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nrdD and *nrdG* Genes Are Essential for Strict Anaerobic Growth of *Escherichia coli*

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In *Escherichia coli* ribonucleotide reduction is catalyzed by two separate enzymes during aerobic and anaerobic growth. The aerobic enzyme is coded by the *nrdAB* genes, the anaerobic enzyme by *nrdDG*. We now show that knock-out mutants of either *nrdD* or *nrdG* cannot grow during strict anaerobiosis, achieved by inclusion of sodium sulfide in the medium. Interestingly, these mutants grow well under microaerophilic conditions by overproducing the aerobic enzyme. Under such conditions wild-type bacteria turn off *nrdAB* and switch on *nrdDG*.

Escherichia coli contains the genetic information for three separate ribonucleotide reductases. The *nrdA* and *nrdB* genes code for the enzyme that provides deoxyribonucleotides during aerobic growth (1). *nrdD* and *nrdG* code for an enzyme that is expressed during anaerobic growth (2). Finally, *nrdE* and *nrdF* code for a cryptic aerobic reductase whose physiological function is unclear (3). *nrdA* and *nrdB* are essential for aerobic growth. Mutations in these genes result in distinct physiological phenotypes that are not complemented by the resident chromosomal *nrdEF* genes. We have now constructed knock-out mutants of *nrdD* and *nrdG* and investigated their growth properties under aerobic, microaerophilic and strict anaerobic conditions.

MATERIALS AND METHODS

Materials. The following *E.coli* bacterial strains and plasmids were used: KK450(*nrdA*$_{ts}$, *nrdB1, thyA, thr, leu,thi, feo, tonA, lacY, supE44, gyrA*), MC1061(Δ*lacX74, hsdR, mcrB, araD139,* Δ*(araABC-leu)7679, galU. galK, rpsL, thi*), UA6055 (MC1061 *nrdE*::Ω Sm^r/Spe^r) (3), pUA338 (*nrdEF* genes) (4), pUA447 (*nrdAB* genes) (5), pHP45ΩCm^r (6), pAE183 (*nrdD* gene) (7), pGP704 (8), and pPX41 (*nrdDG* genes) (9). Strains constructed in this work are: UA6068 (MC1061 *nrdD*::ΩCm^r Rif^r), UA6085 (MC1061 *nrdG*::ΩCm^r Rif^r) UA6074 (KK450 *nrdD*::ΩCm^r Rif^r) and UA6073 (UA6055 *nrdD*::ΩCm^r Rif^r).

Growth conditions. All growth was in Luria-Bertani (LB) broth, with ampicillin (50 μg/ml), spectinomycin (100 μg/ml), rifampicin (75 μg/ml) or chloramphenicol (34 μg/ml) when required.

Three different conditions with respect to oxygen availability were employed: aerobiosis, microaerophilia and strict anaerobiosis. Aerobiosis implied growth in liquid culture on a bacterial shaker or under continuous flushing with air. Microaerophilia ivolved flushing with N_2:CO_2 (96:4) instead of air or growth on plates incubated in Gaspack jars (Becton Dickinson) with Anaerocult A reagent (Merck). Strict anaerobiosis was obtained by growing the cells in vinyl screwcap tubes completely filled with LB containing 3.2 mM sodium sulfide, as is used for growth of obligate anaerobic bacteria (10). In the presence of sulfide a redox potential below −300 mV is obtained. Resazurin (0.2 mg/ml) was added as a redox indicator (colorless below −110 mV). Growth was monitored from the optical density of the culture at 550 nm or from viable cell count by plating dilutions on solid media.

Complementation of hydroxyurea hypersensitivity in KK 450 was determined by plating dilutions of the cultures on agar plates containing different concentrations of hydroxyurea. Colonies were counted after 2 days under aerobic or microaerophyllic conditions.

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Construction of chromosomal knock-out mutants. To construct *E.coli nrdD* knock-out mutants a 3.6 kb chloroamphenicol resistant cassette obtained from plasmid pHP45ΩCm^r was inserted into the internal *Bcl* site of the *E.coli nrdD* gene of pAE183 to give plasmid pUA528. The *Eco*RI-*Xba*I fragment from this plasmid, containing the the inactivated *nrdD* gene was then cloned in the pGP704 plasmid and introduced into the S17-1 (λ*pir*) strain. For its replication the plasmid requires the R6K-specific pir protein product of the *pir* gene (8) and therefore must be maintained in bacteriophage λ*pir* lysogenic strains. The recombinant suicide plasmid pUA529 was transferred into either the Rif^r derivatives of *E.coli* MC1061, UA6055(MC1061 *nrdE*::ΩSm^r/Spc^r) or KK450. Chloramphenicol-resistant transconjugants were screened for loss of vector-mediated ampicillin resistance to detect putative mutants that had exchanged their wild-type gene for the inactivated *nrdD* gene as a consequence of a double crossover event. The presence of the exchange was confirmed by Southern hybridization.

A similar procedure was used to obtain an *nrdG*$^-$mutant. First, a 0.8 kb *Bslt*EII-*Bam*HI fragment from plasmid pPX41 containing the *nrdG* gene was cloned in *Sma*I-cleaved pBluescript SK (Stratagene) to give plasmid pUA530. The ΩCm^r was inserted into an *Sca*I site of the *nrdG* gene which was subsequenctly cloned in pGP704, giving rise to plasmid pUA541 which was finally used for marker exchange with the chromosomal *nrdG* gene of a Rif^r derivative of *E.coli* MC1061.

Assays of ribonucleotide reductases. All assays were done with crude bacterial extracts. The reduction of CDP with DTT as hydrogen donor in air was a measure for the reductase coded by the *nrdAB* genes (11). The activity of the *nrdDG* coded reductase was determined from the anaerobic reduction of CTP with formate (12). In the crude bacterial extract also the aerobic enzyme shows some activity in this assay. To correct for this, assays were made in parallel in air and under nitrogen. The NrdDG proteins are completely inactive in air (13). One unit of enzyme activity is 1 nmol of deoxynucleoside phosphate formed per min.

RESULTS

Mutant strains of *E.coli* lacking either *nrdD* or *nrdG* showed no impairment of growth in air. More surprising, their growth also appeared normal when the cultures were continuously purged with nitrogen. Such ''microaerophilic'' conditions induced in the wild type strain the *nrdDG* genes and turned off *nrdAB* (13). Also colony formation in anaerobic Gas-Pak jars was not impaired. However, inclusion of Na_2S in the anaerobic medium to remove oxygen more completely resulted in cessation of DNA synthesis as indicated by formation of bacterial snakes and loss of colony formation. Na_2S had no such effect on wild type cell growth. Thus under strict anaerobic conditions *nrdD* and *nrdG* are required for the growth of *E.coli.*

What made possible the growth of both *nrdD* and *nrdG* mutants in the absence of Na_2S under the microaerophilic conditions that in the wild type induced the *nrdDG* genes? The answer was obtained from both genetic and biochemical experiments.

For the genetic experiments we used *E.coli* KK450, a strain that due to a mutation in *nrdAB* is hypersensitive to hydroxyurea (14). A comparison of KK450 and KK450*nrdD*$^-$ showed that KK450 was hypersensitive to hydroxyurea only in air, when its growth depended on the *nrdAB* genes, whereas under nitrogen the hypersensitivity was lost, since now the NrdDG enzyme provided deoxyribonucleotides (Fig 1). In contrast, KK450*nrdD*$^-$ was hypersensitive both in air and and in Gaspack jars suggesting that deoxyribonucleotides also during microaerophilia were provided by the hydroxyurea sensitive enzyme coded by the mutant *nrdAB* genes. Further evidence along this line came from experiments in which transformation with plasmid pUA447, carrying *nrdAB*, abolished the sensitivity of KK450*nrdD*$^-$, whereas the corresponding experiment with plasmid *pUA338*, carrying *nrdEF*, was negative (data not shown). The participation of the *nrdEF* system was also excluded from the finding that the double mutant *nrdD*$^-$ *nrdE*$^-$ showed normal growth under microaerophilic conditions.

In the biochemical experiments, extracts from *nrdD*$^-$ or wild type cells were assayed for ribonucleotide reduction under conditions optimal for either the NrdAB proteins (CDP reduction) or the NrdDG proteins (CTP reduction). When the cells were grown in air both types of extracts had the same specific activity for CDP reduction (Table 1). Extracts from cells grown under microaerophilic conditions behaved differently: the wild type strain had now lost the ability to reduce CDP as expected from the switch-off of *nrdAB;* the *nrdD*$^-$ cells showed an increased activity, four-fold more than during aerobic growth (Table 1). That this was caused

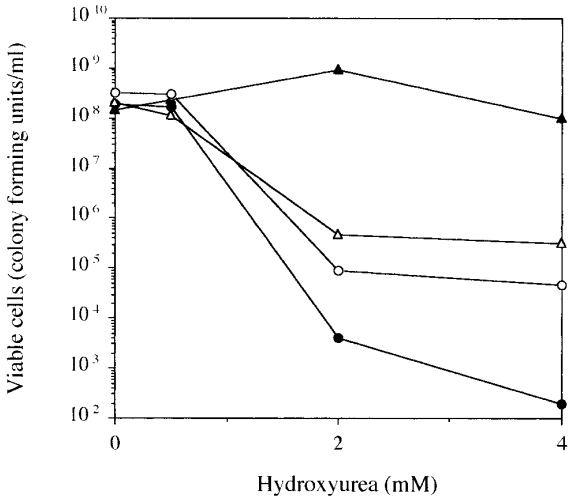

FIG. 1. Effects of hydroxyurea on the growth of strains KK450 and UA6074(KK450*nrdD*::ΩCm^r). Dilutions of cultures of the two strains were plated on rich media containing hydroxyurea at different concentrations and colonies were counted after two days of incubation at 30°C under aerobic (KK450, Δ; UA6074, ○) or microaerophilic (KK450, ▲; UA6074, ●) conditions.

by an increased expression of NrdAB proteins was found from Western blots that demonstrated the overproduction of the NrdA protein in the *nrdD*$^-$ extract, whereas it was absent from the wild type extract (data not shown).

We next grew *nrdD*$^-$ and wild type cells under nitrogen and tested the ability of extracts to reduce CTP under conditions optimal for the assay of NrdDG proteins (Table 1). The assays were done both under nitrogen and in air. Table 1 shows that the wild type cell extract showed activity only during anaerobic incubation indicating that we indeed measured the activity of the NrdDG enzyme. In contrast, air does not affect deoxyribonucleotide production by the mutant cell extract whose activity therefore is due to the NrdAB enzyme. Further confirmation of this came from Western blots that detected the NrdD protein only in the wild-type extract.

DISCUSSION

The aerobic ribonucleotide reductase coded by the *nrdAB* genes contains a tyrosyl radical required for its activity. Generation of this radical requires oxygen. During anaerobic growth

TABLE 1
Ribonucleotide Reductase Activity of Wild-Type and *nrdD*$^-$ Cells Grown under Aerobic or Microaerophilic Conditions

	Enzyme units	
	Aerobic assay	Anaerobic assay
Aerobic growth		
MC1061	0.13	0.11
MC1061*nrdD*$^-$	0.13	0.09
Microaerophilic growth		
MC1061	0.016	0.23
MC1061*nrdD*$^-$	0.47	0.41

dNTPs are produced by a different enzyme coded by the *nrdDG* genes. Here we show that knock-out mutants of *nrdD* and *nrdG* do not grow under strict anaerobic conditions, demonstrating that the two genes are indispensible for the anaerobic growth of *E.coli.*

In *E.coli*, the shift from aerobic to anaerobic growth involves major changes in gene expression, orchestrated by two genetic systems. One involves the Fnr protein (15), the other the ArcA and ArcB products (16). At low partial pressure of oxygen the Fnr protein binds to a specific region (the Fnr box) of certain genes and stimulates their expression (anaerobic genes), whereas the ArcAB proteins decrease the transcription of other genes (aerobic genes). An Fnr box has been found upstream of the *nrdDG* operon (17). Moreover, the transcription of the *nrdAB* operon was found to be decreased at low oxygen tension (18), suggesting involvement of the arcAB system. The two systems appear to set the activities of the *nrdAB* and *nrdDG* operons in relation to the availability of oxygen. Already under microaerophilic conditions the *nrdDG* operon is responsible for the synthesis of dNTPs in wild type bacteria. The *nrdD*$^-$ mutant is, however, unable to make the switch. At the low oxygen tension the less efficient formation of the tyrosyl radical of the NrdB protein is, instead, compensated by an increased formation of the NrdAB proteins, similar to the triggering of transcription of *nrdAB* by hydroxyurea, a scavenger of the tyrosyl radical (19).

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