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Escherichia coli rpiA Gene Encoding Ribose Phosphate Isomerase A

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The *rpiA* gene encoding ribose phosphate isomerase A was cloned from phage 1A2(471) of the Kohara gene library. Subcloning, restriction, and complementation analyses revealed an 1,800-bp *Ssp*I-generated DNA fragment that contained the entire control and coding sequences. This DNA fragment was sequenced and shown to harbor an open reading frame of 219 codons, sufficient to encode a polypeptide with an M_r of 22,845. The synthesis of the *rpiA*-encoded polypeptide was detected by analysis of minicells, which established the subunit M_r as 27,000. The assignment of the correct reading frame was confirmed by amino-terminal analysis of partially purified ribose phosphate isomerase A. Our data indicate that the enzyme is composed of two identical subunits. The 5′ end of the *rpiA*-specified transcript was analyzed by primer extension, which revealed a well-conserved −10 region 34 bp upstream of the presumed translation start codon. Analysis of the 3′ end of the transcript by S1 nuclease mapping showed that transcription termination occurred within an adenylate-rich sequence following a guanylate-cytidylate-rich stem-loop structure resembling a rho factor-independent transcription terminator. Host strains harboring the *rpiA* gene in a multicopy plasmid contained up to 42-fold as much ribose phosphate isomerase A activity as the haploid strain.

Pentose phosphates are important intermediates of the cellular metabolism of *Escherichia coli*. Thus, catabolism of exogeneous pentoses is mediated through phosphorylation (21), and pentose phosphates are formed by the oxidative branch of the pentose phosphate pathway (10). Among the most prominent pentose phosphates is phosphoribosylpyrophosphate, a precursor of the pentose moieties of nucleotides and nucleic acids. Phosphoribosylpyrophosphate is synthesized by transfer of a pyrophosphoryl group from ATP to the C-1 hydroxyl of ribose 5-phosphate (18). Ribose 5-phosphate is also used for the synthesis of sedoheptulose 7-phosphate, a precursor of the heptose moieties of the core region of lipopolysaccharides, and in the synthesis of erythrose 4-phosphate, an intermediate in the biosynthesis of the aromatic amino acids.

E. coli can utilize several pentoses as carbon sources. Among these is ribose, which can be phosphorylated to ribose 5-phosphate for further metabolism. For this purpose, *E. coli* contains a high-affinity uptake system consisting of binding and membrane-bound proteins, as well as a ribokinase, all encoded by the *rbs* gene cluster at 83 min on the linkage map (22). In addition, *E. coli* may extract ribose 5-phosphate from the breakdown of nucleotides or nucleosides either formed during cellular metabolism or exogeneously added to the growth medium (13). Additional catabolism of ribose 5-phosphate requires the nonoxidative branch of the pentose phosphate pathway: ribose 5-phosphate ↔ ribulose 5-phosphate ↔ xylulose 5-phosphate. The former reaction is catalyzed by ribose phosphate isomerase, and the latter is catalyzed by ribulose phosphate 3-epimerase. Finally, the sequential action of transketolase, transaldolase, and transketolase converts pentose phosphates to intermediates of the glycolytic pathway (10). In *E. coli*, two ribose phosphate isomerases (A and B) have been identified (7, 9). The *rpiA* gene for ribose phosphate isomerase A (D-ribose 5-phosphate ketol isomerase; EC 5.3.1.6) is located at 63 min on the linkage map (39). As part of an analysis of the pentose phosphate metabolism, we here present a characterization of the *rpiA* gene.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* K-12 strains used are described in Table 1. Growth media were either NZY broth (containing the following per liter: 10 g of NZ-amin [Struers, Denmark], 5 g of yeast extract [Oxoid], and 5 g of NaCl), or AB minimal medium (6) supplemented with glucose (0.2%) and thiamine (0.5 mg/liter) and, when necessary, serine (40 mg/liter), adenosine (20 mg/liter), or ribose (0.2%). When used, Casamino Acids were added at 0.2%. Cell cultures were incubated in an Aqua Shaker (Adolf Kühner Inc., Birsfelden, Switzerland) at 37°C with aeration by shaking (approximately 200 rpm). Cell growth was monitored in an Eppendorf PCP6121 photometer as A_{436}. An A_{436} of 1 (1-cm light path) corresponds to approximately 3×10^8 cells per ml. Solid media were prepared by adding 1.5% agar (Difco) to minimal medium or NZY broth ingredients.

Plasmids. The plasmids constructed during this work are described in the text. The cloning vectors used were pBR322 (5), pBluescriptSK$^+$ (Stratagene Cloning System), and the *galK* promoter-cloning vector pKO500. The latter resembles the pKO1 plasmid described previously (26), except for the presence of a multiple cloning site in pKO500. In addition, pUC4-K was used to provide a kanamycin resistance-encoding DNA fragment (44). Plasmids were selected and maintained by the presence of ampicillin (50 mg/liter) or tetracycline (5 or 10 mg/liter). Kanamycin was used at 30 mg/liter.

Genetic techniques. Procedures for transformation with plasmid DNA (23) and transduction by bacteriophage P1 (27) or bacteriophage λ (37) were described previously. The Rpi phenotype was scored on minimal medium with or without ribose. In some instances, adenosine was used in place of

TABLE 1. Bacterial strains

Strain	Sex	Genotype[a]	Construction, source, or reference
AS11	HfrC	*thi zwf rpiA1*	38
AT2475	HfrH	*thi-1 serA6 rel-1 lacI22*	CGSC[b]
BOE71	F⁻	*polA1 lysA*	L. Boe
HO340	F⁻	*araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF*	28
HO644	F⁻	*minA minB thi rpsL his lac mtl man xyl tonA*	28
HO791	HfrH	*thi-1 rel-1 lacI22 rpiA1*	P1(AS11) × AT2475, Ser⁺
HO846	F⁻	*recB21 recC22 sbcB15 thr leu proA2 hisG argE thi ara lacY galK xyl mtl rpsL tsx supE* Δ(*iciA-rpiA*-P_{serA})*101*::Kan^r	JC7623, transformation with linearized pHO216, selecting Kan^r
HO847	F⁻	*araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF* Δ(*iciA-rpiA*-P_{serA})*101*::Kan^r	P1(HO846) × HO340, Kan^r
HO880	HfrH	*thi-1 rel-1 lacI22* Δ(*rpiA*)*103*::Tet^r	AT2475, recombination of pHO244 via the *polA1* strain BOE71
HO890	F⁻	*araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF* Δ(*rpiA*)*103*::Tet^r	P1(HO880) × HO340, Tet^r
JC7623	F⁻	*recB21 recC22 sbcB15 thr leu proA2 hisG argE thi ara lacY galK xyl mtl rpsL tsx supE*	19
N99	F⁻	*galK2 proAB*	26
NM522	F′	F *lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/*supE thi* Δ(*lac-proAB*) Δ(*hsdMS*)	11

[a] Δ indicates deletion of the loci given in the parentheses. P_{serA} indicates the *serA* promoter.
[b] CGSC, *E. coli* Genetic Stock Center, Yale University.

ribose (38). Complementation of *rpiA* or *serA* by the virulent recombinant phages of the gene library of Kohara et al. (17) was achieved by transforming the recipient strains (HO791 or AT2475) with pKB800, which carries the *c*I857 allele of bacteriophage λ in addition to the *bla* gene (2). The resulting transformants (HO791/pKB800 and AT2475/pKB800) were then grown at 32°C and transduced to Rpi⁺ or Ser⁺ at 32°C with lysates of the recombinant λ phages. The presence of the plasmid-borne *c*I857 allele served to repress the growth of the recombinant λ phage. The phenotype of *galK*-bearing strains was scored on MacConkey agar supplemented with galactose (1%). To facilitate the genetic analysis of the *rpiA* gene, two deletion alleles were constructed. The *rpiA101* allele was constructed in vitro in pHO216 by deletion of the *rpiA*-containing *Ssp*I DNA fragment of pMM643 and replacing it by the kanamycin resistance-encoding *Hin*cII DNA fragment of pUC4-K. The mutant allele was transferred to the chromosome by homologous recombination using an *recBC sbcB* strain (45). The *rpiA103* allele was obtained in vitro by deleting the overhang of the unique *Bcl*I site within the *rpiA* gene, harbored in pHO217, and inserting the tetracycline resistance-encoding gene of pBR322 (harbored in a blunt-ended *Eco*RI-*Ava*I DNA fragment). The mutation was then transferred to the chromosome by homologous recombination using a *polA* strain (15, 33).

Analysis of plasmid-specified polypeptides. The *E. coli* minicell-producing strain, HO644, was transformed with appropriate plasmids, and minicells were isolated. The plasmid-specified polypeptides were labelled by incubating minicells with [^{35}S]methionine (0.2 μM [4.5 TBq/mmol]) (14) followed by electrophoresis through 12.5% polyacrylamide gels containing sodium dodecyl sulfate (20), staining, and autoradiography.

DNA technology. Plasmid DNA and bacteriophage λ DNA were isolated by standard techniques (4, 8). Purification of plasmid DNA by buoyant density centrifugation in CsCl gradients containing ethidium bromide was performed as described before (34). Single-stranded phagemid DNA was isolated essentially as described by Sambrook et al. (34) with NM522 as the host strain and M13K07 as the helper phage. Hydrolysis of DNA by restriction endonucleases was performed as described by the vendors (Boehringer, Promega, New England Biolabs, and Amersham). Where required, cohesive ends of DNA fragments were converted to blunt ends by incubation with S1 nuclease (Amersham). Ligation of DNA fragments was performed with T4 DNA ligase (Amersham) by a 16-h incubation at 4°C at a DNA concentration of approximately 50 mg/liter. Hydrolysis of terminal 5′-phosphate groups of DNA was performed with calf intestinal alkaline phosphatase (Boehringer).

Nucleotide sequences were determined by the chain termination method with dideoxyribonucleotides and single-stranded DNA as the templates (35). The templates were phagemids containing restriction fragments with overlapping deletions. Polymerization was achieved by the Sequenase DNA polymerase and was conducted as described by the supplier (United States Biochemical Corp.) M13 universal, KS, and T7 primers (Stratagene Cloning System) were used. Labelling was performed with ^{35}S-deoxyadenosine 5′-*O*-(1-thiotriphosphate) (New England Nuclear Corp.). Sequencing reactions were submitted to electrophoresis in buffer gradient gels containing 8 M urea and 6% polyacrylamide as described previously (34), and sequences were read from autoradiograms of the dried gels. The plasmids used are described in Results.

RNA technology. Cells growing exponentially in glucose minimal medium at 37°C were rapidly chilled by the addition of ice and harvested by centrifugation. The RNA was isolated by a hot phenol procedure (41). To analyze the transcription start, the oligonucleotide 5′-GCGTCAATAA AGTGTGCGGC-3′ (purchased at the Danish Center of Microbiology) was labelled at the 5′ end by incubation with [γ-^{32}P]ATP (0.7 μM [100 TBq/mmol]) and T4 polynucleotide kinase (GIBCO BRL) essentially as described by Sambrook et al. (34). For primer extension (41), 5 ng of the labelled primer was hybridized with 5 μg of the RNA by heating to 90°C in the presence of 0.1 M KCl followed by cooling to room temperature. The hybridized nucleic acids were then incubated with the four deoxyribonucleotides and Moloney murine leukemia virus reverse transcriptase at 37°C under the conditions specified by the supplier (GIBCO BRL). To assess the size of the extended polynucleotide, a set of sequencing reactions were performed with the same labelled primer as above, single-stranded DNA of pHO217 as the

template, and the TaqTrack sequencing system (Promega Corp.). The extension and sequencing reactions were loaded adjacent to one another on a 6% polyacrylamide, nongradient, denaturing gel (16). The gels were run and processed as described above.

A probe for S1 nuclease mapping was prepared by digesting 15 pmol of pHO235 DNA with restriction endonuclease *Asp*I followed by incubation with the large fragment of *E. coli* DNA polymerase I (Boehringer) in the presence of [α-^{32}P]dCTP (0.5 μM [25 TBq/mmol]). This treatment resulted in a DNA fragment that was labelled at only one end. The DNA was precipitated, recovered, and incubated with restriction endonuclease *Sac*I, and a 251-bp *Asp*I-*Sac*I DNA fragment labelled at the 3′ end of the *Asp*I site was obtained. The *Sac*I recognition site is located in the vector, and 40 bp of the nonlabelled end of the fragment were vector derived. This *Asp*I-*Sac*I DNA fragment contained the carboxy-terminal encoding end of the *rpiA* gene. S1 nuclease mapping (3) was performed by incubating the probe (100 ng) with RNA (150 μg) at 75°C for 15 min followed by cooling to room temperature over an 18-h period to allow hybridization. S1 nuclease (40 U) (Boehringer) was then added, and the samples were removed after 10 and 30 min of incubation at 30°C or after 24 h of incubation at 4°C. The reaction was terminated by ethanol precipitation. The recovered nucleic acids were applied to a gel like that used for primer extension analysis together with a sample of the probe that had been treated with the specific deoxyguanylate-deoxyadenylate sequencing reaction of Maxam and Gilbert (25), and electrophoresis and processing of the gel was performed as described above for nucleotide sequence determination.

Bacillus subtilis RNA was isolated from strain 168 by the procedure described previously (12).

Preparation of cell extracts. Bacterial cells in 40 ml of minimal medium culture in logarithmic phase were harvested at an A_{436} of 0.8. The pellet was suspended in 0.5 ml of 50 mM potassium phosphate buffer (pH 7.2)–1 mM EDTA and disrupted by sonication for 60 s at 0°C in an ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., London, United Kingdom). The extract was centrifuged at 5,000 × *g* for 15 min at 4°C, and the supernatant fluid, containing 2 to 5 mg of protein per ml, was dialyzed against 50 mM potassium phosphate buffer (pH 7.2)–1 mM EDTA.

Enzyme assays. Ribose phosphate isomerase activity was assayed by a modification of the procedures described previously (1, 9), in which the ribose 5-phosphate-dependent formation of ribulose 5-phosphate is determined by a colorimetric procedure. Extract (25 μl, appropriately diluted in 50 mM potassium phosphate buffer [pH 7.2], 1 mM EDTA, 50 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml) was mixed with 175 μl of a reaction mixture, prewarmed to 37°C, to give final concentrations of 5 mM ribose 5-phosphate and 50 mM Tris-HCl (pH 7.5). A sample with 50 mM potassium phosphate buffer (pH 7.2), 1 mM EDTA, 50 mM dithiothreitol, and 0.1 mg of bovine serum albumin per ml was included as a blank. After a 10-min incubation at 37°C, 2.00 ml of 66% (vol/vol) H_2SO_4, 70 μl of 0.12% carbazole (Fluka) (dissolved in absolute ethanol) and 70 μl of 1.5% (wt/vol) cysteine were added and mixed by vortexing, and incubation continued at 37°C for 30 min, after which the color development was measured as A_{540}. The assayed enzyme activity resulted in an A_{540} of less than 1.0. The amount of ribulose 5-phosphate produced was estimated from incubation of known amounts of commercial ribulose 5-phosphate in the colorimetric determination. Ribose phosphate isomerase activity is expressed as micromoles of ribulose 5-phosphate formed per minute per milligram of protein. β-Lactamase was assayed with nitrocefin (Glaxo) as the substrate essentially as described by O'Callaghan et al. (29). The activity of β-lactamase is expressed as ΔA_{482} per minute per milligram of protein. Protein content was determined by the bicinchoninic acid procedure with chemicals provided by Pierce (40). Bovine serum albumin was used as the standard.

Methods of protein analysis. Ribose phosphate isomerase A was partially purified from strain HO340/pHO217 at 4°C as follows. Twelve grams of cell paste (obtained by overnight growth of the cells in NZY broth supplemented with 0.2% glucose) was suspended in 48 ml of 50 mM potassium phosphate buffer (pH 7.2)–1 mM EDTA, disrupted by sonication, and centrifuged at 15,000 × *g* for 15 min. The extract was made 1% in streptomycin sulfate and centrifuged as before. $(NH_4)_2SO_4$ was added to the supernatant fluid, and the fraction precipitating at 40 to 50% saturation was isolated and dialyzed against 50 mM potassium phosphate buffer (pH 7.2)–1 mM EDTA. A fraction containing ribose phosphate isomerase A at a specific activity of 135 μmol/min/mg of protein was obtained. Part of this fraction (200 μg) was electrophoresed under denaturing conditions (20), followed by electroblotting onto polyvinylidene difluoride membrane (Bio-Rad) (24). The band corresponding to the ribose phosphate isomerase A subunit was excised and submitted to automated Edman degradation by standard techniques on an Applied Biosystems 477A gas phase sequenator at the Department of Protein Chemistry, Institute of Molecular Biology, University of Copenhagen, Denmark.

Materials. D-Ribose, D-ribose 5-phosphate, D-ribulose 5-phosphate, and other fine chemicals were purchased from Sigma Chemical Co. [^{35}S]methionine, ^{35}S-deoxyadenosine 5′-*O*-(1-thiotriphosphate), [γ-^{32}P]ATP and [α-^{32}P]dCTP were obtained from New England Nuclear Corp.

Nucleotide sequence accession number. The sequence of the 1,804-bp *Ssp*I-generated DNA fragment has been submitted to the EMBL nucleotide sequence data bank and has been assigned the accession number X73026.

RESULTS

Cloning of the *rpiA* gene. *E. coli* mutants defective in the *rpiA* gene are ribose auxotrophs, despite the presence of ribose phosphate isomerase B (7, 38). To clone the *rpiA* gene, we took advantage of the fact that the *rpiA* and *serA* genes are very closely linked, with more than 90% linkage established by bacteriophage P1-mediated transduction, at 63 min on the *E. coli* linkage map (39). Furthermore, the nucleotide sequence of the coding region of *serA* has been published (43). By using the restriction map of this nucleotide sequence, we located *serA* at approximately 3,070 kbp on the map established by Kohara et al. (17), as has also been found by others (32). We therefore sought to analyze the Kohara bacteriophages 1A2(471), 6C5(472) and 1H10 (473) for the presence of the *rpiA* gene; the bacteriophage 1A2(471) has been shown to harbor the *serA* gene (30). Indeed, bacteriophage 1A2(471) contained both the *rpiA* and *serA* genes, as shown by complementation of *rpiA1* or *serA6*, i.e., transduction with a selection for ribose or serine prototrophy as described in Materials and Methods.

Figure 1A shows a restriction map of the 3,060- to 3,090-kbp region of the *E. coli* physical map and the extent of the DNA inserted in phage 1A2(471). The DNA from this phage strain was isolated, and the *rpiA* gene was subcloned in a 9.5-kbp *Eco*RI-generated DNA fragment (Fig. 1A) in the

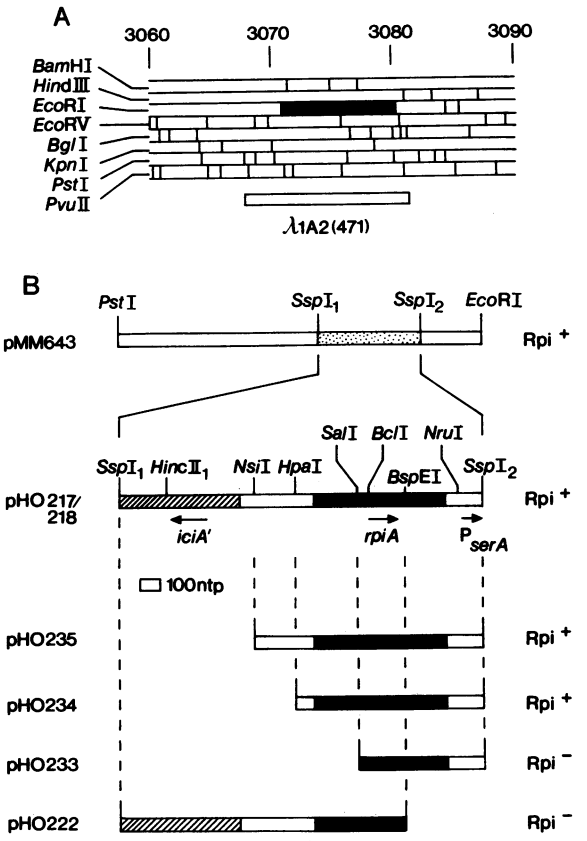

FIG. 1. Cloning, restriction, and deletion analyses of *rpiA*. (A) Physical map of the 3,060- to 3,090-bp region of the *E. coli* chromosome. A restriction map is given as well as the extent of the 1A2(471) clone of the gene library constructed by Kohara et al. (17). The solid bar indicates the 9.5-kbp *Eco*RI DNA fragment that complements *rpiA* (redrawn from Kohara et al. [17]). The 9.5-kbp *Eco*RI DNA fragment was cloned in pBR322, which generated pMM641. (B) The bar at the top indicates the 6.0-kbp *Pst*I-*Eco*RI DNA fragment of pMM643. The *Eco*RI site of pMM643 corresponds to the left-hand *Eco*RI site of the solid bar shown in panel A. The dotted area indicates the $SspI_1$-$SspI_2$ DNA fragment to where the *rpiA*-complementing activity was mapped. The $SspI_1$-$SspI_2$ DNA fragment of pMM643 was cloned in the *Sma*I site of pBluescript SK^+, yielding pHO217 and pHO218. The two plasmids are identical, except for the orientation of the inserts relative to the vector sequences. The inserts of pHO217 and pHO218 were further deleted, and the structures of some of these deletion derivatives are given together with results of a complementation analysis; to the right is indicated the phenotype that each plasmid confers to an *rpiA* mutant strain after transformation [either HO791 (*rpiA1*), HO847 (Δ*rpiA101*), or HO890(Δ*rpiA103*)]. The plasmid pHO222 was derived from pHO217, and pHO233 to pHO235 were derived from pHO218. For sequencing, the following additional deletions were derived: from pHO217, $SspI_2$-*Sal*I (pHO223), $SspI_2$-*Hpa*I (pHO224), $SspI_2$-*Nsi*I (pHO225), and $SspI_2$-*Hinc*II$_1$ (pHO226); from pHO218, $SspI_1$-*Hinc*II$_1$ (pHO236) and $SspI_1$-*Bsp*EI (pHO232). The solid bars indicate the open reading frame of *rpiA*, the hatched regions indicate the open reading frame specified by the *iciA* gene, and *iciA'* indicates the deletion of the carboxy-terminus-encoding end of the gene. P_{serA} indicates the *serA* promoter. Arrows indicate direction of transcription, and ntp indicates nucleotide pairs (base pairs). Relevant restriction endonuclease recognition sites are given above the bars.

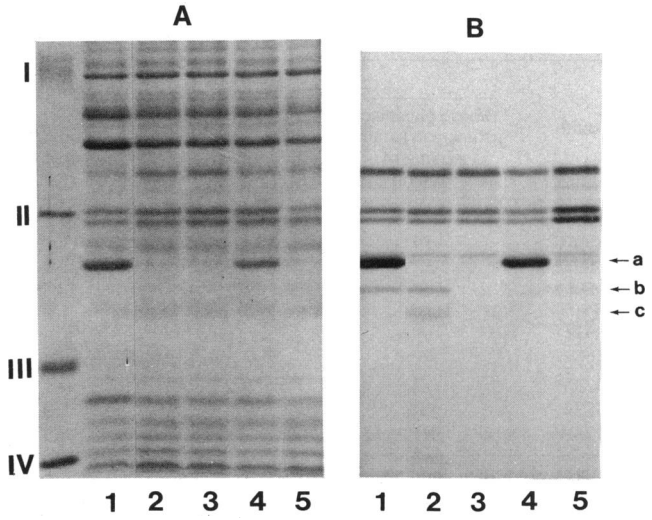

FIG. 2. Polypeptides encoded by plasmids. Minicells containing various plasmids were isolated and analyzed as described in Materials and Methods by ^{35}S labelling and polyacrylamide gel electrophoresis. (A) Coomassie blue-stained gel. (B) Autoradiograph of the gel shown in panel A. Minicells contained the following plasmids: lane 1, pHO217 (*rpiA*$^+$); lane 2, pHO222 (*rpiA*); lane 3, pHO233 (*rpiA*); lane 4, pHO234 (*rpiA*$^+$); lane 5, pBluescriptSK$^+$. The following molecular weight markers were used: I, ovalbumin (45,000); II, carbonic anhydrase (31,000); III, soybean trypsin inhibitor (21,500); IV, lysozyme (14,400). Arrows a, b, and c point to the bands discussed in the text, with M_rs of 27,000, 26,000, and 23,000, respectively.

*Eco*RI site of pBR322 (pMM641). The plasmid pMM641 was digested with restriction endonuclease *Pst*I and ligated to obtain pMM643 harboring a 6.0-kbp *Pst*I-*Eco*RI DNA fragment that also complemented *rpiA1*.

To further locate the *rpiA* gene within the cloned DNA fragment of pMM643, a deletion analysis was performed (data not shown). The dotted DNA region of pMM643 in Fig. 1B indicates the region in which the *rpiA* gene is located, i.e., the *rpiA*-complementing activity is encoded on the right-hand side of the $SspI_1$ site. Furthermore, comparison of our restriction map with that of Tobey and Grant (43) located the amino-terminus-encoding end of the *serA* gene to the right-hand end of the *Pst*I-*Eco*RI fragment of pMM643. The *serA* gene, thus, is transcribed to the right. The $SspI_1$-$SspI_2$ DNA fragment was then subcloned in pBluescriptSK$^+$, and another set of deletions was constructed as shown in Fig. 1B. The results of this analysis revealed a DNA fragment of approximately 850 bp that complemented *rpiA* (pHO234).

Cloning of the *rpiA* promoter. The direction of transcription of *rpiA* was determined by cloning various fragments into the promoter cloning vehicle pKO500, which contains the *galK* gene as a reporter gene. Thus, cloning the *Hpa*I-*Bcl*I fragment in front of *galK* resulted in a plasmid that conferred a Gal$^+$ phenotype to strain N99 (*galK2*), whereas cloning the *Nru*I-*Bcl*I fragment in front of *galK* resulted in a plasmid that retained the Gal$^-$ phenotype of strain N99. In both cases, the *Bcl*I site was proximal to the *galK* gene. Therefore, the *rpiA* gene is transcribed to the right as shown in Fig. 1B, and it can be inferred that both *rpiA* and *serA* are transcribed towards the lower nucleotide numbers on the physical map, i.e., counterclockwise on the genetic map.

Plasmid-encoded polypeptides. The expression of the *rpiA*

TABLE 2. Enzyme activities in strain HO340 harboring various plasmids[a]

Plasmid	Enzyme activity		
	Ribose phosphate isomerase (μmol/min/mg of protein)	β-Lactamase (ΔA_{482}/min/mg of protein)	Ratio (μmol/ΔA_{482})[b]
pBR322	2	296	<0.1
pMM641[c]	55	48	1.1
pMM642[c]	52	38	1.4
pMM643	80	ND[d]	ND[d]
pHO217	18	10	1.8
pHO218	11	11	1.0
pHO222	1	3	0.3
pHO233	2	172	<0.1
pHO234	40	32	1.3
pHO235	83	42	2.0

[a] Cells were grown and enzymes were assayed as described in Materials and Methods.
[b] Ratio of ribose phosphate isomerase to β-lactamase.
[c] The inserts of the plasmids pMM641 and pMM642 are identical except for their orientation relative to the vector sequences.
[d] ND, not determined. The plasmid pMM643 does not encode β-lactamase.

gene was further analyzed in minicells. Figure 2 shows the results of analysis of polypeptides specified by the two *rpiA*-complementing plasmids pHO217 and pHO234, as well as the two noncomplementing plasmids pHO222 and pHO233. The *rpiA*-complementing plasmids both produced a polypeptide with an M_r of 27,000, which was produced neither by the noncomplementing-*rpiA* deletion plasmids nor by the vector (Fig. 2B). This band with an M_r of 27,000 was visible also in the Coomassie blue-stained gel (Fig. 2A). Both pHO217 and pHO222 produced a polypeptide with an M_r of 26,000. The plasmid pHO222 also produced a faint band migrating as a polypeptide with an M_r of 23,000. The conclusions drawn from this experiment are that the polypeptide with an M_r of 27,000 is the ribose phosphate isomerase A subunit. The value obtained here is in reasonable agreement with the value for the size of the subunit calculated from the deduced amino acid sequence (22,845; see below). The polypeptide with an M_r of 23,000 formed from pHO222 is a truncated version of the ribose phosphate isomerase A subunit. Finally, the polypeptide with an M_r of 26,000 does not coincide with the complementing capabilities of the plasmids, and therefore is unrelated to ribose phosphate isomerase A. The polypeptide with an M_r of 26,000 may be specified by the gene for an open reading frame that reads out of pHO217 and pHO222 (left-hand direction in Fig. 1B). Furthermore, the direction of transcription of *rpiA* is rightward in Fig. 1B and thus confirms the results of the promoter cloning described above.

Overexpression of *rpiA* and analysis of ribose phosphate isomerase A. The amount of ribose phosphate isomerase activity specified by the various plasmids was determined in crude cell extracts, and the results are given in Table 2. The complementation data and the enzyme activities are consistent, i.e., plasmids that complement *rpiA* show a 6- to 42-fold increase in ribose phosphate isomerase activity, whereas the noncomplementing plasmids show no increase. The activity of β-lactamase was measured as an estimate of the plasmid copy number. It appears from these results that there were large variations in the plasmid copy number, particularly among the pBluescriptSK$^+$ derivatives pHO217 to pHO235. The ribose phosphate isomerase/β-lactamase ratio is also given in Table 2. The cells harboring *rpiA*-complementing plasmids showed a ratio of 1 or more, whereas strains with noncomplementing plasmids showed a ratio of less than 1. The specific activities of ribose phosphate isomerase given in Table 2 can be ascribed almost exclusively to ribose phosphate isomerase A, because the cells were grown in glucose minimal medium, i.e., under conditions in which there is virtually no ribose phosphate isomerase B activity present (38).

Ribose phosphate isomerase A was partially purified as described in Materials and Methods. Automated Edman degradation established the amino-terminal sequence as Met-Thr-Gln-Asp-Glu-Leu-Lys-Lys-Ala-Val.

Nucleotide sequence of the *rpiA* gene. To sequence the *rpiA* gene, a series of deletions were created in pHO217 and pHO218 (Fig. 1B), and the nucleotide sequence of the entire DNA fragment was determined on both strands with complete overlaps of all cloning junctions. The *Ssp*I$_1$-*Ssp*I$_2$ DNA fragment consists of 1,804 bp. There were three open reading frames, one of which constitutes the *rpiA* gene. The second open reading frame, transcribed divergently from *rpiA*, is encoded by the *iciA* gene as shown by comparison of our nucleotide sequence with that of the *iciA* gene (42). The product of this latter gene is an inhibitor of initiation of DNA replication in vitro. A third open reading frame reading leftward in Fig. 1B and covering approximately the region designated *rpiA* was also detected. However, from the results of the promoter identification analysis described above, we did not attach any importance to this third open reading frame regarding *rpiA*. Moreover, the DNA fragment contained part of the *serA* promoter, as established by sequence identity with the previously published sequence (31). Figure 1B shows a map of the 1,804-bp DNA fragment together with relevant restriction sites.

The nucleotide sequence of the 1,134-bp *Nsi*I-*Ssp*I$_2$ DNA fragment is shown in Fig. 3. The sequence of the open reading frame of *iciA* has been omitted. The deduced amino acid sequence of ribose phosphate isomerase A is shown below the nucleotide sequence. It comprises 219 amino acid residues and has a calculated M_r of 22,845. Inspection of the nucleotide sequence revealed a sequence (AGG) of moderate homology to a Shine-Dalgarno sequence (36) with a 10-nucleotide spacing to the presumed translation initiation codon. In addition, the hexanucleotide sequence TATAAT, perfectly matching a consensus −10 region, was found with a 34-nucleotide spacing to the translation initiation codon. A hexanucleotide resembling a consensus −35 region at a proper spacing to the −10 region is not observed.

The assignment of the correct reading frame of *rpiA* was confirmed by comparison of the deduced amino acid sequence with that obtained from Edman degradation of the partially purified enzyme. The deduced sequence showed identity for the 10 residues determined experimentally.

Transcription of the *rpiA* gene. To further analyze the initiation site of transcription, primer extension was performed on RNA isolated from a strain harboring *rpiA* in a multicopy plasmid, as well as on RNA from a haploid strain. The results (Fig. 4) show that transcription is initiated from guanylate residue 259, spaced 6 nucleotides downstream from the TATAAT sequence. Notably, the transcript obtained from the haploid strain showed the same transcription start point. The level of *rpiA*-specified mRNA, as expected, was much lower in the haploid strain.

Termination of transcription of *rpiA* was analyzed by S1 nuclease mapping, with a DNA fragment labelled at the 3′ end of the *Asp*I site (nucleotides 920 to 928 of Fig. 3), located

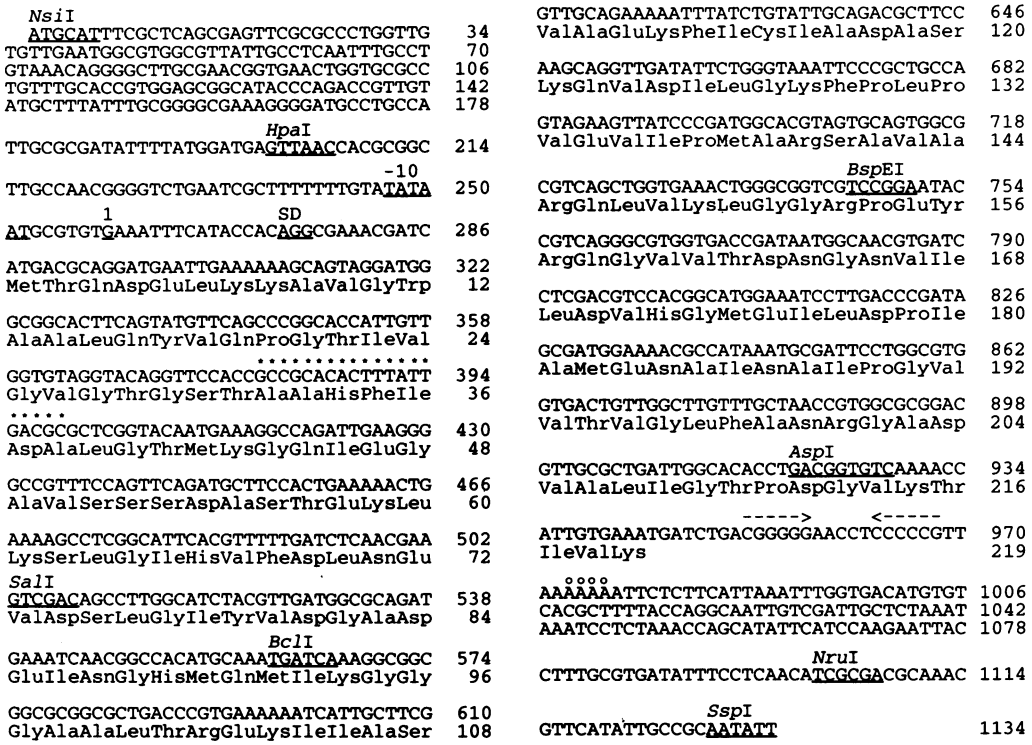

FIG. 3. Nucleotide sequence of the *rpiA* gene. The sequence of the coding strand of the 1,134-bp *Nsi*I-*Ssp*I DNA fragment is shown. Numbers to the right of each line of nucleotides indicate nucleotide positions. The deduced amino acid sequence of ribose phosphate isomerase A is shown below the nucleotide sequence. Numbers to the right of each line of amino acids indicate amino acid positions. Relevant features of the sequence are underlined and include restriction endonuclease recognition sites, a promoter sequence (−10), a transcription start site (indicated by 1 above nucleotide 259), and a Shine-Dalgarno sequence (SD) (36). The asterisks indicate nucleotides 380 to 399, which are complementary to the oligonucleotide used in the primer extension analysis of the 5′ end of the *rpiA*-specified mRNA. The arrows above nucleotides 952 to 957 and 963 to 968 indicate the dyad symmetry possibly involved in termination of the *rpiA*-specified mRNA, and the open circles above adenylate residues 973 to 976 indicate the region of transcription termination.

approximately seven codons before the translation stop codon, used as a probe. RNA was isolated from a strain harboring the *rpiA* gene in a multicopy plasmid. Results of protection of the probe from S1 nuclease degradation by the RNA species are given in Fig. 4B. It appears from these data that transcription termination occurs within the stretch of adenylate residues 971 to 977 that immediately follows the dyad symmetry (nucleotides 952 to 968 of Fig. 3). Thus, increasing incubation time of the hybrids with S1 nuclease tends to increase the intensities of the bands with a 3′ end in this area (lanes 1 to 3 of Fig. 4). As a control, the probe was also annealed to RNA that was isolated from a wild-type *B. subtilis* strain (lane 4 of Fig. 4). In this case, there were no bands present within the adenylate-rich region. In contrast, all of the other faint bands above and below the adenylate-rich region were also present in lane 4 and therefore are unrelated to the *E. coli rpiA* mRNA. The dyad symmetry is located 8 nucleotides beyond the coding sequence of *rpiA*, and the structure resembles a rho factor-independent transcription terminator. The apparent heterogeneity of the 3′ end of the *rpiA* mRNA transcript may be due to either true heterogeneity in the termination process or alternatively to instability of the hybrids at the 3′ end of the RNA molecules. Analysis of mRNA isolated from a haploid strain resulted in the same protection pattern as that observed for mRNA isolated from the plasmid-harboring strain, although the bands were much lower in intensity (data not shown). From the results of the mRNA analysis, it appears that the *rpiA* gene is expressed as a monocistronic transcript and that the length of the *rpiA*-specified mRNA is approximately 715 nucleotides.

DISCUSSION

We have cloned the *rpiA* gene of *E. coli*. That this gene is the structural gene for ribose phosphate isomerase A is shown by the data as follows. (i) The cloned gene restores ribose prototrophy to an *rpiA* (ribose-requiring) strain. (ii) Strains harboring the cloned gene in a multicopy number plasmid contain elevated ribose phosphate isomerase A activity. (iii) The subunit size of ribose phosphate isomerase A determined by gel electrophoresis is in reasonable agreement with that calculated from the deduced amino acid sequence. Furthermore, we determined the primary structure of the *rpiA* gene as well as some of the flanking sequences. Upstream of the *rpiA* gene we identified an open reading frame, encoded by the *iciA* gene, which is involved in regulation of DNA replication initiation (42). Immediately downstream of the *rpiA* gene, the *serA* gene, encoding 3-phosphoglycerate dehydrogenase, was identified.

Ribose phosphate isomerase has been partially purified previously (9), and results of gel filtration revealed an M_r of the native enzyme of 45,000. A comparison with our determination of a subunit M_r of 23,000 to 27,000 indicates that the enzyme is composed of two subunits of equal size. Moreover, we believe, from analysis of ribose phosphate

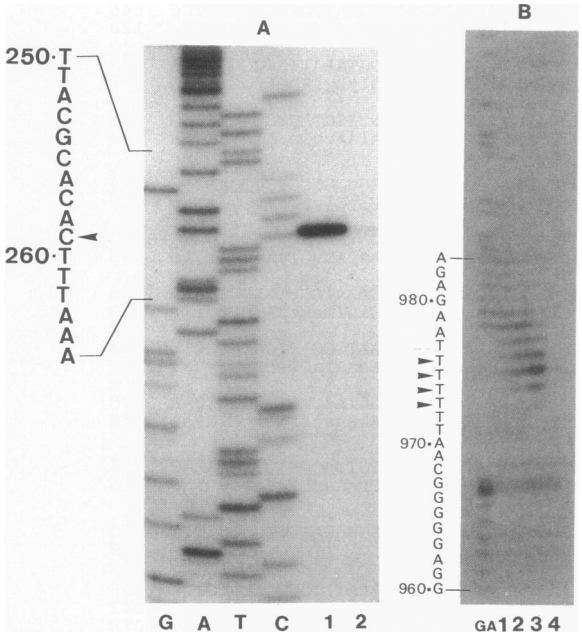

FIG. 4. Analysis of the *rpiA*-specified mRNA. (A) Analysis of the 5′ end by primer extension was performed as described in Materials and Methods. An autoradiogram of a denaturing 6% polyacrylamide gel is shown. Lanes G, A, T, and C are the sequence ladder. Lane 1 shows the extension of the primer on an RNA template isolated from strain HO340/pHO217 (i.e., a strain multiploid for the *rpiA* gene). Lane 2 shows the extension of the primer on an RNA template isolated from strain HO340 (i.e., a haploid strain). The relevant nucleotide sequence of the template strand is given to the left. Numbers indicate nucleotide positions, and the arrowhead indicates the transcription initiation nucleotide. (B) Analysis of the 3′ end by S1 nuclease mapping was performed as described in Materials and Methods. An autoradiogram of a denaturing 6% polyacrylamide gel is shown. Lane GA shows the deoxyguanylate-deoxyadenylate-specific sequencing reactions. Lanes 1 to 3 show protection of the *Asp*I-(*Ssp*I$_2$)-*Sac*I probe, labelled at the *Asp*I site, from S1 nuclease degradation by RNA isolated from strain HO340/pHO217. Lane 4 shows the protection pattern obtained by *B. subtilis* RNA. The following conditions were used for S1 nuclease treatment: lane 1, 10 min at 30°C; lane 2, 30 min at 30°C; lane 3, 24 h at 4°C; lane 4, 10 min at 30°C. The relevant nucleotide sequence of the template strains is given to the left. Numbers indicate nucleotide positions, and arrowheads indicate the most probable transcription termination sites.

isomerase activity in plasmid-harboring strains, that the two subunits are identical. If this were not so, we would not expect the activity of the enzyme to be increased in the plasmid strains.

The reason for *E. coli* having two enzymes with ribose phosphate isomerase activity remains unknown. Our perspectives for future research in this field include characterization of ribose phosphate isomerase B as well as the gene encoding this enzyme.

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