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Ribose Catabolism of *Escherichia coli*: Characterization of the *rpiB* Gene Encoding Ribose Phosphate Isomerase B and of the *rpiR* Gene, Which Is Involved in Regulation of *rpiB* Expression

KIM I. SØRENSEN,† AND BJARNE HOVE-JENSEN*

Department of Biological Chemistry, Institute of Molecular Biology, University of Copenhagen, Copenhagen, Denmark

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Escherichia coli **strains defective in the *rpiA* gene, encoding ribose phosphate isomerase A, are ribose auxotrophs, despite the presence of the wild-type *rpiB* gene, which encodes ribose phosphate isomerase B. Ribose prototrophs of an *rpiA* genetic background were isolated by two different approaches. Firstly, spontaneous ribose-independent mutants were isolated. The locus for this lesion, *rpiR*, was mapped to 93 min on the linkage map, and the gene order *zje*::Tn*10*-*rpiR*-*mel*-*zjd*::Tn*10*-*psd*-*purA* was established. Secondly, ribose prototrophs resulted from the cloning of the *rpiB* gene on a multicopy plasmid. The *rpiB* gene resided on a 4.6-kbp *Hin*dIII-*Eco*RV DNA fragment from phage λ10H5(642) of the Kohara gene library and mapped at 92.85 min. Consistent with this map position, the cloned DNA fragment contained two divergent open reading frames of 149 and 296 codons, encoding ribose phosphate isomerase B (molecular mass, 16,063 Da) and a negative regulator of *rpiB* gene expression, RpiR (molecular mass, 32,341 Da), respectively. The 5′ ends of *rpiB*- and *rpiR*-specified transcripts were located by primer extension analysis. No significant amino acid sequence similarity was found between ribose phosphate isomerases A and B, but ribose phosphate isomerase B exhibited high-level similarity to both LacA and LacB subunits of the galactose 6-phosphate isomerases of several gram-positive bacteria. Analyses of strains containing *rpiA*, *rpiB*, or *rpiA rpiB* mutations revealed that both enzymes were equally efficient in catalyzing the isomerization step in either direction and that the construction of *rpiA rpiB* double mutants was a necessity to fully prevent this reaction.**

For growth on D-ribose as a carbon source, *Escherichia coli* requires the proteins of the high-affinity membrane transport system as well as ribokinase. These proteins are encoded by a set of genes, *rbsA*, *-B*, *-C*, *-D*, and *-K*, located as a cluster at 83 min on the linkage map (35). *E. coli* can also utilize the ribose moiety of nucleosides either synthesized intracellularly or added to the growth medium. The catabolism of ribose 5-phosphate requires the participation of enzymes of the nonoxidative branch of the pentose phosphate pathway (Fig. 1). By this pathway, ribose 5-phosphate, ribulose 5-phosphate, and xylulose 5-phosphate are interconverted in reactions catalyzed by ribose phosphate isomerase and ribulose-5-phosphate-3-epimerase (14, 53). Finally, the conversion of pentose phosphates into the glycolytic pathway intermediates, fructose 6-phosphate and glyceraldehyde 3-phosphate, requires the sequential action of transketolase, transaldolase, and transketolase. Furthermore, ribose 5-phosphate is an important compound of cellular anabolism, as it is utilized as the substrate for the synthesis of phosphoribosyl diphosphate, which serves as a precursor for the biosynthesis of nucleotides and the amino acids histidine and tryptophan (25). Pentose phosphates are also substrates for the synthesis of erythrose 4-phosphate and sedoheptulose 7-phosphate, which are precursors for aromatic amino acids and cell wall heptoses, respectively (14).

* Corresponding author. Mailing address: Department of Biological Chemistry, Institute of Molecular Biology, University of Copenhagen, 83H Sølvgade, DK-1307 Copenhagen K, Denmark. Phone: 45 3532 2027. Fax: 45 3532 2040. Electronic mail address: hove@mermaid.molbio.ku.dk.

† Present address: Department of Microbiology, The Technical University of Denmark, DK-2800 Lyngby, Denmark.

In *E. coli*, two ribose phosphate isomerases (D-ribose-5-phosphate ketol-isomerase; EC 5.3.1.6), A and B, have been identified biochemically (10, 13). The *rpiA* gene for ribose phosphate isomerase A has been located at 63 min on the linkage map (51). Ribose phosphate isomerase A is most likely a homodimeric enzyme with a subunit molecular mass of 22,845 Da (27). This enzyme is constitutively expressed and accounts for at least 99% of the total ribose phosphate isomerase activity of strains grown in nutrient broth (50). Strains defective in the *rpiA* gene are ribose auxotrophs but can use ribose as a carbon source, probably because of the presence of ribose phosphate isomerase B (50). Spontaneous secondary mutations that lead to ribose prototrophy in *rpiA* strains occur at a high frequency. It has been suggested that the regenerated ribose phosphate isomerase activity of such a mutant is due to a mutation in a gene for a regulator protein, permitting constitutive synthesis of ribose phosphate isomerase B, whose synthesis is induced by ribose or ribose-containing compounds in wild-type strains (51). This gene has been designated *rpiR*.

As an approach to understanding the physiology of the *E. coli* ribose phosphate isomerases, we cloned the *rpiB* gene, encoding ribose phosphate isomerase B, and the *rpiR* gene, encoding a regulator of *rpiB* gene expression. This article describes the mapping, cloning, and characterization of these two genes. The functions of ribose phosphate isomerases A and B are discussed in terms of their roles in the pentose phosphate pathway.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. The *E. coli* K-12 strains used are listed in Table 1. Bacterial cultures were grown at 37°C, unless otherwise stated. The medium used was either NZY broth (10 g of NZ-amin per liter,

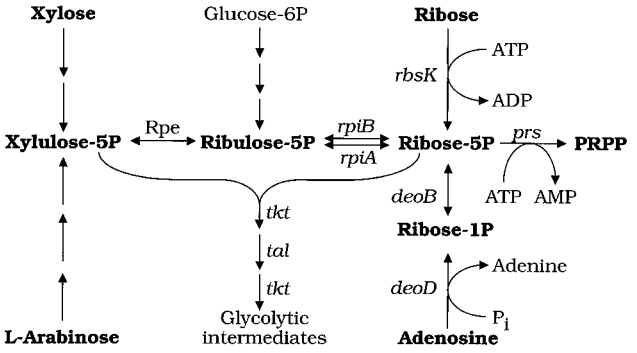

FIG. 1. Metabolism of pentose phosphates in *E. coli*. All compounds are D-stereoisomers unless otherwise noted. Abbreviations: AMP, 5′-AMP; Glucose-6P, glucose 6-phosphate; PRPP, phosphoribosyl diphosphate; Ribose-1P, ribose 1-phosphate; Ribose-5P, ribose 5-phosphate; Ribulose-5P, ribulose 5-phosphate; Xylulose-5P, xylulose 5-phosphate. The following enzymes are indicated by gene designations: phospho(deoxy)ribomutase (*deoB*), purine nucleoside phosphorylase (*deoD*) (19), phosphoribosyl diphosphate synthetase (*prs*) (24, 28), ribokinase (*rbsK*) (14), ribose phosphate isomerase A (*rpiA*) (27), ribose phosphate isomerase B (*rpiB*), transaldolase (*tal*), and transketolase (*tkt*) (14). No gene has been assigned to ribulose-5-phosphate-3-epimerase (Rpe). Ribose 1-phosphate also can be formed by the phosphorolysis of uridine, catalyzed by uridine phosphorylase (*udp*), or by phosphorolysis of xanthosine, catalyzed by xanthosine phosphorylase (*xapA*) (6, 34).

5 g of yeast extract [Oxoid] per liter, and 5 g of NaCl per liter [27]) or AB minimal medium (9) supplemented with thiamine (0.5 mg/ml). Carbon sources were glucose, ribose, xylose, and melibiose (0.2% [each]). Supplements, when required, were added at the following concentrations: amino acids, 50 μg/ml; adenosine, 20 μg/ml; adenine, 15 μg/ml; ampicillin, 100 μg/ml; kanamycin, 30 μg/ml; and tetracycline, 10 or 50 μg/ml. Cell cultures were incubated in an Aqua Shaker (A. Kühner Inc., Birsfelden, Switzerland) with aeration by shaking (215 rpm). Cell growth was monitored in an Eppendorf PCP6121 photometer as A_{436}. An A_{436} of 1 (1-cm path length) corresponds to approximately 3×10^8 cells per ml. Media were solidified by the addition of 1.5% agar (Difco) to minimal medium or NZY broth ingredients. Plasmids pKIS206, pKIS207, pKIS208, pKIS212, pKIS215, and pKIS222, which are derivatives of pUC19 (55), are described in Fig. 2. Plasmid pKIS203 was constructed by cloning the 980-bp *Hin*dIII$_2$-*Eco*RI fragment (Fig. 2) into the *Eco*RI and *Hin*dII sites of pUC19. Plasmid pKIS579, containing the *lacA* and *lacB* genes from *Streptococcus mutans*, was constructed by cloning a 3.3-kbp *Hin*dIII DNA fragment of pYA579 (30) into the *Hin*dIII site of pUC19.

Genetic techniques. Conjugations were performed by mixing exponentially growing NZY broth cultures of donor and recipient strains in a 1:50 ratio, with subsequent incubation at 37°C with very slow shaking for 1 h. The same procedure was used for the transfer of F episomes, except that the donor and recipient strains were grown in minimal medium. When the conjugation of two Hfr strains was performed, the recipient strain was made F^- phenocopy by vigorous shaking as described previously (41). Bacteriophage P1-mediated transduction, penicillin counterselection, and UV mutagenesis were performed essentially as described previously (29, 41). The *mel* marker was scored on MacConkey agar medium supplemented with melibiose (1%) or on minimal medium supplemented with melibiose and adenosine to satisfy the ribose requirement of *rpiA* strains. The *psd*(Ts) marker was scored on minimal medium at 42°C, with 32°C being the permissive temperature. The *purA* marker was scored on medium lacking adenine. Transformation with plasmid DNA has been described previously (39).

DNA procedures. Methods for the isolation of plasmid, single-stranded phage, chromosomal, and bacteriophage λ DNAs were previously described (4, 11, 48). Restriction endonuclease digestion and ligation with T4 DNA ligase were carried out as recommended by the suppliers (Boehringer Mannheim Biochemicals and New England Biolabs, Inc.).

The end labelling of DNA fragments was carried out by incubating DNA, eventually dephosphorylated by calf intestinal alkaline phosphatase treatment, with [γ-^{32}P]ATP (0.7 μM [100 TBq/mmol]) and T4 polynucleotide kinase (Gibco BRL), as previously described (48). The hybridization of a probe to DNA of the entire collection of bacteriophage λ clones of Kohara et al. (32), immobilized on a nylon membrane, was performed as recommended by the membrane supplier (Takara Biochemical, Inc.).

Single-stranded templates for nucleotide sequence determination were obtained by cloning various overlapping restriction endonuclease-generated DNA fragments into the replicative form of bacteriophage M13mp18 or M13mp19 with NM522 as the host strain (17, 58). The entire nucleotide sequence of both strands with complete overlaps of all cloning junctions was determined with the Sequenase DNA polymerase version 2.0 (U.S. Biochemical Corp.), and labelling was performed with ^{35}S-deoxyadenosine 5′-*O*-(1-thiotriphosphate) (New England Nuclear Corp.). The sequence reaction products were separated in 6 or 8% polyacrylamide denaturing gels, and the sequences were read from the autoradiograms of dried gels (48). The primers used were the M13 universal primers, whereas other synthetic oligonucleotides (purchased at Hobolth DNA Syntese, Hillerød, Denmark) were used for the sequencing of overlapping regions.

Enzyme assays. Exponentially growing cells were harvested by centrifugation at an A_{436} of 1.0 and concentrated 10-fold in 50 mM potassium phosphate buffer (pH 7.2)–1 mM EDTA. After disruption for 45 s at 0°C in an ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., London, United Kingdom), the debris was removed by centrifugation. The activities of ribose phosphate isomerase and β-lactamase were determined by previously published procedures (2, 27, 43). Protein content was determined by the method of Lowry et al. (37), with bovine serum albumin as a standard.

Inactivation of the *rpiB* gene. The *rpiB* gene was disrupted by inserting a kanamycin resistance-encoding *Bam*HI DNA fragment from plasmid pUC4-K (55) into the *Bcl*I site in the *rpiB* gene of plasmid pKIS212 (Fig. 2B). The resulting mutant allele (*rpiB137*::Kan^r) harbored in pKIS215 was transferred to the chromosome by homologous recombination with a *polA1* strain and bacteriophage P1-mediated transduction as previously described (5, 26). Insertion of the kanamycin resistance-encoding fragment in the chromosome of HO1458 was verified by PCR (22). The two synthetic oligonucleotides used were complementary to nucleotides of codons 17 to 22 of *rpiB* (5′-GTGCCACTATTTCATG-3′) and nucleotides of codons 8 to 13 of *rpiR* (5′-CCGTTCGGAAGCGCTG-3′) and were annealed to the template at 55°C. After 30 cycles of PCR, a DNA fragment of 1,724 bp was obtained with the DNA of strain HO1458 (*rpiB*::Kan^r) as the template, whereas a DNA fragment of 460 bp was obtained with the DNA of strain HO340 (*rpi*$^+$) as the template. Restriction endonuclease digestion of the 1,724-bp PCR product confirmed that the inserted DNA was the kanamycin resistance-encoding fragment.

Primer extension. Cells were grown exponentially at 37°C in NZY broth containing ribose. At an A_{436} of 1.0, cells were chilled rapidly by the addition of ice and harvested by centrifugation. Total RNA was isolated essentially as previously described (52). The synthetic oligonucleotides used were the same as those used for PCR. Each primer was labelled on the 5′ end as described above. To determine the 5′ ends of transcripts, 10 ng of labelled primer was hybridized to 5 μg of total RNA. The hybridized primer was then extended by the addition of the four deoxyribonucleoside triphosphates and Moloney murine leukemia virus reverse transcriptase (Gibco BRL) (52). The extension products were applied to 8% polyacrylamide sequencing gels adjacent to comigrating DNA sequence ladders that were generated from the same primers on recombinant M13 DNA templates.

Analysis of plasmid-encoded polypeptides. Plasmids were transformed into the *E. coli* minicell-producing strain HO644. Minicells were then prepared and incubated with [^{35}S]methionine (0.2 μM [4.5 TBq/mmol]) for 1 h (24). Polypeptides were extracted and analyzed by electrophoresis through a 15% polyacrylamide gel containing sodium dodecyl sulfate (SDS) (33). After being stained with Coomassie brilliant blue R-250, the gel was submitted to autoradiography.

Computer-assisted sequence analysis. Amino acid sequences were compared by using programs based on the BLAST algorithm (1) at the National Center for Biotechnology Information Services. The BLASTP program was used to screen the amino acid sequence database.

Nucleotide sequence accession number. The sequence of the 2,312-bp *Hin*dIII-*Ssp*I DNA fragment has been submitted to the EMBL data bank and been assigned accession number X82203.

RESULTS

Isolation and characterization of *rpiR* mutants. *rpiR* mutants were isolated as pseudorevertants of ribose-auxotrophic strains harboring allele *rpiA1* (HO791), *rpiA101*::Kan^r (HO847), or *rpiA103*::Tet^r (HO890) by plating cells on minimal medium without ribose. The frequency of the appearance of spontaneous *rpiR* mutants was approximately 10^{-6}. The Hfr strains described by Singer and coworkers (49), each of which contains a Tn*10* insertion approximately 20 min beyond the point of origin of transfer, were used as donors, and strain HO859 (*rpiA101*::Kan^r) was used as the recipient in conjugations. Selection was for tetracycline resistance and kanamycin resistance. The following two Hfr strains donated the *rpiR*$^+$ wild-type allele with high frequency: CAG8160 (a derivative of strain KL14 with point of origin of transfer at 66 min [clockwise transfer]) and CAG5052 (a derivative of strain KL227 with point of origin at 7 min [counterclockwise transfer]). Strain CAG5051 (a derivative of strain HfrH with point of origin of transfer at 97 min [clockwise transfer]) yielded no *rpiR*$^+$ recombinants. Therefore, *rpiR* appeared to be located in the interval from 66 to 97 min on the linkage map. Bacterio-

TABLE 1. Bacterial strains used

Strain	Sex	Genotype[a]	Construction, source, and/or reference
AS11	Hfr	*thi zwf rpiA1*	50
AT2475	Hfr	*thi-1 serA6 rel-1 lacI22*	CGSC[b]
CAG5051	Hfr	(H)(PO1) *relA thi-1 spoT supQ nadA57*::Tn*10*	Singer et al. (49)
CAG5052	Hfr	(KL227)(PO3) *metB1 relA1 btuB3191*::Tn*10*	Singer et al. (49)
CAG8160	Hfr	(KL14)(PO68) *leu relA thi*::Tn*10*	Singer et al. (49)
CAG18488	F$^-$	*zjd-2231*::Tn*10*	Singer et al. (49)
CAG18427	F$^-$	*zje-2241*::Tn*10*	Singer et al. (49)
CAG18555	F$^-$	*zje-3183*::Tn*10kan*	Singer et al. (49)
DC356	F$^-$	*fadR201 adh-81 supF58 zgb-224*::Tn*10 mel-1*	D. Clark via CGSC
EH150	F$^-$	*psd-2*(Ts) *lac tsx fhu supE gal xyl mtl*	E. P. Kennedy (21) via CGSC
HO120	F$^-$	*supF relA spoT rpsL lamB metB purE deoD purA*	SØ312, UV mutagenesis, penicillin counterselection in the absence of adenine
HO340	F$^-$	*araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF*	42
HO480	F$^-$	*polA1 lysA*	26
HO644	F$^-$	*minB thi his rpsL lac mtl man mal xyl tonA*	42
HO791	Hfr	*thi-1 rel-1 lacI22 rpiA1*	27
HO799	Hfr	*thi zwf rpiA1 rpiR111*	AS11, spontaneous ribose independent
HO800	Hfr	As AS11, but *rpiR112*	As HO799
HO809	Hfr	As AS11, but *rpiR113*	As HO799
HO810	Hfr	*thi-1 rel-1 lacI22 rpiA1 rpiR114*	HO791, spontaneous ribose independent
HO811	Hfr	As HO791, but *rpiR115*	As HO810
HO812	Hfr	As HO791, but *rpiR116*	As HO810
HO847	F$^-$	*araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF* Δ(*rpiA*-P$_{serA}$)*101*::Kan^r	27
HO859	Hfr	*thi-1 rel-1 lacI22 rpiR114* Δ(*rpiA*-P$_{serA}$)*101*::Kan^r	HO810 × P1(HO847), Kan^r
HO890	F$^-$	*araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF* Δ(*rpiA*)*103*::Tet^r	27
HO920		*thi-1 rel-1 lacI22 rpiR114* Δ(*rpiA*-P$_{serA}$)*101*::Kan^r	HO859 × P1(CAG18488), Tet^r
HO925	Hfr	*thi-1 serA6 rel-1 lacI22 zgb-224*::Tn*10*	AT2475 × P1(DC356), Tet^r
HO968	F$^-$	*supF relA spoT rpsL lamB metB zgb-224*::Tn*10 serA6*	SØ003 × P1(HO925), Tet^r
HO976	F$^-$	*supF relA spoT rpsL lamB metB rpiA1*	HO968 × P1(AS11), Ser$^+$
HO978	F$^-$	*supF relA spoT rpsL lamB metB rpiA1 rpiA114 zjd-2231*::Tn*10*	HO976 × P1(HO920), Tet^r
HO1007	Hfr	*thi zwf rpiA1 rpiR117*	AS11, spontaneous ribose independent
HO1008	Hfr	*thi zwf rpiA1 rpiR118*	As HO1007
HO1009	Hfr	*thi zwf rpiA1 rpiR119*	As HO1007
HO1010	Hfr	*thi zwf rpiA1 rpiR120*	As HO1007
HO1041	Hfr	*thi zwf rpiA1 rpiR121*	As HO1007
HO1042	Hfr	*thi-1 rel-1 lacI22 rpiA1 rpiR122*	HO791, spontaneous ribose independent
HO1043	Hfr	*thi-1 rel-1 lacI22 rpiA1 rpiR123*	As HO1042
HO1044	Hfr	*thi-1 rel-1 lacI22 rpiA1 rpiR124*	As HO1042
HO1045	Hfr	*thi-1 rel-1 lacI22 rpiA1 rpiR125*	As HO1042
HO1046	Hfr	*thi-1 rel-1 lacI22 rpiA1 rpiR126*	As HO1042
HO1047	F$^-$	*araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF* Δ(*rpiA*-P$_{serA}$)*101*::Kan^r *rpiR127*	HO847, spontaneous ribose independent
HO1048	F$^-$	As HO847, but *rpiR128*	As HO1047
HO1049	F$^-$	As HO847, but *rpiR129*	As HO1047
HO1050	F$^-$	As HO847, but *rpiR130*	As HO1047
HO1051	F$^-$	As HO847, but *rpiR131*	As HO1047
HO1052	F$^-$	*araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF* Δ(*rpiA*)*103*::Tet^r *rpiR132*	HO890, spontaneous ribose independent
HO1053	F$^-$	As HO890, but *rpiR133*	As HO1052
HO1054	F$^-$	As HO890, but *rpiR134*	As HO1052
HO1055	F$^-$	As HO890, but *rpiR135*	As HO1052
HO1056	F$^-$	As HO890, but *rpiR136*	As HO1052
HO1458	F$^-$	*araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF rpiB137*::Kan^r	HO340, homologous recombination of pKIS215 via strain HO480 (*polA1*)
HO1459	F$^-$	*araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF rpiB137*::Kan^r	HO340 × P1(HO1458), Kan^r
HO1460	F$^-$	*araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF* Δ(*rpiA*)*103*::*Tet*^{r} *rpiB137*::Kan^r	HO890 × P1(HO1458), Kan^r
KL729	F$^-$	*leuB tonA lacY supE gal hisG recA1 argG rpsL malA xyl rpsL mtl metB*/F112 *metB*$^+$ *rpiR*$^+$	K. B. Low (36) via CGSC
NM522	F$^-$	*supE thi* Δ(*lac-proAB*) Δ(*hsdMS*)/F *lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$	17
SØ003	F$^-$	*supF relA spoT rpsL lamB metB*	23
SØ312	F$^-$	*supF relA spoT rpsL lamB metB purE deoD*	31
UH-Ac2	F$^+$	*aceE2 trp-26 mel-1*	CGSC

[a] Δ, deletion of the locus given in the parentheses; P$_{serA}$, *serA* promoter; Am, amber mutation; Ts, temperature-sensitive mutation.
[b] CGSC, *E. coli* Genetic Stock Center, Yale University.

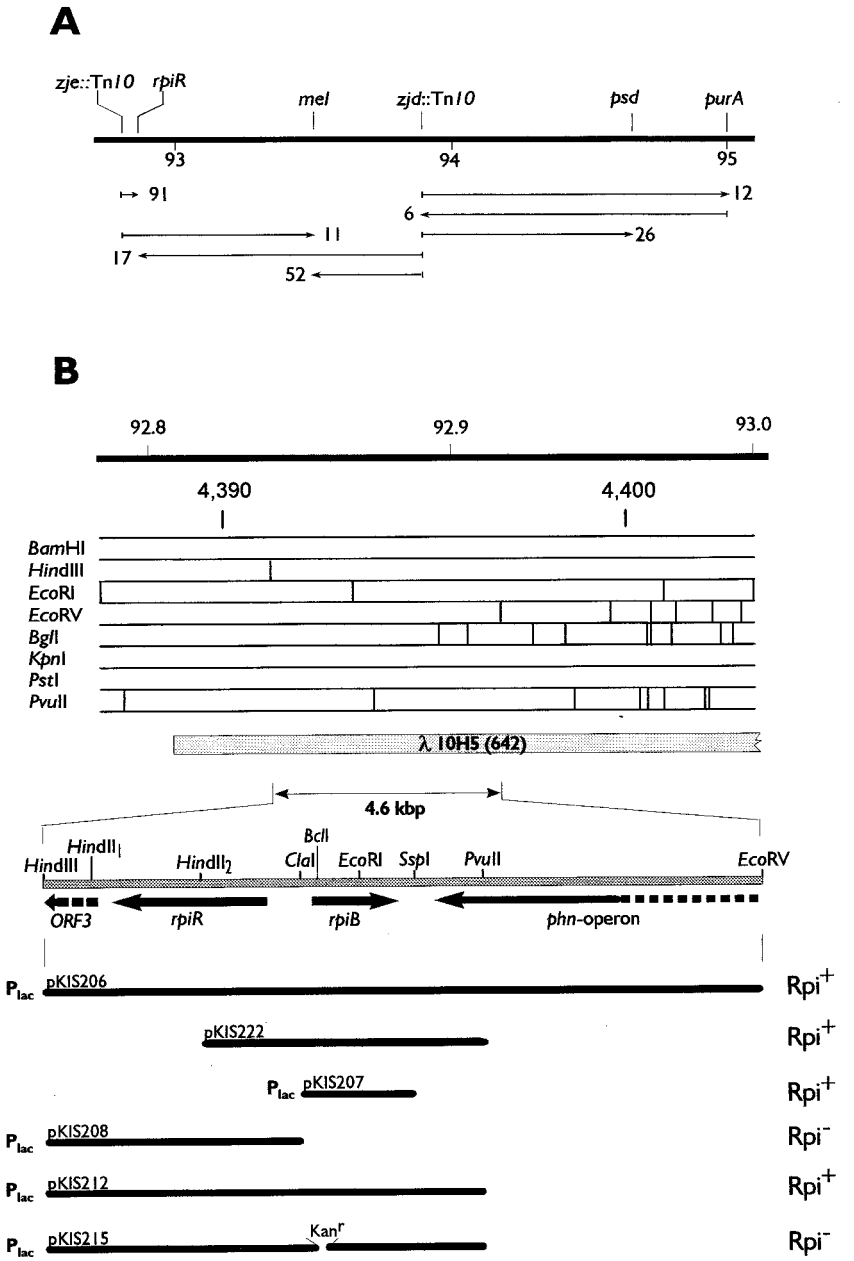

FIG. 2. Mapping and deletion analysis of *rpiB* and *rpiR*. (A) A genetic map of the *rpiR* region of the *E. coli* linkage map is shown as a bar. The *zje*::Tn*10* marker is *zje-2241*::Tn*10*, and the *zjd*::Tn*10* marker is *zjd-2231*::Tn*10*. Numbers below the bar indicate map units (in minutes) (3). Values at the end of arrows are percent cotransduction frequencies. Arrows point toward unselected markers. We were unable to confirm the previously published linkage map position of *zje-2241*::Tn*10* at 94.5 min. There was no linkage of *zje-2241*::Tn*10* with either *psd* or *purA*, located at 94.5 and 95 min, respectively. Rather, *zje-2241*::Tn*10* mapped at approximately 93 min, as shown here. (B) A physical map of the 4,387- to 4,403-kbp region of the *E. coli* chromosome is shown as a bar. Numbers above the bar are units (in minutes) of the genetic map. A restriction map is shown below the bar (redrawn from that of Kohara et al. [32] with the corrections of Makino et al. [38]). Below the restriction map is shown the extent of bacteriophage λ10H5(642). Below this, a restriction map of a 4.6-kbp *Hin*dIII-*Eco*RV DNA fragment is shown, with relevant open reading frames indicated by arrows. Broken arrows indicate open reading frames not completely included on this fragment. The structures of the plasmids used for deletion and enzyme analyses, as well as for analysis of plasmid-specified polypeptides, are shown as black rods. Plasmid pKIS206 was constructed by digesting the DNA of λ10H5(642) with restriction endonucleases *Hin*dIII and *Eco*RV and ligating this DNA to *Hin*dIII-*Hin*dII-digested pUC19 DNA. Plasmid pKIS207 was constructed by ligating *Cla*I-*Ssp*I-digested pKIS206 DNA to *Acc*I-*Sma*I-digested pUC19 DNA, whereas pKIS208 was constructed by ligating *Hin*dIII-*Cla*I-digested pKIS206 DNA to *Hin*dIII-*Acc*I-digested pUC19 DNA. Plasmid pKIS212 was obtained by digestion of pKIS206 DNA with restriction endonuclease *Pvu*II. This treatment removed most of the *phn* operon as well as part of the *lacZ* gene of the vector. Plasmid pKIS215 was obtained by ligating the kanamycin resistance-encoding *Bam*HI-generated DNA fragment of pUC4-K to the unique *Bcl*I site of pKIS212. Plasmid pKIS222 was constructed by digestion of pKIS206 DNA with restriction endonucleases *Pvu*II and *Hin*dII and subsequent ligation. By this treatment, the *Hin*dII$_2$-*Pvu*II DNA fragment was ligated to the *Pvu*II site of the vector, thereby deleting the *lac* promoter (P_{lac}) and part of the *lacZ* gene of the vector along with most of the *phn* operon. On the right, the phenotype that each plasmid confers to strain HO847 (Δ*rpiA101*) is indicated. Rpi$^+$, ribose prototrophy; Rpi$^-$, ribose auxotrophy.

phage P1-mediated transduction of strains with Tn*10* insertions mapped to the 66-to-97-min interval (49) was used for further mapping and placed the *rpiR* gene at approximately 93 min. Additional genetic mapping was performed with strains harboring the mutant alleles *zjd-2231*::Tn*10* (CAG18488), *zje-2241*::Tn*10* (CAG18427), *mel-1* (UH-Ac2), *psd-2* (EH150), and *purA* (HO120). The results of this analysis established the gene order as (*zje-2241*::Tn*10*)-*rpiR*-*mel*-(*zjd-2231*::Tn*10*)-*psd*-*purA* (data not shown). A genetic map of the *rpiR* region together with cotransduction frequencies is given in Fig. 2A. The *rpiR114* mutant allele was used for the mapping experiments described above. However, 25 additional *rpiR* alleles (*rpiR111* to *-113* and *rpiR115* to *-136* [Table 1]) were mapped to the same region. The joint transduction frequencies of these *rpiR* alleles and the two allelic transposon insertions *zje-2241*::Tn*10* (CAG18427) and *zje-3183*::Tn*10kan* (CAG18555) varied between 81 and 93%, suggesting that these mutations were alleles of the same gene. The F112 episome, which carries *metB*$^+$ as well as the *rpiR* region, was transferred from strain KL729 to the ribose-prototrophic strain HO978 (F$^-$ *metB rpiA rpiR114 zjd-2231*::Tn*10*) by selection for Met$^+$ Tet^r. All 90 *rpiA rpiR114*/F112 *rpiR*$^+$ exconjugants analyzed acquired a requirement for ribose, indicating that the wild-type allele was dominant over *rpiR114*.

Cloning of *rpiB* and *rpiR*. To clone the *rpiB* gene, we exploited the fact that a strain defective in the *rpiA* gene is a ribose auxotroph; we expected that the presence of *rpiB* on a multicopy plasmid would suppress this ribose auxotrophy. A chromosomal *Eco*RI DNA library of strain HO810 (*rpiA1 rpiR114*) was generated in pUC19 and transformed into strain HO847 (Δ*rpiA101*). By selecting for ribose prototrophy and ampicillin resistance, we obtained transformants harboring plasmids with a 7.8-kbp insert. The ribose phosphate isomerase B activities in these transformants were only twofold higher than that of the host strain, despite the high copy number of pUC19. Furthermore, subcloning and sequencing revealed that only the promoter and N-terminal coding part of the presumed *rpiB* gene were cloned. This suggested that the increased enzyme activity was not a consequence of plasmid-specified ribose phosphate isomerase B activity but rather was caused by titration of a repressor protein, resulting in increased expression of the chromosomal *rpiB* gene.

To clone the entire gene, a DNA fragment containing the *rpiB* promoter and the N-terminal coding part of the gene was used as a probe. By using an *E. coli* gene mapping membrane containing DNAs of the entire collection of bacteriophage λ clones of the Kohara gene library (32), the probe was shown to hybridize to the DNAs of three λ phages. The inserts of these phages, λ12A6(641), λ10H5(642), and λ12H2(643), overlapped the region at approximately 92.85 min on the linkage map, equivalent to 4,393 kbp on the physical map. A 4.6-kbp *Eco*RV-*Hin*dIII DNA fragment, including sequences 1.7 kbp upstream and 2.6 kbp downstream of the N-terminal coding part of *rpiB*, was cloned from bacteriophage λ10H5(642), yielding plasmid pKIS206 (Fig. 2B). The transformation of pKIS206 DNA into strain HO847 (Δ*rpiA101*) yielded transformants that were ribose prototrophs. To further locate the *rpiB* gene within the cloned DNA fragment, restriction and deletion analyses were performed (Fig. 2B). Plasmids containing the 2,462-bp *Hin*dIII-*Pvu*II DNA fragment (pKIS212), the 1,501-bp *Hin*dII$_2$-*Pvu*II DNA fragment (pKIS222), or the 687-bp *Cla*I-*Ssp*I DNA fragment (pKIS207) were able to suppress the ribose requirement of the *rpiA* strain. Strain HO847/pKIS215 (*rpiB*::Kan^r) was a ribose auxotroph, showing that the insertion of the kanamycin resistance-encoding DNA fragment into this plasmid inactivated the *rpiB* gene. The phenotypes of plasmid-harboring cells were in agreement with the measured activities of ribose phosphate isomerase B (Table 2). Cells harboring plasmids that converted the *rpiA* strain to ribose prototrophy (pKIS206, pKIS207, pKIS212, and pKIS222) had 5- to 28-fold-higher enzyme activities than that of the haploid strain HO847/pUC19, whereas plasmids unable to suppress the ribose requirement of the *rpiA* strain (pKIS208 and pKIS215) specified an enzyme activity similar to that observed in the haploid strain HO847/pUC19. On the basis of these results, we concluded that the coding part of the *rpiB* gene was located on the 687-bp *Cla*I-*Ssp*I DNA fragment.

TABLE 2. Ribose phosphate isomerase B activities of strain HO847 (Δ*rpiA101*) harboring various plasmids[a]

Plasmid	Activity (μmol/min/mg of protein)[b]	
	− Ribose	+ Ribose
pKIS206 (*rpiB*$^+$ *rpiR*$^+$)	1.3	3.1
pKIS207 (*rpiB*$^+$)	6.5	7.1
pKIS208 (*rpiR*$^+$)	—[c]	0.17
pKIS212 (*rpiB*$^+$ *rpiR*$^+$)	1.5	ND[d]
pKIS215 (*rpiR*$^+$)	—	0.27
pKIS222 (*rpiB*$^+$)	6.0	5.6
pUC19	—	0.25

[a] Cells were grown in glucose minimal medium without (−) or with (+) ribose.
[b] Enzyme activities are normalized for plasmid copy number by using the β-lactamase activity of strain HO847/pKIS206 as a reference.
[c] —, no growth.
[d] ND, not determined.

Furthermore, we found that transformants of strain HO810 (*rpiA1 rpiR114*) harboring pKIS208 (*rpiR*$^+$) were ribose auxotrophs. This result showed that the 1,620-bp *Hin*dIII-*Cla*I DNA fragment complemented the mutant *rpiR* allele, indicating that the *rpiR* gene was located on this fragment.

Regulation of *rpiB* gene expression. Comparison of the ribose phosphate isomerase B activities presented in Table 2 showed that cells harboring pKIS206 (*rpiB*$^+$ *rpiR*$^+$) contained an enzyme activity of 1.3 μmol/min/mg of protein when grown without ribose, whereas the activity was 3.1 μmol/min/mg of protein when cells were grown with ribose. This indicated that the presence of ribose caused a 2.5-fold induction of ribose phosphate isomerase B synthesis. Furthermore, in the absence of ribose, cells harboring pKIS222 (*rpiB*$^+$) showed a four- to fivefold-higher ribose phosphate isomerase B activity compared with that of cells harboring pKIS206 (*rpiB*$^+$ *rpiR*$^+$). This result indicates that *rpiB* gene expression is negatively affected by the *rpiR* gene product.

Plasmid-encoded polypeptides. To identify the *rpiB* gene product, plasmids pKIS206 (*rpiB*$^+$ *rpiR*$^+$), pKIS207 (*rpiB*$^+$), pKIS203 (Δ*rpiB* Δ*rpiR*), and pUC19 were introduced into the minicell-producing strain HO644. The encoded polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Both plasmids that supported the growth of an *rpiA* strain in the absence of ribose, pKIS206 and pKIS207, produced a band that was unique for these plasmids (Fig. 3B [arrow a]). This band, which showed higher intensity with pKIS207, represented a polypeptide with an M_r of 16,000. The size and band intensities were in agreement with the subunit size deduced from the amino acid sequence and the difference in the enzyme activities of cells harboring pKIS206 and pKIS207. This band with an M_r of 16,000 was also visible on the Coomassie-stained gel (Fig. 3A) and was assumed to represent the ribose phosphate isomerase B subunit. Plasmid pKIS203 contained the sequence from the *Hin*dII$_2$ site to the *Eco*RI site within the *rpiB* gene. This plasmid produced a unique band that migrated

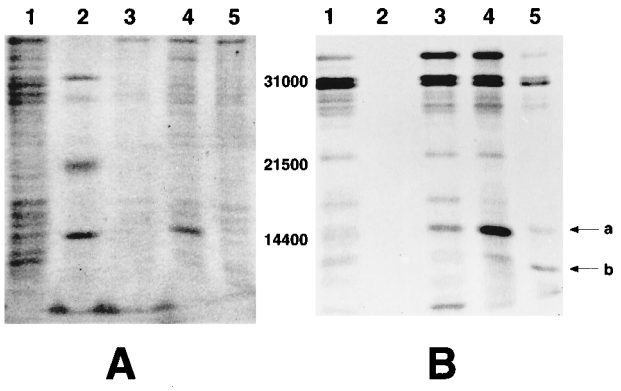

FIG. 3. Polypeptides specified by plasmids. Minicells harboring various plasmids were isolated and analyzed by [^{35}S]methionine labelling and gel electrophoresis in an SDS-containing polyacrylamide gel (15%), as described in Materials and Methods. (A) Coomassie brilliant blue-stained gel; (B) autoradiogram of the same gel. Minicells contained the following plasmids: pUC19 (lanes 1), pKIS206 (*rpiB*$^+$ *rpiR*$^+$) (lanes 3), pKIS207 (*rpiB*$^+$) (lanes 4), and pKIS203 (*rpiB*$^+$) (lanes 5). Lanes 2 contained the following M_r markers: carbonic anhydrase (31,000) soybean trypsin inhibitor (21,500), and lysozyme (14,400). The arrow labelled a points to the band representing the ribose phosphate isomerase B polypeptide, and the arrow labelled b points to a band representing a truncated ribose phosphate isomerase B polypeptide.

as a polypeptide with an M_r of approximately 10,000 (Fig. 3B [arrow b]). This polypeptide most likely represented a truncated form of the ribose phosphate isomerase B subunit. A band representing the *rpiR* gene product was not observed in cells harboring plasmid pKIS206.

Nucleotide sequence of the *rpiB* and *rpiR* genes. We determined the nucleotide sequence of the 2,312-bp *Hin*dIII-*Ssp*I DNA fragment harboring the *rpiB* and *rpiR* genes (Fig. 4). This fragment contained two divergent open reading frames. The open reading frame representing *rpiB* was transcribed clockwise and encoded a polypeptide of 149 amino acids, with a calculated molecular mass of 16,063 Da. A sequence (GGGG AAG) resembling a ribosome binding site (16) was located upstream of the initiation codon (AUG). The sequence TGTG AAGTTTTGCAC (nucleotides 1,670 to 1,684), which immediately precedes the putative *rpiB* ribosome binding site, conforms well to the consensus operator sequence associated with the binding of regulator proteins such as GalR and the cyclic AMP-catabolite gene activator protein complex (12, 40). The region downstream of *rpiB* contained the downstream end of the *phn* operon, as shown by sequence identity with a previously published sequence (38). A second open reading frame, located upstream of *rpiB*, was transcribed counterclockwise and consisted of 296 codons, which was sufficient to encode a polypeptide with a molecular mass of 32,341 Da. Complementation analyses and enzyme activity determinations indicated that this open reading frame represented the *rpiR* gene. The presumed start codon of *rpiR* was preceded by a sequence (AAGAGAA) with good homology to a consensus ribosome binding site. The very AT-rich leader region of *rpiR* contained a large inverted repeat sequence encompassing nucleotides 1,413 and 1,454 as well as several direct repeat sequences. A third open reading frame, ORF3, with the same orientation as that of the *rpiR* gene, began 58 bp after the stop codon of *rpiR* and was preceded by a putative ribosome binding site (GAGAACA).

Transcription of *rpiB* and *rpiR*. The 5′ ends of *rpiB*- and *rpiR*-specified mRNAs were mapped by primer extension (Fig. 5). The transcription start site for *rpiB* mRNA synthesis was mapped to the G at nucleotide 1,607 (Fig. 5A). Consistent with this, we found a hexanucleotide sequence, TAAAAC, with good homology to a consensus −10 region (20) located with proper spacing to the transcription start site (Fig. 4). In addition, we found a hexanucleotide resembling a −35 region with proper spacing from the −10 region.

Several transcription start sites were found for *rpiR* mRNA synthesis (Fig. 5B). The major start point was mapped to the A at nucleotide 1,481 (lower strand), with minor start points at the A's located at nucleotides 1,482 and 1,487. The start points at nucleotides 1,481 and 1,482 might correspond to a promoter with a −10 region at nucleotides 1,489 to 1,494 (ACTAAT), while the start point at nucleotide 1,487 might correspond to a promoter with a −10 region at nucleotides 1,494 to 1,499 (TTTAAA). Regions with homology to a consensus −35 region and in proper spacing to the −10 regions were not found, indicating that DNA sequences upstream and downstream at the −35 region might be important in this promoter (8). Furthermore, we identified a region of dyad symmetry after the *rpiB* gene at nucleotides 2,150 to 2,177 which might represent a rho-independent transcription terminator for the *rpiB* gene (57). No terminator sequence succeeding the *rpiR* gene was found.

Sequence similarities. The predicted amino acid sequences of ribose phosphate isomerase B, RpiR, and the ORF3-encoded protein were analyzed for amino acid similarities to the sequences available in peptide databases, as described in Materials and Methods. We were unable to find significant amino acid similarity between ribose phosphate isomerases A and B. Instead, ribose phosphate isomerase B showed 53% identity to an uncharacterized polypeptide of *Bacillus subtilis* (15) and approximately 40% identity to both *lacA*- and *lacB*-encoded subunits of the galactose 6-phosphate isomerases from *Staphylococcus aureus* (46), *S. mutans* (47), and *Lactococcus lactis* (54). Galactose 6-phosphate isomerase catalyzes the interconversion of galactose 6-phosphate and tagatose 6-phosphate. This reaction is unknown in *E. coli*. Transformation with the multicopy plasmid pKIS579, harboring the *lacA* and *lacB* genes from *S. mutans* (30), resulted in the suppression of the ribose requirement of *E. coli* HO847 (Δ*rpiA101*). When cells were grown in the absence of ribose, the ribose phosphate isomerase activity of strain HO847/pKIS579 (*lacA*$^+$ *lacB*$^+$)$_{S.\ mutans}$ was 0.7 μmol/min/mg of protein, compared with 6.0 μmol/min/mg of protein for strain HO847/pKIS222 (*rpiB*$^+$). This showed that galactose 6-phosphate isomerase is able to use a pentose phosphate as a substrate.

The *rpiR* gene product was 24% identical and 46% similar to a protein of similar size (molecular mass, 31,254 Da) encoded by the ORFB gene, which is located upstream of the *nagH* gene in *Clostridium perfringens* (7). The function of the ORFB-encoded protein is unknown. Inspection of the deduced amino acids 50 to 69 of RpiR revealed similarities to the α-helix-β-turn-α-helix motifs found in the DNA-binding domains of transcription repressors and activators (45). The deduced amino acid sequence of the N-terminal part of ORF3 was more than 30% identical to similar sequences of each of the D-ribose-binding proteins (RbsB) of *B. subtilis*, *E. coli*, and *Salmonella typhimurium* (18, 56).

Growth analysis of *rpiA*, *rpiB*, and *rpiA rpiB* strains. The phenotypes, i.e., growth on carbon sources, of strains harboring *rpiA* and *rpiB* mutations individually or in combination were analyzed (Table 3). In accordance with previous observations, strain HO890 (*rpiA*::Tet^r) was ribose prototrophic (51) and slightly impaired in the utilization of ribose compared with that of the wild-type strain (HO340). In contrast, the presence of the *rpiB*::Kan^r allele (strain HO1459) had no effect. Thus, ribose phosphate isomerases A and B appeared to be able to

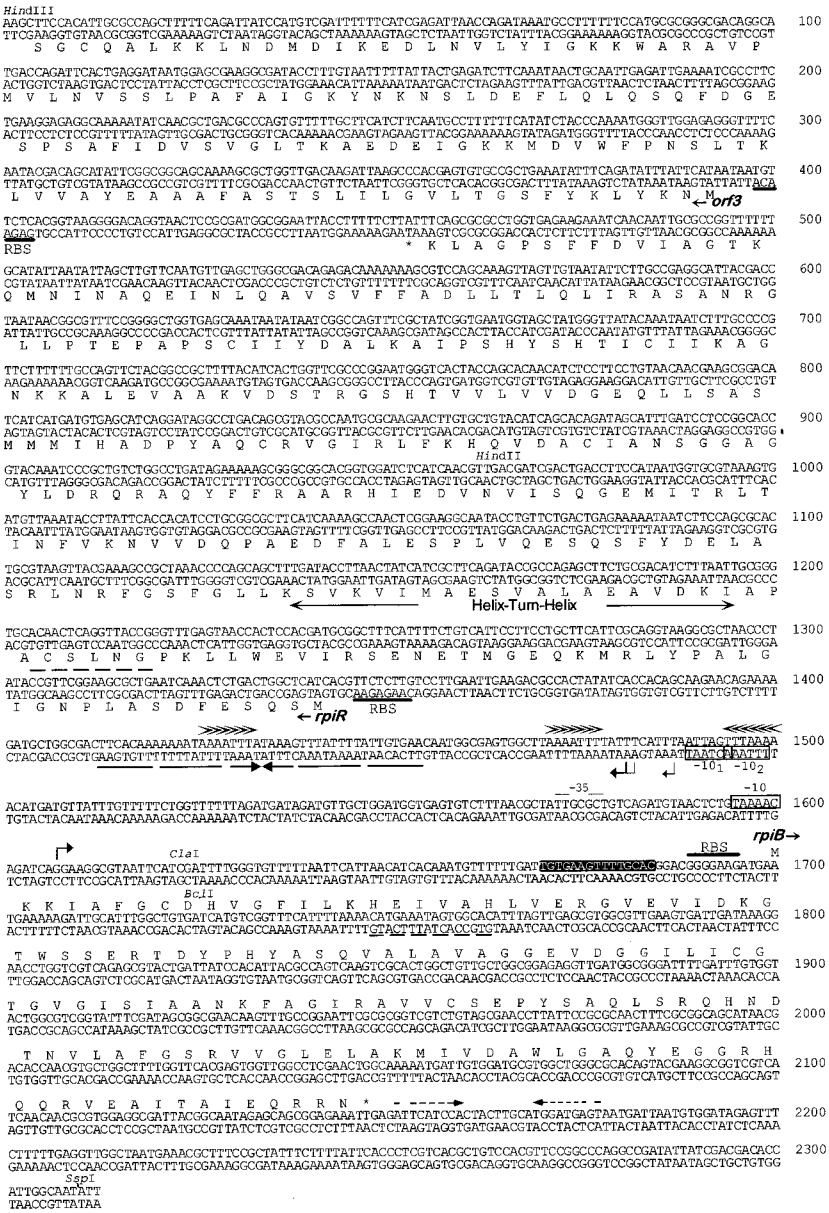

FIG. 4. Nucleotide sequence of the 2,312-bp *Hin*dIII-*Ssp*I *rpiR-rpiB* DNA fragment. The number at the end of each line indicates the nucleotide position. The deduced amino acid sequences of the open reading frames are indicated by one-letter code. Stop codons are indicated by asterisks. Relevant restriction endonuclease recognition sites are given above the nucleotide sequence. Ribosome binding sites (RBS) are indicated by bold underlining, −10 regions are boxed, and the putative −35 region of the *rpiB* promoter is overlined. Small right-angled arrows indicate transcription start sites found by primer extension analysis; the most frequently used start sites are shown as bold arrows. The convergently pointing arrows below nucleotides 1,413 to 1,454 and arrowheads in the *rpiR* promoter region denote direct and indirect repeats, respectively. Dashed lines indicate the positions of annealing of the oligonucleotides used in primer extension analysis (Fig. 5). The dashed arrows above nucleotides 2,150 to 2,177 indicate a putative transcription terminator (57). Divergent arrows below part of the RpiR amino acid sequence illustrate a helix-turn-helix motif. A region that exhibits homology with the consensus operator binding sequence (nucleotides 1,670 to 1,684) (12, 40) is indicated by white letters in a black box.

convert ribose 5-phosphate to ribulose 5-phosphate. In addition, both enzymes were able to catalyze the reverse reaction. The *rpiB*::Kan^r *rpiA*$^+$ strain (HO1459) grew without ribose, demonstrating that ribose phosphate isomerase A catalyzed the conversion of ribulose 5-phosphate to ribose 5-phosphate. Although the *rpiA*::Tet^r *rpiB*$^+$ strain (HO890) was ribose auxotrophic, it readily mutated to ribose prototrophy. These *rpiA rpiB*$^+$ *rpiR* strains retained the mutant *rpiA* allele, demonstrating that ribose phosphate isomerase B catalyzed the conversion of ribulose 5-phosphate to ribose 5-phosphate. However, the double mutant, *rpiA*::Tet^r *rpiB*::Kan^r (HO1460), was severely impaired in growth under all conditions tested. This strain required ribose for growth. In addition, a compound which can be converted to xylulose 5-phosphate, e.g., xylose or glucose, was required. Growth on rich medium was also impaired, and the addition of ribose to rich medium caused a severe inhibition of growth (data not shown). It is likely that this inhibition is caused by an accumulation of ribose 5-phosphate. The results presented above demonstrated that two mutations were necessary to prevent the reversible isomerization of ribulose

TABLE 3. Growth rates of *rpi* strains[a]

Strain	Allele[b]		Doubling time (min) in minimal medium supplemented with:				
	rpiA	*rpiB*	Ribose	Xylose	Ribose + xylose	Glucose	Ribose + glucose
HO890	−	+	188	>600	98	>600	88
HO1459	+	−	145	108	107	59	62
HO1460	−	−	>600	>600	150	>600	89
HO340	+	+	148	104	106	60	60

[a] Growth rates were determined as described in Materials and Methods.
[b] +, wild type; −, mutant.

5-phosphate and ribose 5-phosphate, thus confirming the results of biochemical analysis, which showed the existence of two enzymes catalyzing this reaction (13, 51).

DISCUSSION

The subunit molecular mass of ribose phosphate isomerase B was established as 16,063 Da. Gel filtration revealed the M_r of the native enzyme to be 32,000 to 34,000 (13), indicating that this enzyme is a homodimer. The subunit molecular mass of ribose phosphate isomerase A was calculated to be 22,845 Da (27). Both enzymes appeared to catalyze the same reaction. It is therefore surprising that there was no sequence homology between the two enzymes. This, together with the fact that *S. mutans* galactose 6-phosphate isomerase revealed a high degree of similarity to *E. coli* ribose 5-phosphate isomerase B and that the *S. mutans* enzyme substituted for the *E. coli* pentose phosphate isomerase, seems to suggest that the pentose phosphate isomerase activity of *E. coli* ribose phosphate isomerase B is a secondary function. However, it remains to be established that *E. coli* ribose phosphate isomerase B can use a hexose phosphate as a substrate. Expression of the *rpiB* gene appeared to be negatively regulated by the product of the upstream *rpiR* gene. We observed fivefold-lower ribose phosphate isomerase B activity in a strain harboring plasmid-borne *rpiB* and *rpiR* genes than that in a strain harboring a plasmid-borne *rpiB* gene. This effect is similar to results presented before, which revealed a sevenfold increase in the expression of ribose 5-phosphate isomerase B activity after the addition of ribose or nucleosides, both of which are catabolized via ribose 5-phosphate (13, 51). Furthermore, the ribose 5-phosphate isomerase activities in *rpiR* strains are more than 100-fold higher than those of uninduced wild-type strains, indicating that the addition of ribose is not enough to fully induce *rpiB* gene expression. Finally, derepression of *rpiB* gene expression has so far been observed only in strains harboring an *rpiA* mutation (13).

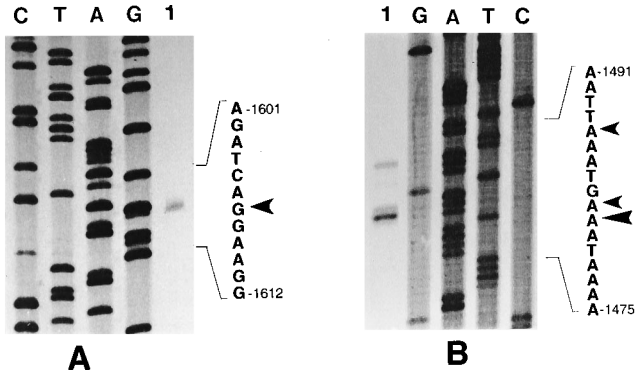

FIG. 5. Analyses of the 5′ ends of *rpiB*- and *rpiR*-specified mRNA transcripts. Primer extension analysis was performed with RNA templates isolated from strain HO847 (Δ*rpiA101*)/pKIS206 (*rpiB*$^+$ *rpiR*$^+$), as described in Materials and Methods. The positions of the annealing of primers are shown in Fig. 4. Autoradiograms of denaturing 6% polyacrylamide gels are shown. (A) Analysis of the 5′ end of *rpiB*-specified mRNA. Lanes C, T, A, and G are sequence ladders. Lane 1 contained the extension product. A portion of the sequence of the coding strand is given on the right, with the arrowhead pointing at the transcription initiation guanylate residue. (B) Analysis of the 5′ end of *rpiR*-specified mRNA. Lanes G, A T, and C are sequence ladders. Lane 1 contained the extension product. A portion of the sequence of the coding strand is given on the right, with arrowheads pointing at the transcription initiation adenylate residues. The larger arrowhead points at the most frequently used position. There was a minor increase in the mobility of the extension product compared with those of the sequence ladders. This effect was caused by the use of an unphosphorylated primer in sequencing reactions, whereas the primer was phosphorylated for the extension reaction.

Several results indicated that the *rpiR* gene was weakly expressed. (i) A titration effect on the expression of the chromosomal *rpiB* gene is observed by cloning the *rpiB* promoter region in *trans* on a multicopy plasmid, and a plasmid-borne *rpiB* gene is expressed efficiently despite the presence of the chromosomal *rpiR* gene. (ii) Analysis of minicells harboring a plasmid-borne *rpiR* gene reveals no polypeptide of the expected size (molecular mass, 32,341 Da). (iii) None of the *rpiR* promoters have consensus −35 regions.

The inducer for ribose phosphate isomerase B and the mechanism by which RpiR regulates *rpiB* gene expression remain to be elucidated. A helix-turn-helix motif characteristic of many regulatory proteins was found in the N-terminal part of RpiR, indicating a DNA-binding function. The regulatory mechanism may involve the unusually AT-rich intergenic region, which contains one large inverted repeat sequence, several direct repeat sequences, and a region with homology to a consensus operator sequence (Fig. 4). Obviously, the roles of these potential promoter elements for *rpiB* and *rpiR* gene regulation await more detailed study.

The entire nucleotide sequence of the *Hin*dIII-*Ssp*I DNA fragment is also AT rich. The A+T content (55%) is significantly higher than the approximately 49% A+T content found to be the average for the whole *E. coli* genome (44). This could indicate that these genes were transferred to *E. coli* from an ancestor with a more A+T-rich genome, such as *B. subtilis*, *S. mutans*, or *S. aureus*, all of which encode proteins with very high homologies to ribose phosphate isomerase B.

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