

## Genetic and Biochemical Characterization of *Salmonella enterica* Serovar Typhi Deoxyribokinase

LISE TOURNEUX,<sup>1</sup> NADIA BUCURENCI,<sup>1†</sup> COSMIN SAVEANU,<sup>1‡</sup> PIERRE ALEXANDRE KAMINSKI,<sup>2</sup> MADELEINE BOUZON,<sup>2</sup> ELISABETH PISTOTNIK,<sup>1</sup> ABDELKADER NAMANE,<sup>1</sup> PHILIPPE MARLIÈRE,<sup>2</sup> OCTAVIAN BÂRZU,<sup>1</sup> INÈS LI DE LA SIERRA,<sup>3</sup> JAN NEUHARD,<sup>4</sup> AND ANNE-MARIE GILLES<sup>1\*</sup>

Laboratoire de Chimie Structurale des Macromolécules,<sup>1</sup> Unité de Biochimie Cellulaire,<sup>2</sup> and Unité de Biochimie Structurale,<sup>3</sup> Institut Pasteur, 75724 Paris Cedex 15, France, and Center for Enzyme Research, Institute of Molecular Biology, University of Copenhagen, Copenhagen K, Denmark<sup>4</sup>

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We identified in the genome of *Salmonella enterica* serovar Typhi the gene encoding deoxyribokinase, *deoK*. Two other genes, vicinal to *deoK*, were determined to encode the putative deoxyribose transporter (*deoP*) and a repressor protein (*deoQ*). This locus, located between the *uhpA* and *ilvN* genes, is absent in *Escherichia coli*. The *deoK* gene inserted on a plasmid provides a selectable marker in *E. coli* for growth on deoxyribose-containing medium. Deoxyribokinase is a 306-amino-acid protein which exhibits about 35% identity with ribokinase from serovar Typhi, *S. enterica* serovar Typhimurium, or *E. coli*. The catalytic properties of the recombinant deoxyribokinase overproduced in *E. coli* correspond to those previously described for the enzyme isolated from serovar Typhimurium. From a sequence comparison between serovar Typhi deoxyribokinase and *E. coli* ribokinase, whose crystal structure was recently solved, we deduced that a key residue differentiating ribose and deoxyribose is Met10, which in ribokinase is replaced by Asn14. Replacement by site-directed mutagenesis of Met10 with Asn decreased the  $V_{max}$  of deoxyribokinase by a factor of 2.5 and increased the  $K_m$  for deoxyribose by a factor of 70, compared to the parent enzyme.

Deoxyribokinase (dRK) catalyzes the ATP-dependent phosphorylation of 2-D-deoxyribose to 2-D-deoxyribose-5-phosphate (dRib-5P). The enzyme was identified in bacterial species (*Lactobacillus plantarum* and *Salmonella enterica* serovar Typhimurium) capable of using deoxyribose as a sole carbon and energy source (6, 7, 8). The product of the reaction, dRib-5P, is subsequently cleaved to acetaldehyde and glyceraldehyde-3-phosphate by dRib-5P aldolase. This enzyme together with thymidine phosphorylase, phosphopentomutase, and purine nucleoside phosphorylase is also involved in the catabolism of 2'-deoxyribonucleosides. In *Escherichia coli* and serovar Typhimurium, the genes encoding these four enzymes, *deoC*, *deoA*, *deoB*, and *deoD*, constitute the *deo* operon located at approximately 99 min on the respective linkage maps. Expression of the *deo* operon is controlled negatively by the *deoR*-encoded repressor, DeoR. The actual inducer of the *deo* operon is dRib-5P (4, 16). The gene encoding dRK, *deoK*, together with a locus encoding a putative deoxyribose permease (*deoP*), was tentatively mapped at about 20 min on the serovar Typhimurium chromosome (8). Expression of *deoK* is also inducible, but the inducer is free deoxyribose rather than dRib-5P (8), indicating that *deoK* is not part of the *deo* regulon. *E. coli* K-12 cannot grow with deoxyribose as the sole carbon source, presumably because it lacks dRK activity.

With the genome sequencing projects for *S. enterica* serovars Typhi, Typhimurium, and Paratyphi being under way, we aimed to identify the *deoK* gene locus in the released data bank

by determining the N-terminal amino acid sequence of the partially purified enzyme from a nonpathogenic bacterial strain.

### MATERIALS AND METHODS

**Chemicals.** Nucleotides, restriction enzymes, T4 DNA ligase, T7 DNA polymerase, and coupling enzymes were from Boehringer Mannheim. Vent DNA polymerase was from New England Biolabs. Oligonucleotides were synthesized by the phosphoamidate method, using a commercial DNA synthesizer (Cyclone Bioscience).

**Bacterial strains, plasmids, growth conditions, and DNA manipulations.** *E. coli* MG1655, NM554 (14), and BL21(DE3) (22) strains were used for complementation, cloning experiments, and recombinant protein overproduction, respectively. The *deoK* gene from serovar Typhi strain Ty2 was amplified by PCR using the following upstream and downstream primers: 5'-GCAAGCTTTGTGACGATTCTGAGAGGGAG and 5'-CCCAGCTTTATTCGTTCAACGAAAGATACTCATT. The resulting 0.9-kb amplicon was cut with *Hind*III (at the sites underlined in the primer sequences) and ligated with the plasmid pSU19 (2) cut with the same enzyme. The *deoKP* region from serovar Typhi strain Ty2 was similarly amplified by PCR using the upstream and downstream primers 5'-GC AAGCTTTGTGACGATTCTGAGAGGGAG and 5'-TAGGATCCTAGACGCGGCGACTC. The resulting 2.3-kb amplicon was cut with *Hind*III and *Bam*HI (at the sites underlined in the primer sequences) and ligated with the plasmid pSU19 cut with the same enzymes. The *ribK* gene from serovar Typhimurium was amplified by PCR using the upstream and downstream primers 5'-CGGGATCTCGGAACCCCGAATATGAAAACCGC and 5'-CGGAATCAAGCGTTACCCCTGC. The resulting 0.95-kb amplicon was cut with *Bam*HI and *Eco*RI (at the sites underlined in the primer sequences) and ligated with plasmid pSU19 cut with the same enzymes. The three recombinant plasmids were introduced in the *E. coli* strain MG1655 by calcium chloride transformation. Mineral medium MS (15) supplemented with 20 mM deoxyribose and solidified with 15 g of agar per liter was used to test the recombinant strains.

For recombinant protein overproduction, the *deoK* gene from serovar Typhi strain Ty2 was amplified by PCR. The two synthetic upstream and downstream oligonucleotides used for amplification were 5'-GGGGGAATTC AAG AAGGA GTATTAACATGGATATCGCGGTTATTGGCTA-3' and 5'-CCCAAGCTTTATTCGTTCAACGAAAGATACTCATT-3'. The PCR product harboring the *Eco*RI and *Hind*III restriction sites (underlined in the oligonucleotide sequences) was inserted between the same sites of plasmid pET24a (Novagen). The resulting plasmid pBLT5510 harbors the *deoK* gene under the control of a hybrid promoter-operator region, consisting of the T7 promoter followed by a *lac* operator. Replacement of Met10 with an Asn residue by site-directed mutagenesis was performed by PCR using the pBLT5510 recombinant plasmid as a matrix and the following oligonucleotides: 5'-GTTATTGGCTCTAACAACGT TGACCTTATCACCTAC-3' and 5'-GTAGGTGATAAGGTCAACGTTGTTA

\* Corresponding author. Mailing address: Laboratoire de Chimie Structurale des Macromolécules, Institut Pasteur, 28, rue du Dr. Roux, 75724 Paris Cedex 15, France. Phone: 33 (1) 45 68 89 68. Fax: 33 (1) 40 61 39 63. E-mail: amgilles@pasteur.fr.

† Permanent address: Institutul Cantacuzino, 70100 Bucharest, Romania.

‡ Permanent address: Department of Biochemistry, University of Medicine and Pharmacy, Cluj-Napoca, Romania.

GAGCCAATAAC-3' (modified codon is in boldface). The sequence of the wild-type and of the mutated *deoK* gene was verified by the dideoxynucleotide sequencing method (18). For overexpression, plasmids pBLT5510 and pPAK5511 (harboring the mutated *deoK* gene) were introduced into the *E. coli* strain BL21 (DE3)/pDIA17.

**Analytical methods.** dRK activity was determined according to the method of Schimmel et al. (19) by coupling dRib-5P formation to NADH oxidation using dRib-5P aldolase and glycerophosphate dehydrogenase-triose phosphate isomerase as coupling enzymes or by coupling ADP formation to NADH oxidation during regeneration of ATP by phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase. The reaction medium (0.5-ml final volume) contains 50 mM Tris-HCl (pH 7.4); 10 mM MgCl<sub>2</sub>; 0.2 mM NADH; 1 mM ATP; 200 mM ammonium chloride; 1 U each of dRib-5P aldolase, glycerophosphate dehydrogenase, and triose phosphate isomerase or 50 mM Tris-HCl (pH 7.4); 10 mM MgCl<sub>2</sub>; 0.2 mM NADH; 1 mM ATP; 1 mM phosphoenolpyruvate; 200 mM ammonium chloride; and 1 U each of pyruvate kinase and lactate dehydrogenase. The reaction was started in each case with dRK at appropriate dilutions with 50 mM Tris-HCl (pH 7.4) and 2 mM 2-D-deoxyribose (or 10 mM D-ribose in the second assay), and the absorption decrease at 334 nm was monitored with an Eppendorf PCP6121 photometer with the thermostat set at 30°C. Protein concentration was measured according to the method of Bradford (3). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (12). The protein bands from SDS-PAGE were electroblotted onto a Problott membrane filter (Applied Biosystems) and detected by staining with Coomassie blue. The N-terminal amino acid sequences of protein from excised bands were determined by a protein sequencer (Applied Biosystems, Inc.). Ion spray mass spectra were recorded on an API 365 triple-quadrupole mass spectrometer (Perkin-Elmer-Sciex, Thornhill, Canada). Samples dissolved in water-methanol-formic acid (50/50/5 [vol/vol/vol]) were introduced at 5 µl/min with a syringe pump (Harvard Apparatus, South Natick, Mass.). The mass spectrometer was scanned continuously from *m/z* 900 to 1,700 with a scan step of 0.1 and a dwell time per step of 2.0 ms, resulting in a scan duration of 16.0 s. Data were collected on a Power Macintosh 8600/200 and processed through the Biotoobox 2.2 software from Sciex.

## RESULTS AND DISCUSSION

**N-terminal sequence of dRK.** Strain SL35 of serovar Typhimurium was grown in medium containing Casamino Acids (19). When optical density at 600 nm reached 1.5, the medium was supplemented with deoxyribose (1 g/liter), and the growth was continued for another hour. Bacteria collected for 20 min by centrifugation at 10,000 × *g* and 4°C were tested for dRK activity in the sonicated extract. The specific activity of dRK in crude extract (0.12 U/mg of protein) indicated that it represented ~0.15% of total bacterial proteins. The specific activity of dRK was 50-fold enriched by loading 100 mg of proteins onto a 6- by 1.5-cm Blue-Sepharose column equilibrated with 50 mM Tris-acetate (pH 6.0). dRK collected in the flow-through was immediately loaded onto a 4- by 1.5-cm DEAE-Sepharose column equilibrated with the same buffer. The column was washed with 0.2 M NaCl in 50 mM Tris-acetate (pH 6.0), and dRK was eluted with 0.5 M NaCl in the same buffer. The enzyme (6.4 U/mg of protein) identified by SDS-PAGE as a 33-kDa band (19) was electroblotted and microsequenced. The 60 N-terminal amino acid residues MDIAVIGSNMVDLITYTNQMPKEGETLEAPAFKIGCGGKGANQAVAAAKLNSKVLMLTKV were used in a Blast search against the serovar Typhi genomic database at the Sanger Centre (www.Sanger.ac.uk).

**Organization of the *deoK* region in serovar Typhi.** The *deoK* gene of serovar Typhi starting at position 70749 of the contig 473 (Sanger Centre database) encodes a 306-amino-acid protein. A Blast search (1) on complete and unfinished genomes showed about 35% identity with ribokinase (RK) from serovars Typhi and Typhimurium and *E. coli* (Fig. 1). Upstream of the *deoK* gene, three open reading frames (ORFs) were identified, and putative functions have been assigned (Fig. 2). The first exhibits 40% identity with the *deoR* gene of *E. coli* and is preceded by ORFs encoded by the *ilvN* and *ilvB* genes. We named this gene *deoQ* and suggest that it encodes a repressor specific for *deoK*. Downstream of the *deoK* gene, we found an ORF sharing 34.5% sequence identity with the *fucP* gene en-

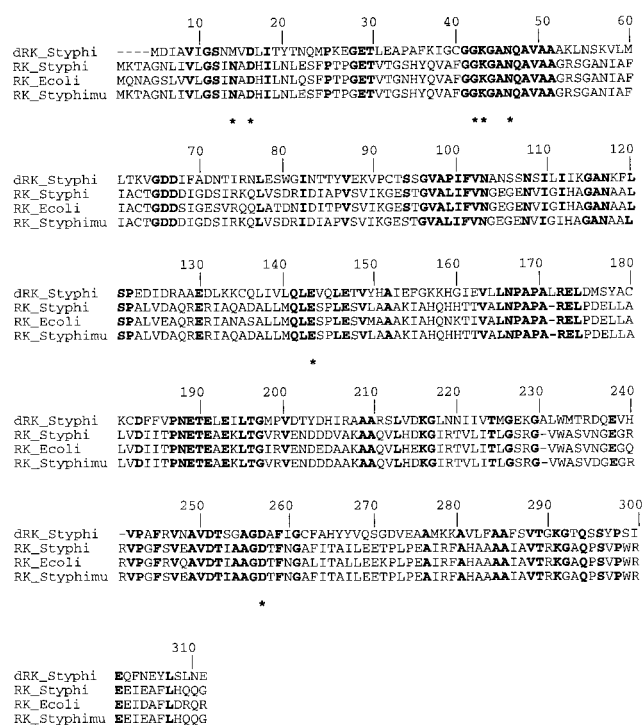


FIG. 1. Alignment of amino acid sequences of dRK from serovar Typhi and RK from serovars Typhi and Typhimurium and *E. coli* using CLUSTAL W (23). Strictly conserved residues, expressed in one-letter code, are in boldface. Residues from *E. coli* RK identified in binding of ribose are marked by asterisks. The numbering above the alignment corresponds to the *E. coli* sequence.

coding the fucose permease of *E. coli*. We hypothesized that this putative permease corresponds to the 2'-deoxyribose transporter described by Hoffee (8) and named the corresponding gene *deoP*. The *deoP* gene is followed by an ORF encoding a protein of 337 amino acid residues with no equivalent in *E. coli* and by the *uhpA* and *uhpB* genes. In the 154-bp intergenic segment situated between the unknown gene and the *uhpA* gene, we identified a putative rho-independent terminator of the *deo* genes, overlapping the putative *uhpAB* promoter. This intergenic space as well as the identified promoter and terminator is different from those described for serovar Typhimurium (9). The 4,384-bp DNA fragment inserted between the *uhpA* and *ilvN* genes containing *deoK*, *deoP*, and *deoQ* may represent a case of horizontal gene transfer. On the genetic maps of *E. coli* and serovar Typhimurium, *uhpA* and *ilvN* are located at approximately 83 min. Assuming that *deoK* and *deoP* in serovar Typhi are located between *uhpA* and *ilvN*, the previously reported position of *deoK* and *deoP* at 20 min on the serovar Typhimurium chromosome most likely is wrong.

***deoK*, a selectable genetic marker in *E. coli*.** *deoK* from serovar Typhi could be used as a selective marker in *E. coli*, which lacks dRK activity. A recombinant *E. coli* strain bearing serovar Typhi *deoK* can grow in mineral medium containing 20 mM deoxyribose (Fig. 3C). This result indicated that the putative permease encoded by *deoP* was not required for catabolizing deoxyribose and that phosphorylation of deoxyribose was the limiting step in the utilization of this compound by *E. coli*. A likely explanation is that the ribose or arabinose ATP binding cassette transport systems can take up deoxyribose. When the *deoP* gene was inserted together with *deoK*, growth of *E. coli* with deoxyribose was significantly enhanced, corroborating the identification of *DeoP* as a putative permease (Fig.

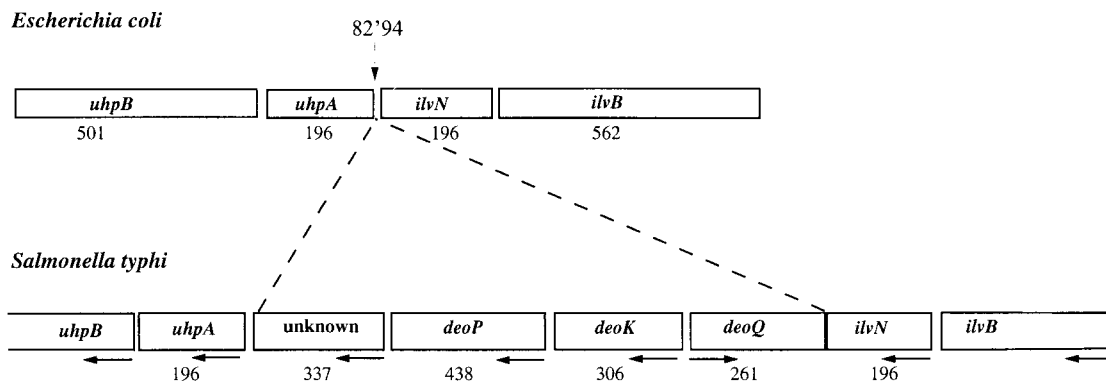


FIG. 2. Organization of the *deoK* region in the serovar Typhi genome and comparison with the equivalent region on *E. coli* genome. Boxes correspond to genes, and numbers below boxes indicate the size of the gene product (number of amino acid residues). Horizontal arrows indicate the directions of transcription, and the vertical arrow locates the deleted locus on the genome of *E. coli*.

3D). Interestingly, overexpression of the putative RK gene *rbsK* from serovar Typhimurium strain LT2 also allowed growth of *E. coli* with deoxyribose (Fig. 3B). Considering the high homology between serovar Typhi and serovar Typhimurium *rbsK* and serovar Typhi *deoK* and *rbsK* gene products (Fig. 1), this result favors a scenario of evolutionary improvement of a marginal dRK activity from an RK enzyme.

**Overproduction of serovar Typhi dRK.** Plasmid pBLT5510 harboring the *deoK* gene from serovar Typhi was introduced into the *E. coli* strain BL21(DE3)/pDIA17. This strain (22) expressed the *lacI* gene on plasmid pDIA17 (13) and the T7 RNA polymerase gene on the chromosome. Overproduction was carried out by growing bacteria at 37°C in 2YT medium (17) supplemented with kanamycin (50 µg/ml) and chloram-

phenicol (30 µg/ml). When the optical density at 600 nm reached 1.5, isopropyl-β-D-thiogalactoside (final concentration, 1 mM) was added to the medium. The bacteria were harvested by centrifugation 3 h after induction. SDS-PAGE followed by Coomassie blue staining indicated that recombinant dRK represented over 20% of total bacterial proteins (Fig. 4). The molecular mass of dRK measured by electrospray ionization mass spectrometry ( $33,228.14 \pm 2.18$  Da) was in agreement with that calculated (33,229.08) from the sequence. The recombinant dRK phosphorylated both deoxyribose and D-ribose with nearly identical  $V_{max}$  values. However, the  $K_m$  for deoxyribose (0.1 mM) was considerably lower than that for ribose (2 mM). These values were similar to those obtained with partially purified enzyme from serovar Typhimurium.

**Structure-function relationship of serovar Typhi dRK.** The molecular mass of native dRK corresponds to a dimer (19), as is the case for the *E. coli* RK and possibly for the serovar Typhi

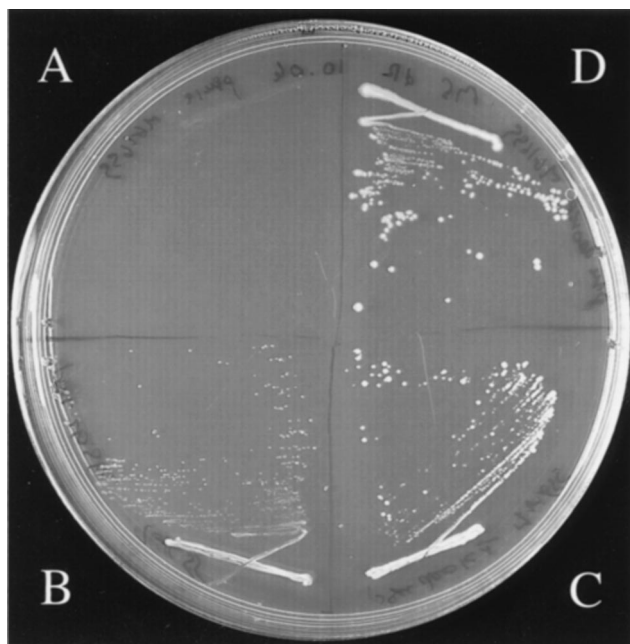


FIG. 3. Acquisition of deoxyribose-catabolizing genes by *E. coli*. The figure shows the growth pattern on deoxyribose (20 mM)-containing plates after 3 days at 37°C of *E. coli* K-12 strain MG1655 transformed with the plasmid pSU19 bearing various inserts: none (A), the *rbsK* gene amplified from serovar Typhimurium strain LT2 and encoding a putative RK (B), the *deoK* gene amplified from serovar Typhi and encoding dRK (C), or the *deoKP* locus amplified from serovar Typhi and encoding both dRK and a putative deoxyribose permease (D).

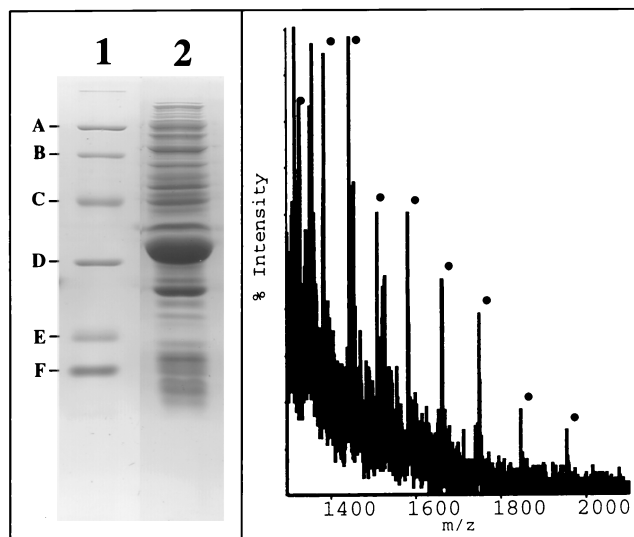


FIG. 4. SDS-PAGE (12.5% polyacrylamide) analysis (left panel, lane 2) and electrospray ionization mass spectrum (right panel) of a bacterial extract over-producing dRK from serovar Typhi. The molecular weight markers for SDS-PAGE analysis (lane 1) are, from top to bottom, phosphorylase *a* (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and lysozyme (14,400) (A to F, respectively).



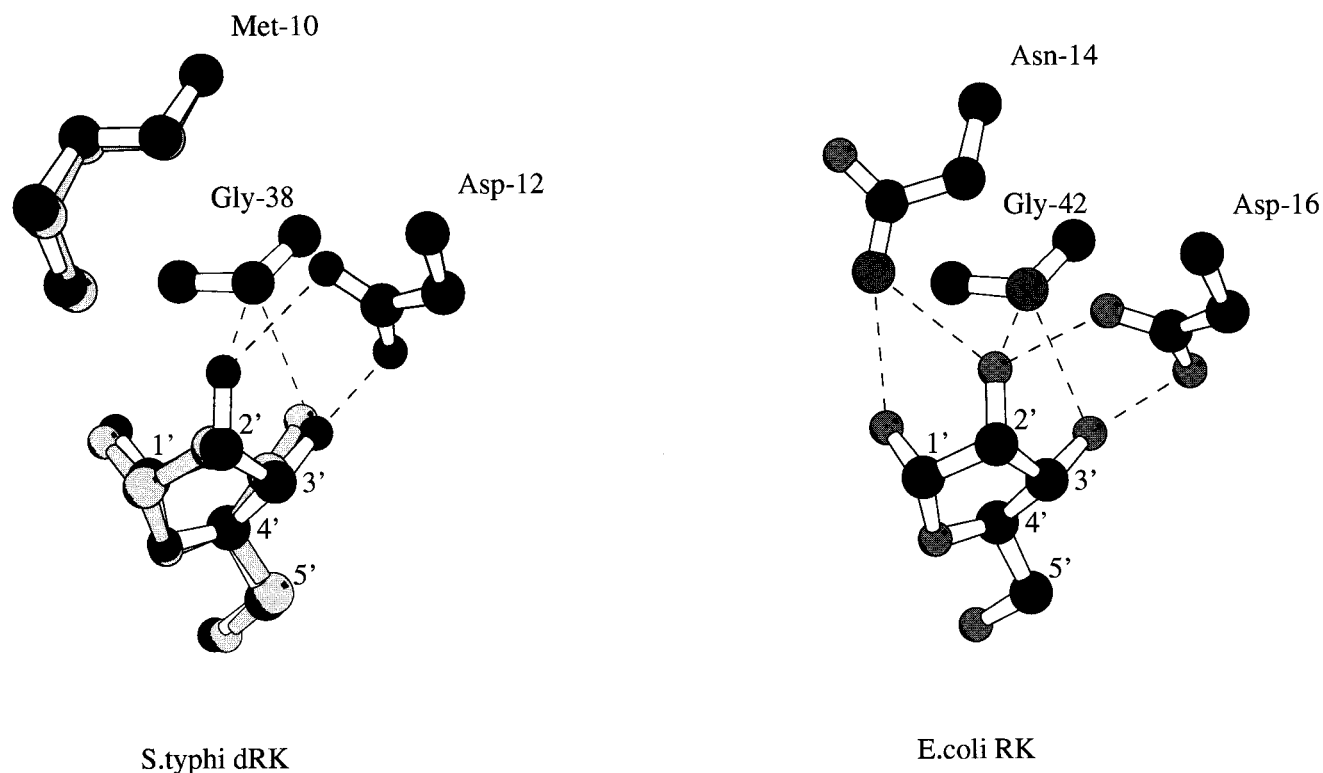


FIG. 5. Schematic drawing of hydrogen bonding interactions in the substrate binding site. (Left) Superposition of ribose (black) with deoxyribose (gray) and relative positions of equivalent residues in the dRK-ribose and dRK-deoxyribose models. (Right) The three residues involved in the interaction with the ribose O-2' atom in the *E. coli* RK (Protein Data Bank code 1rkd) are indicated. Ribose carbon atoms are numbered, and dotted lines correspond to hydrogen bonds. The two complexes were modeled by homology with the X-ray crystal structure of the ternary complex of the *E. coli* RK with the ribose and ADP (21). The side chain of residues not conserved in the *E. coli* RK structure was changed using the O program (10), and then the coordinates were energy minimized with the procedure implemented in X-plor (5). The figure was prepared using MOLSCRIPT (11).

enzyme. The crystal structure of *E. coli* RK, recently solved at 1.8-Å resolution (20, 21), shows that the substrate ribose is maintained in the binding site by an intricate network of H bonds. Seven residues conserved among the RKs from *E. coli*, serovar Typhi, *Haemophilus influenzae*, and *Schizosaccharomyces pombe* are involved in this H-bond network binding (Fig. 1). Six of them (Asp16, Gly42, Lys43, Asn46, Glu143, and Asp255) exist in the sequence of serovar Typhi dRK while the Asn14 is replaced by a methionine residue (Met10). This methionine is also present in a probable dRK from serovar Typhimurium. The presence of a methionine instead of an asparagine residue lowers the strength of ribose binding to dRK compared to that to RK because of the loss of two H-bond interactions (involving O-1' and O-2' ribose atoms) (Fig. 5). Moreover, the Met10 side chain and substrate positions are quite different when the three-dimensional structure models of dRK-ribose and dRK-deoxyribose are compared (Fig. 5, left). The methionine side chain should move to accommodate the hydroxyl linked to the ribose C-2 atom, and the ribose is moved aside from the Met10 residue in the dRK-ribose model. This difference may thus explain why dRK exhibits a better  $K_m$  value for deoxyribose ( $K_m = 0.1$  mM) than for ribose ( $K_m = 2$  mM). To check this hypothesis, we replaced, by site-directed mutagenesis, Met10 in dRK with an Asn residue. The resulting protein has a molecular mass of  $33,209.52 \pm 1.86$  Da, in agreement with that calculated (33,211.99) from the sequence. The Met10 Asn variant of dRK exhibits  $\sim 40\%$  of the  $V_{\max}$  value of the wild-type protein with both deoxyribose and ribose as substrates. The  $K_m$  for deoxyribose was 70-fold higher in the modified dRK compared to the parent enzyme, while under the

same conditions the  $K_m$  for ribose was only twofold increased in the Met10Asn variant compared to the wild-type dRK.

**Concluding remarks.** The various genome sequencing projects revealed that half of the putative proteins encoded by the newly found genes have no known function. On the other hand, well-characterized proteins isolated in the pregenomic era (almost 100 in the case of *E. coli*) are awaiting assignment to their corresponding genes. Partial purification of these proteins, coupled to gel electrophoresis and N-terminal sequencing or/and mass spectrometric analysis, might allow rapid and unequivocal assignment of a gene to a protein, as described in this work.

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