Escherichia coli contains the genetic information for three separate ribonucleotide reductases. Two of them (class I enzymes), coded by the nrdAB and nrdD genes, respectively, contain a tyrosyl radical, whose generation requires oxygen. The NrdAB enzyme is physiologically active. The function of the nrdD gene is not known. The third enzyme (class III), coded by nrdDG, operates during anaerobiosis. The DNA of Lactococcus lactis contains homologous to the nrdD genes. Surprisingly, an nrdD mutant of L. lactis grew well under standard anaerobic growth conditions. The ribonucleotide reductase system of this mutant was shown to consist of an enzyme of the NrdEF-type and a small electron transport protein. The coding operon contains the nrdEF genes and two open reading frames, one of which (nrdH) codes for the small protein. The same gene organization is present in E. coli. We propose that the aerobic class I ribonucleotide reductases contain two subunits, one coded by nrdAB, active in E. coli and eukaryotes (class la), the other coded by nrdE, present in various microorganisms (class lb). The NrdEF enzymes use NrdH proteins as electron transporter in place of thioredoxin or glutaredoxin used by NrdAB enzymes. The two classes also differ in their allosteric regulation by dATP.

Ribonucleotide reductases are essential enzymes that catalyze the reduction of ribonucleoside di-or triphosphates and thereby provide the building blocks required for DNA replication and repair. Three different classes of enzymes are known (1), each with a distinct protein structure but all requiring a protein radical for catalysis and all regulated by similar allosteric effects.

Class I reductases are aerobic enzymes present in all higher organisms and certain microorganisms, among them Escherichia coli (2). This bacterium actually has the potential to produce two separate class I enzymes. One of them, coded for by the nrdA and -B genes (3) is the functional enzyme during the growth of E. coli and has been the prototype for all class I enzymes. The second enzyme is coded for by the nrdE and -F genes (4), first discovered in Salmonella typhimurium (5), and is normally not fully functional. Expression of the chromosomal nrdD genes thus is not sufficient to complement mutations in nrdAB (5). The two enzymes show a limited sequence similarity but contain certain strategic amino acids in identical positions. They differ to some extent in their allosteric regulation and with respect to their hydrogen donors (6). A functionally active reductase of the NrdEF-type was recently found in Mycobacterium tuberculosis (7). Mycoplasma genitalium contains the nrdEF genes but not the nrdAB genes (8).

All class I enzymes consist of two proteins that are named R1 and R2 for NrdAB enzymes (2) and R1E and R2F (6) for the NrdEF enzymes. Each protein has specific functions: R1 and R1E contain the binding sites for both substrates and allosteric effectors and carry out the actual reduction of the ribonucleotide. R2 and R2F contain dithiol iron centers (9) and the tyrosyl radical (10) required for catalysis. Generation of the tyrosyl radical requires oxygen (9) and class I enzymes are therefore believed not to function in the absence of oxygen.

In line with this concept, anaerobically growing E. coli contains a third and completely different reductase (11) that has a glycyl radical (12) but no tyrosyl radical. This enzyme, coded by nrdD (12) and nrdG (13), is the prototype for a whole group of class III enzymes whose presence in several other anaerobically growing organisms can be inferred from specific DNA sequences. Such sequences have been found in the DNA from E. coli phage T4 (14), Lactococcus lactis,9 and Haemophilus influenzae (15). The glycyl radical of this group of enzymes is formed by a complicated activation reaction requiring S-adenosylmethionine and a reducing enzyme system (16). The exquisitely oxygen sensitive of this radical limits the function of class III enzymes to bacteria growing in the absence of oxygen.

A third group of reductases operates with a radical that does not require oxygen for its generation and is not oxygen sensitive. These enzymes use adenosylcobalamin as radical generator and function during both aerobic and anaerobic conditions. They are found in many different microorganisms and form a class II (17, 18).

The reduction of ribose requires a source of electrons. With class I and II enzymes, two small proteins named thioredoxin and glutaredoxin fulfill this function (19). They contain two active cysteine thiols that reduce by dithiol interchange two cysteines in the active center of the reductase. The reduced forms of both small proteins are regenerated by separate enzyme systems: thioredoxin, by a specific thioredoxin reductase...
Ribonucleotide Reduction in *L. lactis*

**Fig. 1. Separation of two protein fractions required for CDP reduction.** The material after ammonium sulfate precipitation was chromatographed on DEAE-cellulose as described under "Experimental Procedures." Fractions were analyzed for protein (+), DE1 activity (○), and DE2 activity (●). Each protein fraction was analyzed in the presence of an excess of the other fraction.

+ NADPH; glutaredoxin, by glutathione, glutathione reductase, and NADPH.

*L. lactis* subsp. cremoris is a member of the family of lactic acid Gram-positive bacteria that grow anaerobically but tolerate low concentrations of oxygen. Recently two open reading frames homologous to the nrdD and -G genes of *E. coli* were discovered and sequenced in *L. lactis* subsp. cremoris MG1363, suggesting the activity of an anerobic reductase in this bacterium. By marker exchange with an internally deleted nrdD gene an nrdD− mutant was constructed that surprisingly grew normally under standard anaerobic conditions. This suggested the presence of an additional reductase able to reduce ribonucleotides under anaerobic conditions supporting the growth of the mutant.

In this paper we characterize the ribonucleotide reductase of the mutant and identify it as a class I enzyme of the NrdEFtype. We also identify an apparently new type of hydrogen donor for this enzyme.

**EXPERIMENTAL PROCEDURES**

Materials—Wild type and nrdD strains of *L. lactis* MG1363 were kindly provided by G. Buist (University of Groningen). *E. coli* DH5α was from Clontech Laboratories Inc., plasmid pGEM-T from Promega Corp., and plasmid pBluescript SK(+) (pBSK) from Stratagene. Oligonucleotide primers were obtained from MWG-Biotech (Germany). The following materials were from Boehringer Mannheim: Taq DNA polymerase, DE52 anion-exchanger was from Whatman.

Ribonucleotide Reductase Activity Assay—As described below two separate proteins were found to be required for enzyme activity. The activity of each protein was determined in the presence of an excess of the second one. Standard conditions implied the use of 0.5 mM CDP (30 cpm/pmol), 0.3 mM dATP, 1 mM DTT, 10 mM MgCl₂, and 50 mM Tris-HCl, pH 8.0, in a final volume of 0.05 ml. Incubation was at 30 °C for 20 min and the amount of dCDP formed was determined after dephosphorylation to dCMP (21). One unit is defined as the formation of 1 nmol of dCDP/min, specific activity is units/mg protein.

Separation of Two Active Proteins—Bacteria were grown anaerobically to mid-logarithmic phase (OD₆₀₀ of 1.1) in 14 liters of M17 Broth, giving 20–40 g (wet weight) of tightly packed cell pellet. All manipulations during the following protein purification were done at close to 4°C. In a typical experiment, 5.2 g of bacterial pellet was sonicated in 8 ml of 50 mM Tris·HCl, pH 7.5, 1 mM phenylmethanesulfonyl fluoride, 1 mM EDTA, 10 mM DTT and centrifuged at 45,000 rpm in a 65 Beckman rotor for 60 min. Nucleic acids were removed by centrifugation after slow addition of 0.15 volume of 10% streptomycin sulfate to the supernatant solution. Solid ammonium sulfate was added during a 1-h period to the supernatant solution to 70% saturation. The resulting precipitate was collected by centrifugation, dissolved in a small volume of buffer A (50 mM Tris·HCl, pH 7.5, 10 mM DTT), and dialyzed against buffer A overnight with one change of buffer. The dialyzed solution was then added to a 30-ml column of DE52 equilibrated with buffer A. The column was first eluted at a rate of 0.5 ml/min with a linear KCl gradient (0–0.2 × KCl in buffer A, 60 × 30 ml), followed by additional elution with 60 ml of 0.2 × KCl in buffer A. Final elution was made with 40 ml of 0.4 × KCl in buffer A. Fractions (3 ml) were collected and analyzed for protein and reductase activity. This chromatographic step separated two fractions (Fig. 1), each inactive by itself but together providing reductase activity. From here on, the two fractions (DE1 and DE2) were purified separately.

Purification of DE1—Pooled chromatographic fractions were concentrated in Centricon-10 tubes (Amicon) to a final volume of 0.8 ml and chromatographed in two separate runs (0.4 ml each) on a column of Superdex-75 HR 10/30 (Pharmacia), equilibrated with 30 mM Tris·HCl, pH 7.5, 0.4 mM KCl, 1 mM DTT on a Pharmacia FPLC machine, run at 0.5 ml/min. DE1 activity emerged in a position of the chromatogram corresponding to an extrapolated apparent molecular mass of 10 kDa when compared with standard proteins (eg with white albumin, trypsin inhibitor, and lysozyme). The active protein was concentrated in Centricon-10 tubes. For determination of the N-terminal amino acid sequence, DE1 was further purified by chromatography on a microbore mixed C2/C18 reverse phase column attached to a SMART micropurification system (Pharmacia). The sample (0.05 ml) was added to the column equilibrated with 1% trifluoroacetic acid in water. Elution was made with a flat gradient of isopropyl alcohol (35–45%) in 1% trifluoroacetic acid and monitored at 214 nm. A major absorbing peak was eluted close to 40% isopropl alcohol, followed by three minor peaks. The materials from each peak were lyophilized and dissolved in 50 mM Tris·HCl, pH 8.0. The material in the major peak contained DE1 even though most enzyme activity was lost during this step. It was used for determination of the N-terminal sequence. On denaturing gel electrophoresis the material gave a single silver staining band, at approximately 10 kDa.

Purification of DE2—Appropriate fractions from the DE52 chromatogram were pooled, dialyzed against buffer A, and concentrated in Centricon-30 tubes to a final volume of 5 ml. MgCl₂ was added to a final concentration of 15 mM and the material was adsorbed to a 2-ml column of DATP-Sepharose (22). The column was first washed with 5 ml of buffer A, containing 25 mM MgCl₂, followed by 5 ml of the same buffer containing 1 mM ATP. Active DE2 was eluted from the column with the same buffer containing 5 mM DATP in place of ATP and concentrated in Centricon-30 tubes.

N-terminal Sequence of DE1—The material from reverse phase chromatography was first alkylated with 4-vinylpyridine in vapor phase by the following procedure. The solution was applied to a glass fiber filter of the type used for amino acid sequence determination and exposed to concentrated β-mercaptoethanol for 1 h by placing the filter above 25 μl of β-mercaptoethanol in an Eppendorf tube. The filter was then immediately placed at 56 °C over a 1:1 mixture of concentrated 4-vinylpyridine and acetonitrile.
dine and ammonia in a second Eppendorf tube for an additional hour. Finally a standard amount of Biobrene (Applied Biosystems) was applied to the filter which was subsequently subjected to automated Edman degradation in an Applied Biosystems 491 amino acid sequenator.

Partial Peptide Sequences from DE2—The dATP-eluete from the last purification step of DE2 was concentrated to a final volume of 0.05 ml in a Centricon-30 tube. After denaturation and alkylation of cysteines, the material was electrophoresed on a 7.5% SDS-polyacrylamide gel. Two bands were separated, one migrating together with an S. typhimurium RIE marker (6), the other just below an S. typhimurium R2F marker. The separated Coomassie-stained bands were excised and treated as described (23). Briefly, the gel pieces were washed with 0.2 M ammonium bicarbonate, 50% acetonitrile to remove the stain and de-replication and to replace the gel buffer with digestion buffer. The gel was thoroughly dried under a stream of nitrogen and was incubated overnight after addition of 0.5 μg of modified trypsin, sequence grade (Promega Corp.) to the moistened gel. Generated fragments were extracted with 0.1% trifluoroacetic acid, 60% acetonitrile and separated by reversed phase liquid chromatography on a μRPC C2/C18 SC 2.1/10 column, operated in the SMART system from Pharmacia. The peptides were eluted by a linear gradient of acetonitrile in 0.05% trifluoroacetic acid. Fractions containing non-homogeneous peptides were rechromatographed on a Sephasil C8 SC2.1/10 column, using the same conditions as above. Peptides selected for sequence analysis were analyzed in an Applied Biosystems model 470A sequencer (Foster City, CA).

Isolation of Partial Sequences of the L. lactis nrdEF Operon by PCR—Genomic DNA from L. lactis cremoris MG1363 was extracted (24) and amplified by PCR with primers designed on the basis of the determined N-terminal and internal amino acid sequences. Genomic DNA (0.1 μg) was incubated in a total volume of 50 μl together with 75 pmol of each primer, all four dNTPs (0.2 mM), 5 μl of 10 × PCR buffer (Boehringer Mannheim), and 1.5 units of Taq polymerase. The reaction was run with the following program: 3 min at 94 °C, 1 cycle/1 min at 94 °C, 1 min at 52°C, and 2 min at 72°C, 35 cycles/7 min at 72°C, 1 cycle. Purification and cloning of the chromosomal DNA with two restriction enzymes its unknown end is ligated to an oligomer (or vector) of known sequence that can be used to design the second, generic PCR primer. In our experiment, we ligated 50 ng of EcoRI-HindII-digested chromosomal L. lactis DNA in 10 μl at 15 °C overnight with 250 ng of pBSK plasmid DNA, after digestion with EcoRI and dephosphorylation. In the following PCR amplification, the pUC/M13 Reverse primer (PrRev) served as generic primer, whereas PrE (5'-TCCATCCTCTAAATCCTC-3') was the specific primer that binds the 5'-3' DNA strand 89 base pairs downstream the DE1 gene. Amplification was carried out as described above using the following program: 3 min at 94 °C, 1 cycle, 1 min at 94 °C, 1 min at 52 °C, and 2 min at 72 °C, 35 cycles/7 min at 72 °C, 1 cycle. Purification and cloning of products was done as described above.

Other Methods—Protein was determined (26) with bovine serum albumin as standard. Analytical protein gel electrophoresis was done with the Phastgel system (Pharmacia) on SDS-10–15% polyacrylamide gradient gels with Coomassie or silver staining. Mini Protean II (Bio-Rad) with a SDS-7.5% polyacrylamide gel was used for preparative purposes. DNA manipulations and Southern hybridizations were done by standard procedures (27). Digoxigenin labeling of probes was done with the DIG DNA labeling and detection kit (Boehringer Mannheim). Nucleotide sequences were determined with the dideoxy method with fluorescent primers and the Automated Laser Fluorescent DNA sequencer (Pharmacia). Computer analyses were made with the University of Wisconsin Genetics Computer Group package (version 8.0-Open VMS).

RESULTS

Evidence for Ribonucleotide Reductase Activity in Extracts from a L. lactis nrdD Mutant—The starting point for our work was the demonstration that extracts from the nrdD strain that has been grown anaerobically in media containing N₂CO₃ (96:4) could reduce CDP to dCDP. A growth curve from such a strain was indistinguishable from that of the wild type (data not shown). Under standard conditions for the R1:R2 enzyme from E. coli we found that the extracts reduced CDP equally well during aerobic and anaerobic conditions, excluding the activity of a class I reductase (11). The activity was not increased by addition of adenosylcobalamin, suggesting that it was not due to a class I reductase (17, 18). Positive evidence for a class I reductase came from the strong inhibition by

| Purification | Protein | Total units | Specific activity |
|--------------|---------|-------------|------------------|
|              |         | DE1 | DE2 | DE1 | DE2 |
| Extract      | mg      |     |     |     |     |
| Ammonium sulfate | 146  | 123 | 153 | 0.87 | 1.04 |
| DE1          | 127     | 61  | 30  | 0.48 | 0.23 |
| DEAE         | 13      | 11  | 11  | 0.83 | 0.16 |
| Superdex-75  | 0.10    |     |     |      |      |
| DE2          |         |     |     |      |      |
| DEAE         | 18      |     | 15  | 0.82 | 1.04 |
| dATP-Sepharose | 0.042  |     | 8.3 | 198  |
Fig. 3, A, effect of ATP and dATP on CDP reduction. Incubation was with 12 μg of DE1 and 25 μg of DE2 (DEAE fractions), replacing the standard concentration of 0.3 mM dATP by the indicated concentrations of either ATP (○) or dATP (●); B, effect of DTT on CDP reduction. DE2 (77 μg, DEAE fraction) was incubated under standard conditions except for the concentration of DTT shown on the abscissa, with (●) or without (○) 1.5 μg of DE1 (Superdex-75 fraction). μU, milliunits.

hydroxyurea (50% inhibition by 0.7 mM hydroxyurea in the standard assay, data not shown) and the finding that CDP was preferred as substrate over CTP (2). Enzyme activity was sensitive to repeated cycles of freezing and thawing. Extracts from cells grown to late logarithmic phase (OD600 more than 1.4) showed little activity.

Purification of the L. lactis Ribonucleotide Reductase System—Purification of the enzyme activity started from the crude extract of the nrdD strain as described under "Experimental Procedures." The two protein fractions DE1 and DE2 were separated by DEAE chromatography. Full enzyme activity required the presence of both fractions during the assay (Fig. 2). DE1 by itself showed no activity, DE2 by itself was marginally active. Each fraction could then be purified separately with results summarized in Table I. This purification is far from ideal since the recovery of each activity was very low (8.6% for DE1 and 5.4% for DE2). However, at the end of the procedure the two proteins had reached a high state of purity which made it possible to obtain partial amino acid sequences from each protein. Instead of improving on the yield we therefore decided first to carry out a preliminary characterization of the catalytic activity of the highly purified proteins and then to switch our efforts to the DNA level aiming at a cloning of genes and the final construction of overproducing strains.

Characterization of the Catalytic Activity—Several characteristics of CDP reduction by the crude bacterial extract already suggested that the activity depended on a class I enzyme. To distinguish between an NrdAB- and NrdEF-like enzyme (2, 6) we investigated with the purified proteins the allosteric regulation and the nature of the hydrogen donor for the reaction.

In enterobacteriaceae CDP reduction by the "classical" NrdAB enzyme requires ATP and is inhibited by dATP (1, 2), whereas the same reaction when catalyzed by the NrdEF enzyme is strongly stimulated by dATP, with ATP giving only a marginal effect (6). As shown in Fig. 3A, CDP reduction by the L. lactis enzyme system is strongly stimulated by dATP but not by ATP. From this point of view, the L. lactis enzyme thus behaves like an NrdEF enzyme.

With all NrdAB enzymes that were investigated in detail both thioredoxin and glutaredoxin are potential hydrogen donors (19). With the NrdEF enzyme from S. typhimurium thioredoxin was essentially inactive, whereas glutaredoxin was active (6). However, compared to NrdAB enzymes, the K_m for glutaredoxin was 1 order of magnitude higher for the NrdEF enzyme. High concentrations of DTT can function as an artificial hydrogen donor for all class I enzymes investigated so far (19).

DE2 from L. lactis had by itself very low CDP-reductase activity that depended on the presence of DTT (Fig. 3B). The "background" activity, without addition of DTT, seen in Fig. 3B is explained by the presence of a small amount of DTT in the DE2 preparation, required for stabilization of the protein. When DE1 was added together with DE2, DTT gave a strong stimulation of the reaction. This effect, together with the small molecular mass of DE1 suggested that this protein functioned as an intermediary between DTT and the actual ribonucleotide reductase present in DE2, similar to thioredoxin and glutaredoxin in other class I and class II systems.

Peptide Sequences Identify DE1 as a "Redoxin" and DE2 as an NrdEF Ribonucleotide Reductase—As described under "Experimental Procedures," partial peptide sequences were obtained for both DE1 and DE2 and are summarized in Table II.

For DE1, reverse phase chromatography had given a product that was homogeneous on electrophoresis on a denaturing SDS gel, with an apparent molecular mass of 10 kDa. The 40-step long N-terminal sequence shown in Table II is in accordance with the sequence deduced from the base sequence of the gene described below with the exception of Cys-19 that from the DNA sequence is expected to be Trp. Cys-10 and Cys-13 correspond to the cysteines in the Cys-X-X-Cys sequence characteristic for all glutaredoxins and thioredoxins (19). Overall, the N-terminal sequence presents good alignments with that of glutaredoxins (see "Discussion") and definitely identifies DE1 as a glutaredoxin-related redoxin that functions as an intermediate between DTT and DE2 to provide the electrons required for the reduction of ribose.

The DE2-peptides in Table II were obtained by trypsin di-
gestion of the proteins from two bands of an SDS gel as described under “Experimental Procedures.” These bands had mobilities close to those for class I R1E and R2F proteins, and the peptides are labeled accordingly in Table II. Computer comparison of the peptides obtained from the larger protein gave the best alignments with known amino acid sequences in the peptides are labeled accordingly in Table II. Computer comparison of the peptides obtained from the larger protein gave the best alignments with known amino acid sequences in the S. typhimurium sequence, with underlined residues common to S. typhimurium and L. lactis. These results identify the ribonucleotide reductase from L. lactis as an NrdEF enzyme.

Partial Cloning of the Genes of the L. lactis Reductase System—On the assumption that the genes of the nrdEF operons of L. lactis and S. typhimurium are organized in the same general way, we constructed four primers to amplify almost the whole region of the L. lactis operon by two PCR amplifications, as indicated in Fig. 4. A fragment of 2.8 kb was isolated using primers corresponding to peptide 1 (PrA) and peptide 4 (PrB), and a fragment of 1.3 kb with primers for peptide 4 (PrC) and peptide 5 (PrD). The two fragments were cloned in vector pGEM-T, giving rise to plasmids pUA559 and pUA560, respectively. DNA sequencing of the extremes of both fragments from pUC/M13 Universal primers confirmed the presence of the L. lactis nrdEF operon by a high homology with the S. typhimurium operon (data not shown). In this step we also determined the 3′-end sequence of the gene encoding DE1.

Cloning and Sequencing of the Full-length DE1 Gene—To obtain a chromosomal fragment containing the whole DE1 coding region and its putative promoter upstream region we used single specific primer PCR (25) as described under “Experimental Procedures.” Restriction analysis of plasmid pUA559 identified a HindII and an EcoRI site at 0.7 and 1.3 kb, respectively, from the PrA extreme of the fragment cloned into the plasmid. The 0.7-kb fragment obtained by digestion of pUA559 with HindII and HindIII was purified, digoxigenin-labeled, and used as a probe in a Southern analysis of L. lactis genomic DNA. This showed that the DE1 gene was contained in 1.6-kb EcoRI-EcoRI and 1.05-kb HindII fragments (see Fig. 5A). Total L. lactis DNA digested with EcoRI-HindIII was ligated to EcoRI-digested and dephosphorylated pBSK plasmid. The ligated material was used directly for single specific primer PCR amplification (25) with primers PrRev and PrE giving rise to a 0.8-kb fragment which was cloned in pGEM-T plasmid (pUA561). The fragments present in several independent clones were sequenced in both strands to ascertain that no Taq polymerase-induced mutations were present.

The nucleotide sequence of the 680-base pair fragment present between the chromosomal EcoRI site and the PrE primer binding site is shown in Fig. 5B and has been deposited in the EMBL database under accession number X92690. The DE1 gene is formed by 219 nucleotides, encoding a putative protein of 72 residues with a predicted molecular mass of 8.3 kDa. A ribosome binding site is located 7 base pairs upstream of the ATG-triplet coding for the first methionine. Also several putative TATA boxes are found. Downstream of this gene, the 5′-extreme of an additional ORF is found, also preceded by a less conserved ribosome binding site. The amino acid sequence predicted by this ORF shows a considerable similarity (56%) to that of the ORF2 present in the nrdEF operons of E. coli and S. typhimurium (4), with, respectively, 32.4 and 29.4% identities. The G + C content of the entire sequenced fragment is 31.3%, that for the DE1 coding region is 34.3%, close to the 37% established for L. lactis (28). Also the codon usage of the DE1 gene is in agreement with that established for L. lactis (data not shown). Both results indicate that the incorporation of the gene for DE1 into the L. lactis genome is not a recent event.
Consensus shows by DE1 strongly suggests that it is a similar redoxin protein. We concentrationsofglutaredoxin1canfullfilthisfunctionforthe R1proteinofclassIenzymesoftheNrdAB-type(19).High taredoxins transfer electrons from NADPH via glutathione to considerablehomologytovariousglutaredoxinsequences.Glu- Barbe´, unpublished data.

As described under “Discussion,” the DE1 sequence shows considerable homology to various glutaredoxin sequences. Glutaredoxins transfer electrons from NADPH via glutathione to the R1 protein of class I enzymes of the NrdAB-type (19). High concentrations of glutaredoxin 1 can fulfill this function for the R1E protein of S. typhimurium (6). The amino acid sequence of DE1 strongly suggests that it is a similar redoxin protein. We propose the designation nrdH for the gene coding for DE1.

**DISCUSSION**

The fact that an nrdD mutant of L. lactis was able to grow anaerobically may suggest that the gene is dispensable for anaerobic growth of the bacteria. A similar result was recently obtained with E. coli containing an interrupted nrdD gene. It then came as a surprise to find that the anaerobic growth of the L. lactis mutant was supported by a class I ribonucleotide reductase. The general wisdom is that these enzymes require oxygen for the generation of their tyrosyl radical (2). We could not demonstrate any other reductase activity in mutant extracts and it is therefore a fair conclusion that the class I enzyme provided the deoxyribonucleotides for DNA replication under our anaerobic growth conditions.

The most probable explanations for this apparent paradox is that small amounts of oxygen remained during the "anaerobic" incubation and that the NrdD enzyme has a very high affinity for oxygen, sufficient to make possible the generation of the tyrosyl radical under those conditions. When in recent experiments sodium sulfide was added to the medium to scavenge traces of oxygen the growth of the nrdD mutant was inhibited severely, whereas the wild type strain grew normally. This suggests that a functional nrdD gene is indeed required under stricter anaerobic conditions.

We then found also that extracts from wild type L. lactis contained the same kind of activity as the the nrdD mutant leading to the general conclusion that the active ribonucleotide reductase of L. lactis belongs to the NrdD group of reductases. These enzymes differ in several respects from the NrdD enzymes originally discovered in E. coli and also found in all eukaryotes. The differences are large enough to justify a definition of two subgroups of class I enzymes, with NrdD enzymes forming subclass Ia and the NrdD enzymes subclass Ib. Members of each subclass are primarily recognized from their amino acid sequence. As to known functional differences, they concern the allosteric effect of dATP and the nature of the small protein that shuttles electrons from NADPH to the reductase.

dATP is a general inhibitor for class Ia enzymes but is a positive effector for CDP reduction by class Ib enzymes (2, 6). In this respect they behave as class II enzymes (29). Furthermore, the reductin identified in this paper as an electron transporter for the L. lactis reductase is different from the thioredoxin and glutaredoxin used by class Ia enzymes (19).

This reductin was separated from the L. lactis reductase proper by chromatography on DEAE-cellulose early during purification. The reduction of CDP by the reductase then showed an almost absolute requirement for DTT. This is an usual behavior for class I (and II) reductases, since in all cases known so far high concentrations of DTT can at least partially short-circuit the specific redoxin and directly reduce reductase thios of the reductase. With its 72 amino acids, the new reductin is smaller than any other similar electron transport protein. The two cysteines in positions 10 and 13 harbor the redox-active thios that carry out the transsthiolelation required for the maintenance of the active thios of the reductase. The gene for the reductase (nrdH) forms part of the nrdEF operon.

On searching for amino acid sequence homology it became apparent that the protein coded by nrdH presents a considerable degree of similarity with various forms of glutaredoxins and glutaredoxin-like proteins (Fig. 6). All alignments were made such that the two reductase-cysteine of the various proteins occupy identical positions. It then appears that the sequence of the new reductin shows 48.6% similarity and 27.8% identity with that of glutaredoxin 3 (30) and 40.3% similarity and 16.7% identity with that of glutaredoxin 1 (31). However, the greatest similarity is found with the GRX1 products of the nrdEF operons of E. coli (63.9% similarity, 36.1% identity) and S. typhimurium (62.5% similarity, 33.3% identity). The genes for the three proteins also occupy identical positions within the operon. We propose that ORF1 is a nrdH gene and that all three genes have a similar redoxin function for class Ib reductases.

Whereas the amino acid sequence classifies the NrdH proteins as a glutaredoxin-like protein, they can hardly be classified as glutaredoxins. As the name implies, glutaredoxins use glutathione for the reduction of the disulfide bond between the two cysteines of the active center during the shuttling of electrons. However, extracts of L. lactis contain no glutathione. Furthermore, the NrdH proteins do not contain the amino acid sequences of glutaredoxin 1 (boxed in Fig. 6) responsible for

4 X. Garriga, A. J ordan, I. Gibert, R. Eliasson, P. Reichard, and J. Barbé, unpublished data.
5 F. Åslund, unpublished data.
6 Y. Aharonowitz, personal communication.
glutathione binding (32). Also the sequence in the active center (Cys-Met/Val-Gln-Cys) differs from the typical Cys-Pro-Tyr-Cys of glutaredoxins. The sequence also differs from the Cys-Gly-Pro-Cys of thioredoxins. Naming the new redoxin must await the outcome of experiments now in progress that aim at the definition of the enzyme system that reduces the disulfide bond in the active site.

Several other glutaredoxin-like proteins have been described in the literature, often with unknown functions. Among them the protein from Methanobacterium thermoautotrophicum (33) may be involved in ribonucleotide reduction. Also in this case the protein does not contain the amino acids thought to be required for glutathione binding and the microorganism lacks glutathione. Another potential glutaredoxin-like protein corresponds to the ORF2 gene of the rubredoxin operon of Clostridium pasteurianum (20). In this case, the neighbor gene of the operon (ORF1) codes for a thioredoxin reductase-like protein.

The first class Ib enzymes were discovered in S. typhimurium and E. coli as "silent" enzymes whose physiological function is still not understood. The genes are poorly transcribed and chromosomal gene expression is not sufficient to complement mutants in the genes coding for the active class Ia enzymes. Recently, ribonucleotide reductase-like microorganisms were characterized as class Ib enzymes and our work now adds L. lactis to this group. Among microorganisms, class Ia enzymes have so far been found only in Enterobacteriaceae and the closely related H. influenzae. It seems possible that members of class Ib are the prevalent class I enzymes of microorganisms.

Acknowledgment—We are indebted to Lena Hernberg for the N-terminal sequence of the NrdH protein.

REFERENCES

1. Reichard, P. (1993) Science 260, 1773-1777
2. Fontecave, M., Nordlund, P., Eklund, H., and Reichard, P. (1992) Adv. Enzymol. Relat. Areas Mol. Biol. 65, 147-183
3. Carlson, J., Fuchs, J. A., and Messing, J. (1984) Proc. Natl. Acad. Sci. U. S. A. 35, 2494-2497
4. Jordan, A., Aragall, E., Gibert, I., and Barbe, J. (1996) Mol. Microbiol. 19, 777-790
5. Jordan, A., Aragall, E., Gibert, I., and Barbe, J. (1999) J. Bacteriol. 176, 3420-3427
6. Jordan, A., Pontis, E., Atta, M., Krock, M., Gibert, I., Barbe, J., and Reichard, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12892-12896
7. Yang, F., Lu, G., and Rubin, H. (1994) J. Bacteriol. 176, 6738-6743
8. Fraser, H. C., Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., Bult, C. J., Kerlavage, A. R., Sutton, G., Kelley, J. M., Fritschman, J. L., Weidman, J. F., Small, K. V., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T. R., Saudek, D. M., Phillipps, C. A., Merrill, J. M., Tomb, J. -F., Dougherty, B. A., Bldt, K. F., Hu, P. -C., Lucier, T. S., Peterson, S. N., Smith, H. O., Hutchison, C. A., III, and Venter, J. C. (1995) Science 269, 397-403
9. Peterson, L. E., Ehrenberg, A., Sjöberg, B-M., and Reichard, P. (1980) J. Biol. Chem. 255, 6706-6712
10. Larsson, A., and Sjöberg, B-M. (1986) EMBO J. 5, 2037-2040
11. Reichard, P. (1993) J. Biol. Chem. 268, 8833-8836
12. Sun, X., Ollagnier, S., Schmidt, P. A., Atta, M., Mulliez, E., Lepape, L., Eliasson, R., Gräslund, A., Fontecave, M., Reichard, P., and Sjöberg B-M. (1996) J. Biol. Chem. 271, in press
13. Sun, X., Eliasson, R., Pontis, E., Andersson, J., Buist, G., Sjöberg, B-M., and Reichard, P. (1995) J. Biol. Chem. 270, 2443-2446
14. Young, P., Öhman, M., Xu, M. Q., Shub, D. A., and Sjöberg, B-M. (1994) J. Biol. Chem. 269, 20229-20233
15. Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Dougherty, B. A., Merrick, J. M., McKenney, K. K., Sutton, G., FitzHugh, W., Fields, C., Gocayne, J. D., Scott, J., Shirley, R., Liu, L.-I., Glodek, A., Kelley, J. M., Weidman, J. F., Phillips, C. A., Spriggs, T., Hedblom, E., Cotton, M. D., Utterback, T. R., Hanna, M. C., Nguyen, T. D., Saudak, D. M., Brandon, R. C., Fine, L. D., Fritchman, J. L., Fuhrmann, J. L., Geoghegan, N. S. M., Ghmeh, C. L., McDonald, L. A., Small, K. V., Fraser, C. M., Smith, H. O., and Venter, J. C. (1995) Science 269, 496-512
16. Harder, J., Eliasson, R., Pontis, E., Ballinger, M. D., and Reichard, P. (1992) J. Biol. Chem. 267, 25484-25552
17. Blakley, R. L., and Barker, H. A. (1964) Bioch. Biophys. Res. Commun. 16, 291-297
18. Booker, S., and Stubbe, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8352-8356
19. Holmgren, A. (1889) J. Biol. Chem. 264, 13963-13966
20. Mathieu, I., Meyer, J., and Moulis, J-M. (1992) Appl. Environ. Microbiol. 58, 2971-2976
21. Reichard, P. (1958) Acta Chem. Scand. D, 12, 2048
22. Berglund, O., and Eckstein, F. (1972) Eur. J. Biochem. 28, 492-496
23. Hofman, U., Wernstedt, C., Ganzer, J., and Heldin, C.-H. (1995) Anal. Biochem. 224, 451-455
24. Leenhouts, K. J., Kok, J., and Venema, G. (1989) Appl. Environ. Microbiol. 55, 394-400
25. Shyamala, V., and Ames, G. F.-L (1993) Methods Mol. Biol. 15, 339-348
26. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
27. Sambrook, J., Fritsch, E. P., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Chopin, A. (1933) FEMS Microbiol. Rev. 12, 21-39
29. Chen, A. K., Bhat, A., Hopper, S., Abrams, R., and Franzen, J. S. (1977) Biochemistry 16, 654-661
30. Åslund, F., Nordstrand, K., Berndt, K. D., Nikkola, M., Bergman, T., Porsling, H., Jornvall, H., Otting, G., and Holmgren, A. (1996) J. Biol. Chem. 271, 6736-6745
31. Höög, J.-O., Jornvall, H., Holmgren, A., Carlquist, M., and Persson, M. (1983) Eur. J. Biochem. 136, 223-232
32. Bushweiter, J. H., Billiter, M., Holmgren, A., and Würthrich, K. (1994) J. Mol. Biol. 235, 1535-1597
33. McFarlan, S. C., Terrel, C. A., and Hogenkamp, H. P. L. (1992) J. Biol. Chem. 267, 10561-10569