSGSRENANLMYNPESIHKWVADETMKCYALLAIPRKHIAD <u>AHIKGDIHIH</u> DLEYAATRPVCLQHDLRFFFKVGLKVD VYNITNLIENGSRENANIHNPETVHKYVADEALKQYTLLHIPSRLAD <u>AHMSGDIHIH</u> DLEYFARPLN-CLQHDLRFIHGLRVD -NASLLNENANKDSKVIP-TQRDLLAGIVAKHYARHLPRDVVE <u>AHERGDIHYH</u> DLDYSFFPMNCMLJDLKGMLTQFFKMG -NVELLNENANKDSKVIP-TQRDLLAGIVAKHYAKHNILPRDVVE <u>AHERGDIHYH</u> DLDYSFFPMNCMLJDLGMLSGCFKMG -ARWGSLDVLENANRYFGSGFFAYVMEEALNNSLSLIPFIGLK- <u>AHLSGDIHIH</u> DLEYSLYIPYCTGHSIARLLEKGLKTP -NKDLLNENANKDSRVP-TQRDLMAGIVSKHIAKN-MVPSFIMK <u>AHESGIHYH</u> DIDYSPALPFTNCCLVDLKGMLEGKFKG	202 257 267 191 193 90 95
A_EUT M_JAN M_THE E_COL H_INF P_HOR PH_T4	PGKVEAGPPKRMSSAVGQIVNFLGTLQNEWAGAQAFSSFDTYMAPFVRKDAMSYAQVRQCIQELIYNLNVP-SRWGTQTPFTN GTGLHTSVSKPAKHPEVAIQHAAKVMMAAQTNMSGGQSIDEFNVWLAPYVRGLSYEKIKQLWQMFIYELNQMYVARGGQTIFSS GTGDHTSVAGPPKHLETLMNHAGEIMLASQQNMSGGQAMSLMNVFVAPFASGLSYEKIKQAVQMFIFNLMNAYAARGSQVPFTS NAEIEPPKSISTATAVTAQIIAQVASHIYGGTINRIDEVLAFFVTASYNKHRKTAEEMNIFDAERVANSHTKECVDAFQS TITSKPAKHFDTFVDHVANYLITLQHYFSGAQAFSSVEWYAGPFIRREGLNSRKVKQNIQRLIYNLMYP-TRIGLQTPFTN NAQIETPKSIGVATAIMAQITAQVASHQYGTTFANVDKVLSPYVKRTYAKHIEDAEKWQIADALNYAQSKTEKDVYDAFQA	284 349 351 273 275 170 177
A_EUT M_JAN M_THE E_COL H_INF P_HOR PH_T4	LTFDWTCPEDLREQVPVIGGQEMPFAYGELQKEMDLINRAYIEVMTAGDAK-GRVFTFPIPTYNITR-DFPWESENAKRLFEMTAR INLELEIPEFLKDKPAVIA-GTTRGTYGDYEBEAKLILEALVDVMMEGDAM-GKPFLFPNFIIKLKENAFKDENKELMYKIHQLSAK INLEFGVPEFLEDEPAYGPRGEYAGVYGDFAEEAKLLTRAFTEVLLEGDAD-GKPHLFPNFIYSLREDTRGEPDEELSLVHELASK LEYEVNTLHTANGGTPFVTFGFGLGTSWESRLIQSILRNIAGLGKNRKTAVFPKLVFAIRDGLNHKKGDPNYDIKQLALECASK FTVTLDAFRKMLEGDYAIYNGKKVAPLGEYEKEAKMFIIALSEVLREGDAL-GQPFTFFPLTLMVTAKMIWDDEVFEAIFTTAAK YEYEVNTLFSSNGQTPFVTTFGFGLGTD-WTERMIQKAILKNRIKGLGRDGITPIFPFLVLWFVEBGVNLYKDDPNYDIKQLALECAS	368 434 437 359 361 255 263
A_EUT M_JAN M_THE E_COL H_INF F_HOR FH_T4	YGLPYFQNFINSELEPNMVRSMCCRLQLDLR	434 495 498 422 423 338 329
A_EUT M_JAN M_THE E_COL H_INF P_HOR PH_T4	DEAALLRALDRLLELGKQSLENKRKLIQHLMDQGLFPYTKRYLGTLRNHFSTLGVNGVNEMIRNFTADRHDIT DDTKLFEILHERLEILKEALLIKHEVTKERLYVDNLMPFLTQEFDGESYYRVENTTKTFGFVGLNEMLKYHLGBELHE- DDDELFEYLDBYIDMAVEVLRIRRSQAQRCLDYHLLPFLSQEIDGERYYRIENATMSFGFTGLNEMLEYHLGAGIQ DEATFWKLLDERLVLARKALMTRIARLEGVKARVAPILYMEGACGVRLNADDDVSEIFKNGRASISLGYIGIHETINALFGGEHYYD NEEDFYRTLDERLAIAKKALMTRIARLEGVKARVAPILYMEGACGVRLKADENVAQIFKNGRASISLGYIGIHETINALYNGKHIFD DDDKFWEEYERILEIVRITTDWFRERYIKLISSYPHMYSMIKEYLEEFPMSHFNTIGILGLEFAVAIYMNSPELWK TQFNEQKFVELFNERMDLCFEALMCRISSLKGVKATVAPILYQEGAFGVRLKADEDUIIELFKNGRSSVSGUHELLNILVG	507 573 574 509 510 414 412
A_EUT M_JAN M_THE E_COL H_INF P_HOR PH_T4	SEWGHAFALRLLEHVRERIVAFQQETGHLYNLEATPAEGATYRFAREDRKRWPDIRQAGTAEQPYYTNSSQLPVGWTE SKDAVKFGEKVIEYIREYADKLKEETGLRWTVTQTPAE-TAGRFARLDYKYYKEETISVVRGDLNDVDSLYYTNSSHVRVDAPI SFEANRFGLRVIEHINERAAELKKETGWRWSLUGTPAESTAHFFAMLDHEHYPE-AVLQGTEGAYYYTNSSHVRVDAPU NEQLRAKGIAIVERLRQAVDQWKEETGYGFSLYSTPSENLCDRFCRLDTAEFGVVPGVTDKGYYTNSFHLDVEKKV DEQLRAKGIAIVERLRQAVQWKEETGYAFSLYSTPSENLCDRFCRLDTAEFGVVPGVTDKGYYTNSFHLDVEKKV EGNRRDWIESARLMKKMVEFATAKAREWMRATGTPWNVEEVPGESAAAKLAIRDMKEFPELKDVLEDPDNPIYST-SIAPYYGSLE RDIGREILTKMNAHLKQWTERTGFAFSLYSTPAENLCVRFCKLDTEKYGSVKDVTDKGWYTNSFHVSVEENI	585 656 653 585 586 499 484
A_EUT M_JAN M_THE E_COL H_INF P_HOR PH_T4	DPFEALARQEDLQARYTGGTVLHLYMGECLSSGEACRELVRRALTRFRLPYITVTPTFSICPTHCINCGSEDV TLGEKVRIEEKFHPLCNGGHIMHIWNIESAADPEVLMDITKKIT-KTHIGFWTYTKNLSVCNRCGISMGGLRDRCINCGSEDV DLVEKIRIEEKFHPLTPGGHIFNNWLGEAKPDFAALEGLTRRICRRSDIGFWAYSNALSFCLRCKTLMRGLQDSCARCGERDEV NPYDKIDFEAPYPPLANGGFICYGEYPNIQHNLKALEDWDVSYQHVPYGTNTFIDECYECGFTGEFECTSKGFVCPKCGNHDSTVV LGDRVKIEEMYQGSF-TCGVMHHIFLGEE-PDFEALAQLTKKLMR-TKLVYWSYTPAITVCNSCKASFTGLYTRCRCGSKDV TPFEKISREAPYPHIATGGHISVVELPDWKNNLKGLEAVWDYAAQHLDYFGVNMPVDKCFTCGSTHEMTPTENGFVCSICGETDFKKM	649 740 737 673 673 579 572
A_EUT M_JAN M_THE E_COL H_INF P_HOR PH_T4	AKFSRITGYLQGHPFCPRCDEEILARKRDQLAA AKFSRITGYLQNISNWNRAKQKELEDRKLPRI EWYDRITGYVQQVGRAKSSSGGWNRGKQQELLDRRIDL SVTRRVCGYLGSPDARPFNAGKQEEVKRVKHLONGQIG SVTRRVCGYLGSPDARPFNAGKQEEVKRVKHL EIWSRIIGYYRPLRNWNPYRKEFWSKHYVA NTIRRTCGYLGNPNERGFNLGKNKEIMHRVKHQ	676 771 776 712 707 611 605

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.

A_EUT	MSASRASTASAASSRSPMPSPPDACEAAAGRRRDAASLRVGGMTALTTIDFPGRLAAVV	59
M_THE	MNSAVGAELFMCDDGVSLRPGLGGTESVLESWRYIMGKLRVGRFLISSRVMORGRR-FVI	59
M_JAN	MKALVSGIVDLSTIDYPKKASAVI	24
P_HOR	MLVSGWKEVSMVDVHGKTTFTL	22
E_COL	MNYHQYYPVDIVNGPGTR-CTL	21
H_INF	MNYLQYYPTDVINGEGTR-CTL	21
PH_T4	MNYDRIYPCDFVNGPGCR-VVL	21
A_EUT M_THE M_JAN P_HOR E_COL H_INF PH_T4	FCQGCPWRCGYCHNPGLLDARA-PSSIAWAD-VLAFLQARQGLLDGVVFSGGEPTL-Q WPQGCPIRCRGCLNPEFHDEDG-GHLIETARLVDMIRDLRDEIEGVTFTGGEPLA-Q FLYGCNMKCPYCHNLKFMLEHKRGMTVEEIFNDIDFLFADAIVISGGEPTL-Q WLCGCNLRCFFCHMWRIAQGEGC-FKLNREELIAEVDANSF-LVDCFHTTGGEPLI-Q FVSGCYHECPGCYNKSTWRVNS-GQPFTKAMEDQIINDLNDTRIKRQGISLSGDPLHPL FVSGCHACKGCYNQKSWSFSA-GVLFDDVMEQQIINDLKDTRIKRQGITLSGGDPLHPL FVTGCLHKCEGCYNRSTWNARN-GQLFTMNTVKELASHLSKSYIQGLTLTGGDPLYPQ ** * * *	114 114 76 77 80 80 78
A_EUT	GALAGAIA-DVQARGFEVALHTAGMYPDRLPAILPQLDWIGLDLK-APLHRYD	165
M_THE	AMELVKLAGAVKSMGLTVVCFTGYEMDEILKGNIEGGLELL	155
M_JAN	KDAVIEIARYAKEKGFPVKIDTNGTHPEVIELIKNKLIDYVAIDVK-CRFDKYK	130
P_HOR	WKELRNLLVDVRRYLPISLNSNLTLVKPLERVIEFLDHVATDLKVPPTELYGLPRES	134
E_COL	NVPDILKLVQRIRAECPGKDIWVWTGYKLDELNAAQMQ	118
H_INF	NVETLLPFVQRVKRECPDKDIWVWTGYKLDELDKQQRA	118
PH_T4	NREEISNLVSWVKARFPEKDIWLWTGYKFEDIKQLE	114
A_EUT M_THE M_JAN P_HOR E_COL H_INF PH_T4	AITRVPGSGERAWESLRHWVASD-VAGECRTTWHAGMFDIAELHALAESI EFVDVLIDGPYIEEKSAPL-LWRGSTNQDVYFLTERYAEFRDRVMACSEMEAE EFVKCREDGEEINKILKIIDLCKKNNVFVECRTFVPKVMDEEDIEDIAKTV SIKLWKLFLDGLSIVSNYSIPLELRIPVSRGFKVEDIKPWIEEGIERINTDF VVDLINVLVDGKFVQDLKDP-SL-IWRGSSNQVVHHLR MLPYIDVLIDGKFIQEQADP-SL-VWRGSANQIIHRFKL MLKYVDVIIDGKY-EKNLPT-KKLWRGSDNQRLWSNTDGVWKHD *	214 207 183 186 154 155 156
A_EUT	AALGVKHWALQECRGSGASASLGRDDISRLAARFSSFTLRKA	256
M_THE	LKVGADGV-YMTGIFDVEFWEELRRLGDGS	237
M_JAN	KDCDLYAIQQFEPKDAYDEEFKKLPMPKENELRELGKIAKKYIDNVVIRTINGTFEI	240
P_HOR	YVVLNPLVGPPLTDPRDKEWCAEHCWPRNEVEKLKDLLKSLGIEKVIVKSYP	238

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

Α



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 (\bullet), *nrdDG* deletion mutant HF413 (\blacktriangle), complemented mutant HF413(pGE291) (∇), and NrdD G650A exchange mutant HF456 (\blacksquare) 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. eutro-phus*.

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 (\bigcirc and \bullet) and the *nrdDG* deletion mutant HF413 (\triangle and \blacktriangle) 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	nrdDp-lacZ	29	4,156
pGE385	nrdDGp-lacZ	17	4,628
pGE386	$\Phi(nrd\hat{D}-lacZ)$	0	5,986
pGE387	$\Phi(nrdG-lacZ)$	0	157
pGE388	$\Phi(\Delta nrdD-nrdG-lacZ)$	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'-deoxyadenosylradical 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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