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Cloning and Sequencing of the Genes from *Salmonella typhimurium* Encoding a New Bacterial Ribonucleotide Reductase

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A plasmid library of *Salmonella typhimurium* was used to complement a temperature-sensitive *nrdA* mutant of *Escherichia coli*. Complementation was obtained with two different classes of plasmids, one carrying the *E. coli nrdAB*-like genes and the second containing an operon encoding a new bacterial ribonucleotide reductase. Plasmids harboring these new reductase genes also enable obligately anaerobic *nrdB*::Mud1 *E. coli* mutants to grow in the presence of oxygen. This operon consists of two open reading frames, which have been designated *nrdE* (2,145 bp) and *nrdF* (969 bp). The deduced amino acid sequences of the *nrdE* and *nrdF* products include the catalytically important residues conserved in ribonucleotide reductase enzymes of class I and show 25 and 28% overall identity with the R1 and R2 proteins, respectively, of the aerobic ribonucleoside diphosphate reductase of *E. coli*. The 3′ end of the sequenced 4.9-kb fragment corresponds to the upstream region of the previously published *proU* operon of both *S. typhimurium* and *E. coli*, indicating that the *nrdEF* genes are at 57 min on the chromosomal maps of these two bacterial species. Analysis of the *nrdEF* and *proU* sequences demonstrates that transcription of the *nrdEF* genes is in the clockwise direction on the *S. typhimurium* and *E. coli* maps.

In both eukaryotic and prokaryotic cells, the biosynthesis of deoxyribonucleotides (dNTPs) from the corresponding ribonucleotides is catalyzed by ribonucleotide reductases (RR), with the exception of dTTP, whose synthesis is derived from dCTP and dUDP. RR plays a crucial role in the balanced supply of DNA precursors. Imbalances of the dNTPs have been associated with different genetic effects, besides modifying the sensitivity of cells to DNA-damaging agents (26). Three classes of RR have been described so far (for a review, see reference 35). Class I enzymes are aerobic and exist in higher organisms and in some prokaryotes. *Escherichia coli* ribonucleoside diphosphate reductase (RDP reductase) is the best known of this group. The *nrdA* and *nrdB* genes encode the α and β polypeptide chains, which form the R1 (α_2) and R2 (β_2) subunits of the RDP reductase. The *E. coli* RDP enzyme contains a stable radical located at Tyr-122 as an essential component for catalysis (27). Class II enzymes, present in many prokaryotes, employ adenosyl cobalamin as a radical generator (3). Finally, the only enzyme of class III identified is the anaerobic RTP reductase of *E. coli*, encoded by the *nrdD* gene (39). This enzyme requires *S*-adenosylmethionine, which probably is a glycine radical generator (for a review, see reference 36).

The *nrdAB* genes of *E. coli* have been proposed as genes of vital importance because for many years only conditional-lethal *nrdAB* mutants were found (34, 40). Some years ago, it was shown that null *nrdB* mutants, obtained as a consequence of a Mud1 insertion, are viable in the absence of oxygen (21), since under these conditions, an anaerobic RTP reductase is active (13). In this respect, *E. coli* cells growing under either fermentative or nitrate-respiring conditions have a lower basal level of *nrdAB* gene transcription (6). Several regions involved in either positive or negative control of the *nrdAB* genes of *E. coli* have been detected upstream of the coding region (41, 42). The activity of the *E. coli* RDP reductase is increased when DNA replication is inhibited (12, 16). Furthermore, DNA damage enhances the transcription of *nrdAB* genes (18). This increase is related to the SOS response (18).

We were interested in cloning the *nrdAB* genes of *Salmonella typhimurium* to determine if these genes have the same control regions as those of *E. coli*. Nevertheless, during these experiments we isolated not only the *nrdAB* genes but also an operon encoding a new RR. The DNA sequences and locations of these new RR-encoding genes and their predicted amino acid sequences are reported in this paper.

The bacterial strains and plasmids used and their relevant characteristics are shown in Table 1. *E. coli* and *S. typhimurium* strains were grown at either 30, 37, or 42°C in Luria-Bertani medium, Terrific Broth, or AB minimal medium containing 0.2% glucose and 0.4% Casamino Acids (38). Anaerobic growth was in either plates or filled flasks for liquid cultures, both being incubated in anaerobic jars with the GasPak system (Becton Dickinson). Heterologous triparental matings were done as described previously (4). Complementation of temperature sensitivity was determined by either plating samples of a liquid culture or streaking single colonies on minimal medium plates at both 30 and 42°C. Abolition by the chromosomal insert of the *nrdB1* mutation, which in the KK450 strain decreases R2 activity (15), was tested by hydroxyurea resistance (34). This characteristic was determined by plating equal portions of appropriately diluted exponential-phase cultures of the KK450 strain on minimal agar plates containing hydroxyurea at 1 mg/ml. After 3 days of incubation, the presence or absence of growth in these plates was analyzed. Restriction enzyme digestions, subcloning procedures, plasmid extractions, gel electrophoresis, DNA labelling with digoxigenin, and hybridization procedures were carried out by standard methods (38). *E. coli* DH5αF′ cells were transformed either by electroporation (9) or by using frozen competent cells (20). Prior to DNA sequencing, a set of exonuclease III-mediated nested deletions of the isolated *S. typhimurium* insert was created by using the Erase-a-Base system (Promega Corporation). The DNA sequence of each one of these clones was determined by the dideoxy method with fluorescent primers and the Auto-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source (reference)
E. coli K-12 strains		
DH5αF′	*recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 *deoR* φ80d*lac*ZM15 F′	Laboratory stock
KK450	*nrdA*(Ts) *nrdB1 thyA thr leu thi deo tonA lacY supE44 gyrA*	B. M. Sjöberg (34)
H1491	MC4100 *aroB nrdB*::Mud1	K. Hantke (21)
UA4851	KK450 Rif^r	This study
Plasmids		
F′143		B. Bachmann
pPS2	Tc^r NrdAB$^+$	B. M. Sjöberg (30)
pRK404	Tc^r Mob$^+$	G. Ditta (8)
pRK2013	Km^r Tra$^+$ Mob$^+$	G. Ditta (8)
pBluescript SK(+)	Amp^r	Laboratory stock
pUA326	pRK404 containing a 4.9-kb fragment carrying the *nrdEF* genes	This study
pUA335	pBSK containing a 4.9-kb *Xma*I-*Pst*I fragment carrying the *nrdEF* genes	This study
pUA338	pBSK containing a 4-kb fragment by 3′ exonuclease III deletion from pUA335	This study
pUA341	pBSK containing a 3.45-kb *Not*I-*Acc*I fragment from pUA338, under P_{lac} control	This study
pUA343	Like pUA341 but with the 3.45-kb *Not*I-*Acc*I fragment in inverted orientation	This study
pUA344	pBSK containing a 3.2-kb fragment by *Cla*I-*Cla*I deletion from pUA341	This study
pUA345	pBSK containing a 3.2-kb fragment by *Sac*I-*Sac*I deletion from pUA341	This study
pUA395	pBSK containing a 3.6-kb fragment by 3′ exonuclease III deletion from pUA335	This study
pUA396	pBSK containing a 4.7-kb fragment by 5′ exonuclease III deletion from pUA335	This study

matic Laser Fluorescent DNA Sequencer (Pharmacia). The entire nucleotide sequence was determined with both DNA strands. Computer analysis was carried out with the University of Wisconsin Genetics Computer Group package (version 7.2). Sequences from several RR were obtained from the Swiss-Prot data bank.

Isolation of genes encoding a new RR. A library of *S. typhimurium* LT2 chromosomal DNA was constructed by cloning size-fractioned, partially digested *Sau*3AI restriction fragments with an average size of 5 to 8 kb into the *Bam*HI site of the broad-host-range plasmid pRK404 (8) and then was electrotransformed into *E. coli* DH5αF′. To isolate *nrdAB* genes from *S. typhimurium*, the library was transferred by triparental mating into the temperature-sensitive *nrdA*(Ts) mutant of *E. coli* (strain UA4851), plating exconjugants onto rifampin-, tetracycline-, and thymidine-supplemented Luria-Bertani agar plates at 42°C. Several temperature-resistant clones were observed after 2 days of incubation. Plasmid DNA of each of these exconjungants was extracted and retransformed into the KK450 strain to confirm the temperature-resistant phenotype. Restriction and Southern analysis (with a 5.5-kb *Eco*RI-*Pst*I fragment of the pPS2 plasmid containing the *nrdAB* genes of *E. coli* as a probe [34]) of plasmid DNA isolated from these retransformed clones indicated the presence of two kinds of clones. One kind hybridized with the *nrdAB* genes from *E. coli* (Fig. 1A, lane 3), whereas the second did not (Fig. 1A, lane 4). Only two types of RR genes have been described for enterobacteria, i.e., the *nrdAB* and *nrdD* genes from *E. coli*, encoding the aerobic and anaerobic enzymes, respectively. Since the anaerobic RR does not complement *nrdAB* mutants under aerobic conditions, these other clones would contain the genes for a new aerobic RR. For this reason, we decided to concentrate on the smallest chromosomal insert (about 4.9 kb) of this bacterium which did not hybridize with the *nrdAB* genes from *E. coli*. The plasmid carrying this insert was designated pUA326.

The hydroxyurea hypersensitivity of the *nrdB1* mutant strain KK450 was also abolished by the chromosomal fragment contained in the pUA326 plasmid (Fig. 2). The O_2 sensitivity of the *nrdB*::Mud1 mutant of *E. coli* H1491 (21) was also suppressed by pUA326. Thus, the efficiency of plating of H1491 (pUA326) cells in the presence of oxygen was 0.75. Furthermore, Southern blot hybridization of total DNA from *E. coli* and *S. typhimurium* digested with *Eco*RI-*Pst*I and probed with a 2.8-kb *Cla*I-*Hin*dIII fragment of the 4.9-kb insert corroborated that the cloned fragment is present in the *S. typhimurium* chromosome as well as in the *E. coli* chromosome (Fig. 1B), although the species showed different banding patterns: the chromosomal DNA of *E. coli* exhibited a band at 11 kb (Fig.

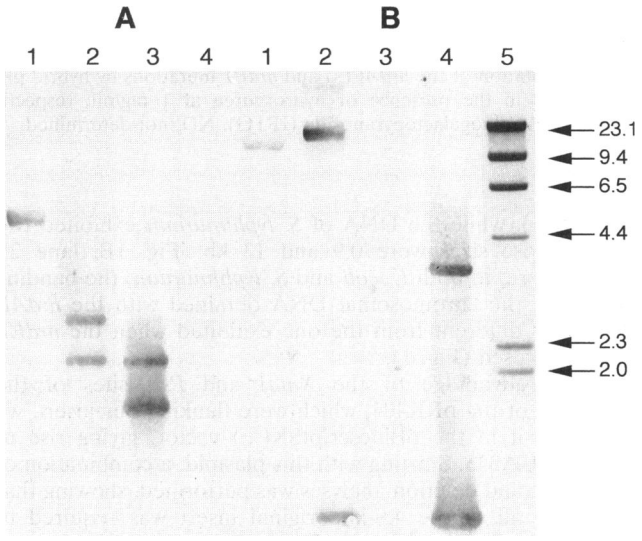
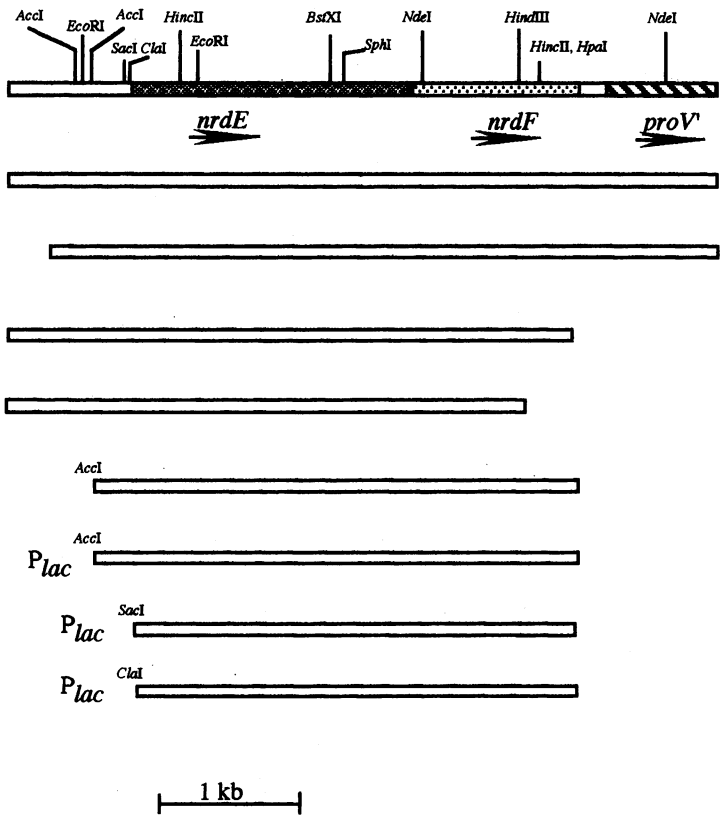


FIG. 1. Southern hybridization of chromosomal DNA from *E. coli* (lanes 1), *S. typhimurium* (lanes 2), a pRK404 plasmid derivative containing the *nrdAB*-like genes from *S. typhimurium* (lanes 3), and plasmid pUA326 carrying the *nrdEF* genes from *S. typhimurium* (lanes 4), all doubly digested with *Eco*RI and *Pst*I. DNA was hybridized with either the 5.5-kb *Eco*RI-*Pst*I fragment of the pPS2 plasmid carrying the *nrdAB* genes from *E. coli* (A) or the 2.7-kb *Cla*I-*Hin*dIII internal fragment of the *nrdEF* genes from *S. typhimurium* contained in the pUA326 plasmid (B). Lane 5 of panel B is a *Hin*dIII digest of digoxigenin-labelled lambda DNA as a molecular weight marker. Numbers on the right are sizes in kilobases.



Plasmid	Complementation $nrdA_{(ts)}$	Complementation *nrdB1*
pUA326	0.67	0.3
pUA335	0.93	0.32
pUA396	2.1×10^{-6}	$<1 \times 10^{-7}$
pUA338	0.70	0.38
pUA395	2×10^{-5}	$<5.5 \times 10^{-8}$
pUA343	1.5×10^{-6}	4.5×10^{-7}
pUA341	0.10	0.015
pUA345	0.13	0.015
pUA344	0.11	0.020
pBSK	$<4.1 \times 10^{-9}$	1.25×10^{-8}
F'143	1	ND

FIG. 2. Physical map and complementation analysis of the inserts contained in plasmid pUA326 and their derivatives, obtained by either subcloning or nested deletions of the original 4.9-kb fragment of the *S. typhimurium* chromosome abolishing temperature sensitivity of the KK450 mutant of *E. coli*. P_{lac} denotes plasmids at which the *lac* promoter has been inserted immediately upstream of the chromosomal fragment. The complementation of the *nrdA*(Ts) and *nrdB1* mutations by hybrid plasmids was derived from the efficiency of plating either on minimal medium at 42°C or in the presence of hydroxyurea at 1 mg/ml, respectively. P_{lac}-mediated complementation was carried out in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG). ND, not determined.

1B, lane 1), while the DNA of *S. typhimurium* exhibited two bands whose sizes were 0.9 and 13 kb (Fig. 1B, lane 2). Furthermore, in both *E. coli* and *S. typhimurium*, the banding pattern of the chromosomal DNA obtained with the *nrdAB* probe was different from the one exhibited when the *nrdEF* probe was used (Fig. 1).

Taking advantage of the *Xma*I and *Pst*I sites of the polylinker of the pRK404, which were flanking our insert, we subcloned it in the pBluescriptSK(+) vector, giving rise to plasmid pUA335. Starting with this plasmid, a combination of subcloning and deletion analyses was performed, showing that practically all of the 4.9-kb original insert was required to support the growth of the KK450 strain at 42°C (Fig. 2).

Nucleotide and amino acid sequences of the new RR genes. The nucleotide sequence of the 4.9-kb fragment of plasmid pUA335 is presented in Fig. 3. Several open reading frames can be identified in the insert, although the deduced products of only the two largest have some identity with other RR enzymes. The sequence between positions 836 and 2980 (consisting of 2,145 bp) is suggested to correspond to one subunit (designated *nrdE*) of the new reductase (Fig. 3). This protein has been obtained in pure form and shown to have the sequence ATTTPERVMQXTMD at its N-terminal end (24). This is in complete agreement with the amino acid sequence deduced from the 42 bp starting at position 839. We suggest that translation starts with Met at the UUG triplet (positions 836 to 838), with eventual loss of this Met. In fact, it is known that the UUG codon also specifies initiation in bacteria, although at a low frequency (7). The loss of the N-terminal methionine has also been reported to occur for the R1 and R2 subunits of the aerobic RR of *E. coli* (5). The second open reading frame, called *nrdF*, is 960 bp long (Fig. 3, positions 2991 through 3950). The predicted molecular weights of the deduced proteins of the *nrdE* and *nrdF* genes were 80,519 and 36,281, respectively. The *nrdE* and *nrdF* open reading frames were separated by a small noncoding region of 10 bp. Putative ribosomal binding sites were located 16 bp upstream of the initiation codon for *nrdE* and 12 bp upstream of that for *nrdF*. No canonical TATA box preceded by a −35 promoter consensus sequence was detected upstream of the *nrdE* gene. However, the subcloning and deletion analysis shown in Fig. 2 (plasmids pUA396, pUA341, and pUA343) indicated that the 836 bp upstream of the *nrdE* open reading frame contains the promoter from which the transcription of these genes occurs. Downstream from the stop codon of the *nrdF* gene (TAA), a putative transcriptional terminator was detected as a perfect

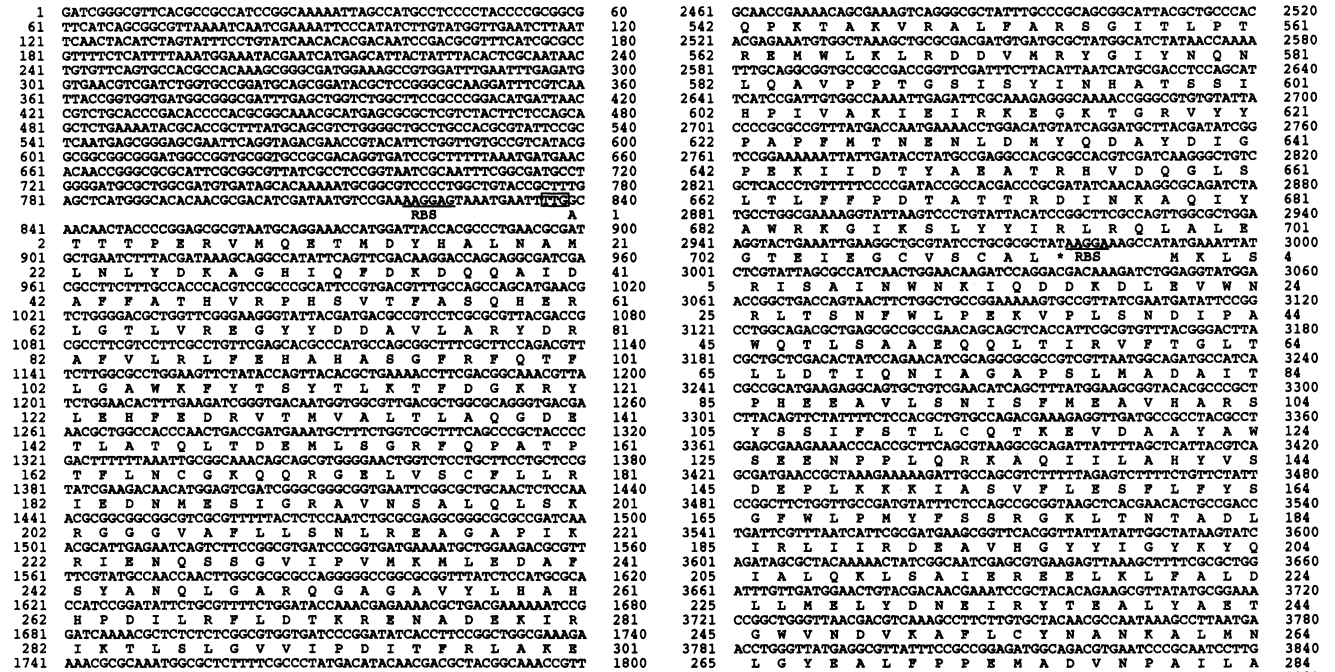


FIG. 3. Nucleotide and deduced amino acid sequences of the *nrdEF* genes of *S. typhimurium* LT2. The putative ribosome binding site (RBS) sequences are underlined. A 12-bp palindrome which may serve as a transcription terminator is indicated by inverted arrows. The ribosome binding site and the first coding triplet of the first gene (*proV*) of the *proU* operon of *S. typhimurium* (33) are boldface and underlined. The TTG triplet proposed as the translation start of the *nrdE* subunit is boxed.

inverted repeat of 12 nucleotides separated by 8 nucleotides (positions 3958 to 3989), followed by a run of AT base pairs.

The sequence of the 3′ end of our 4.9-kb fragment corresponds to the 5′ regions of the *proU* operons of *S. typhimurium* and *E. coli*, indicating that in the chromosomes of both species, the *nrdEF* genes are upstream of the *proU* operon and, consequently, are located at 57 min on the chromosomal maps of these two bacteria (Fig. 3). In agreement with this, plasmid F′143 (which contains the region around 57 min of the *E. coli* chromosome) abolishes the temperature sensitivity of the KK450 mutant (Fig. 2). From previously known data about the *proU* operon (19, 33), it is possible to conclude that the transcription of *nrdEF* genes is in the clockwise direction on the *S. typhimurium* and *E. coli* maps.

Comparison of the *nrdEF* predicted protein sequences with those of other aerobic RR of class I shows a limited identity (Table 2). Thus, the *nrdE* and *nrdF* products have, respectively, 25 and 28% overall amino acid sequence identities with the R1 and R2 subunits of the aerobic RR of *E. coli*. Amino acid sequence alignments of the class I RR from different species have shown that they are homologous within three groups: the

TABLE 2. Amino acid identity of the large and small subunits of the class I RR from different species with products of the *nrdEF* genes, respectively, of *S. typhimurium*[a]

Species	% identity of RR subunit with:	
	nrdE product	*nrdF* product
E. coli	25.0	27.7
Bacteriophage T4	23.3	22.1
Human	27.5	24.3
Mouse	27.6	24.3
S. cerevisiae	17.8	22.3
Vaccinia virus	25.8	19.2
HSV-1	24.7	20.9
HSV-2	27	21.2
Varicella-zoster virus	26.2	26.8
EBV	23.2	22.3
Rir3-Yeast	24.5	

[a] The sequences were aligned by using the Gap program of the University of Wisconsin Genetics Computer Group package. HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; EBV, Epstein-Barr virus; Rir3-Yeast, product of the *RNR3* gene of *S. cerevisiae*, encoding a duplicated R1 subunit.

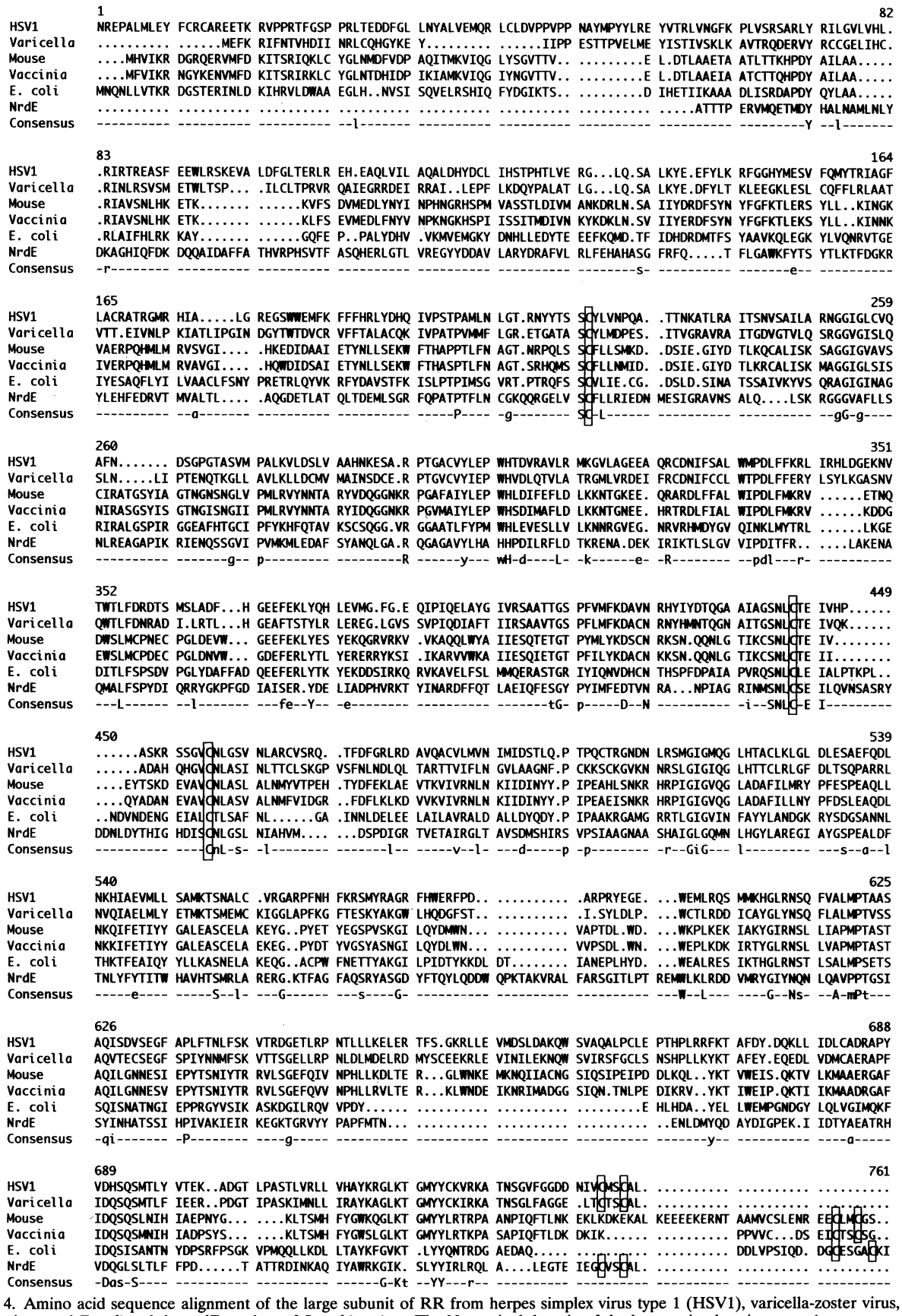


FIG. 4. Amino acid sequence alignment of the large subunit of RR from herpes simplex virus type 1 (HSV1), varicella-zoster virus, mouse, vaccinia virus, and *E. coli* and the *nrdE* product of *S. typhimurium*. The N-terminal domain of the herpes simplex virus type 1 sequence, which is unrelated to all other sequences shown here, has been omitted. The numbering refers to the *E. coli* sequence. A residue is given in the consensus sequence if it is present in at least all but one of the sequences and is in capital letters if it is completely conserved. The five specific cysteine residues representing the essential thiols of the R1 protein (11) are boxed.

FIG. 5. Amino acid sequence alignment of the small subunit of RR from herpes simplex virus type 1 (HSV1), varicella-zoster virus, mouse, vaccinia virus, and *E. coli* and the *nrdF* product of *S. typhimurium*. The N-terminal domains of the mouse and herpes simplex virus type 1 sequences, which are unrelated to all other sequences shown here, have been omitted. The numbering refers to the *E. coli* sequence. A residue is given in the consensus sequence if it is present in at least all but one of the sequences and is in capital letters if it is completely conserved. Conserved amino acid residues involved in the iron ligands, the hydrogen-bonding network, or the R1-binding surface, as well as the tyrosyl radical and its environment (31), are boxed.

eukaryotic proteins, the herpesvirus-type virus proteins, and the *E. coli* and bacteriophage T4 proteins (31). Among these three groups there is a low level of homology, i.e., between 20 and 30% identity. It is worth noting that the homology of the *nrdEF* products with all of these RR is in this range of identity (Table 2), suggesting that NrdEF proteins may belong to a fourth group.

Several consensus residues of aerobic RR of class I are conserved in both the *nrdE* and *nrdF* deduced products, including the five specific cysteine residues representing the essential thiols (35) of the R1 protein, Cys-225, Cys-439, Cys-462, Cys-754, and Cys-759 (Fig. 4). Likewise, the iron ligands (Asp-84, Glu-115, His-118, Glu-204, Glu-238, and His-241), the tyrosyl radical and its environment (Tyr-122, Phe-208, Phe-212, and Ile-234), the hydrogen-bonding network (Asp-237 and Trp-48), and the R1-binding surface (Asp-58, Arg-236, and Tyr-356) of the R2 protein (14) are also preserved in the *nrdF* protein (Fig. 5). These homologies suggest that this new RR belongs to class I.

The results presented in this paper clearly show that in both *S. typhimurium* and *E. coli* there are two sets of genes encoding the function of aerobic synthesis of dNTPs. Other examples of duplication of the same function in which the homologous genes map to distinct loci in *E. coli* include *argI* and *argF* (2, 43) and *tufA* and *tufB* (1, 44). The *arg* genes have diverged to the point that the two enzymes are biochemically distinguishable (28, 29) despite the fact that their amino acid sequences differ by not more than 5% (28). The *tuf* genes are nearly identical, and the only difference between the 339-residue structural proteins is a single substitution at the C terminus (17). Since the gene products of the *tuf* loci are functionally indistinguishable (30), it has been suggested that the duplication provided an emergency backup when demand for the translational elongation factor EF-Tu was high (1). In this respect, and because of the great importance of the RR to any organism, it seems reasonable that more than one *nrd* system is available in bacterial cells to guarantee the production of dNTPs required for DNA replication.

In *Saccharomyces cerevisiae* the R1 gene of RR is duplicated (for a review, see reference 10). This organism contains the genes for the large subunit (*RNR1*) and the small subunit (*RNR2*) but in addition a third gene (*RNR3*). RNR1 and RNR3 proteins are 80% identical in the portions of their genes that have been sequenced (10). *RNR1* and *RNR2* mutations are lethal, whereas *RNR3* mutants have no obvious growth defects and are not sensitive to hydroxyurea. Also, similarly to the case with the *nrdEF* genes in *nrdAB* mutants of *E. coli*, an *RNR1* mutant cannot be complemented by the *RNR3* chromosome copy, but the *RNR3* gene can abolish the deficiency of *RNR1* mutants when introduced in a high-copy-number vector (10). On the other hand, NrdAB and NrdEF proteins are very divergent despite the fact that both seem to be fully functional. In fact, preliminary analysis showed that extracts of *E. coli* *nrdA*(Ts) cells containing a multicopy plasmid carrying *nrdEF* genes have, at the restrictive temperature, RR activity (25). In this respect, it is also worth noting that the principal functional residues of RR enzymes of class I are conserved in the NrdEF proteins. Complementation data for several deleted plasmids

(Fig. 2) also indicate that both *nrdEF* genes must be present to suppress the *nrdAB*-defective phenotype, indicating that some interaction must exist between NrdE and NrdF proteins to form an active RR. Furthermore, R2 and NrdE proteins cannot form a functional complex in vivo, since plasmid pUA395 lacking the 3′ end of the *nrdF* gene was unable to abolish a *nrdA*(Ts) phenotype (Fig. 2).

This work raises a very intriguing question: why does the *nrdA*(Ts) KK450 mutant of *E. coli* die at 42°C if it has another gene for aerobic RR? The answer to this question is not known, but the following alternatives appear to be excluded by our results: (i) an additional mutation in the *nrdEF* genes of this strain, (ii) the *nrdEF* genes being repressed genes belonging to either a cryptic prophage or a plasmid integrated in the bacterial chromosome, and (iii) expression of the *nrdEF* genes requiring a high gene dosage. The first explanation must be discarded since the temperature sensitivity of the *nrdA*(Ts) mutation when present in other genetic backgrounds is also abolished by cloned *nrdEF* genes. The second possibility does not account for the fact that the *nrdEF* genes are present in the same locus of both *E. coli* and *S. typhimurium*, since in this case the hypothetical prophage or plasmid would already be present in the ancient ancestor of these two bacterial species, which are believed to have diverged between 120 and 160 million years ago (32). Moreover, there are characteristics that help to identify genes acquired by a bacterial species from a foreign source such as a phage or a plasmid. One of these characteristics is the G+C content, and another is the codon usage (37). The G+C content (52.9%) as well as the codon usage of the *nrdEF* genes are within the range of values typical for *S. typhimurium* genes. Our results with plasmid F′143 (Fig. 2) and the low-copy-number plasmid pHSG575 carrying these genes (data not shown) lead us also to discard the third hypothesis.

Finally, the remaining possibility is that the structure of the region of the chromosome containing *nrdEF* genes has a negative effect, as with the expression of other genes in *E. coli*, such as *bgl* and *tonB*, which is influenced by supercoiling (22). Likewise, in the *proU* operon (which is downstream of the *nrdEF* genes), the only *trans*-acting mutations isolated so far affect DNA supercoiling (23). Further work is in progress to better characterize the new aerobic RR as well as the regulation of its expression.

Nucleotide sequence accession number. The nucleotide sequence in Fig. 3 has been assigned accession number X73226 in the EMBL data library.

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