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Molecular Cloning and Characterization of Genes Required for Ribose Transport and Utilization in *Escherichia coli* K-12

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We isolated spontaneous and transposon insertion mutants of *Escherichia coli* K-12 that were specifically defective in utilization or in high-affinity transport of D-ribose (or in both). Cotransduction studies located all of the mutations near *ilv*, at the same position as previously identified mutations causing defects in ribokinase (*rbsK*) or ribose transport (*rbsP*). Plasmids that complemented the *rbs* mutations were isolated from the collection of ColE1 hybrid plasmids constructed by Clarke and Carbon. Analysis of those plasmids as well as of fragments cloned into pBR322 and pACYC184 allowed definition of the *rbs* region. Products of *rbs* genes were identified by examination of the proteins produced in minicells containing various *rbs* plasmids. We identified four *rbs* genes: *rbsB*, which codes for the 29-kilodalton ribose-binding protein; *rbsK*, which codes for the 34-kilodalton ribokinase; *rbsA*, which codes for a 50-kilodalton protein required for high-affinity transport; and *rbsC*, which codes for a 27-kilodalton protein likely to be a transport system component. Our studies showed that these genes are transcribed from a common promoter in the order *rbsA rbsC rbsB rbsK*. It appears that the high-affinity transport system for ribose consists of the three components, ribose-binding protein, the 50-kilodalton RbsA protein, and the 27-kilodalton RbsC protein, although a fourth, unidentified component could exist. Mutants defective in this transport system, but normal for ribokinase, are able to grow normally on high concentrations of the sugar, indicating that there is at least a second, low-affinity transport system for ribose in *E. coli* K-12.

Most of the active transport systems in gram-negative bacteria can be grouped into two distinct types (13, 45). One type, exemplified by the lactose transport system in *Escherichia coli*, consists of a single transmembrane protein that couples the transport of ligand to proton motive force. An osmotic shock treatment that releases periplasmic proteins from gram-negative cells has little effect on the activity of such systems. The other type of transport system consists of several components, a ligand-binding protein located in the periplasm as well as proteins associated with the cytoplasmic membrane. Release of the periplasmic protein by osmotic shock greatly reduces transport activity. The energy source for shock-sensitive systems appears to be oxidation-reduction reactions that are not yet fully characterized (30). There is a large number of different shock-sensitive systems in *E. coli*, each specific for transport of a particular amino acid, ion, or sugar. Among the many periplasmic, ligand-binding proteins that are components of these transport systems, three sugar-binding proteins, for galactose, maltose, and ribose, perform additional roles as chemoreceptors that mediate tactic responses to their respective ligands (2, 25, 26).

There is a substantial body of knowledge about the maltose system (7, 29, 35), and recent studies have defined the components of the galactose system (21, 42). In contrast, the transport system involving the ribose-binding protein has been less extensively characterized. Anderson and Cooper (3) analyzed two mutants of *E. coli* K-12 that were unable to grow on ribose as the sole carbon source and showed that one was defective in ribokinase (EC 2.7.1.15) activity and the other was defective in ribose transport. They named the respective mutations *rbsK* and *rbsP* and found that both were located at 84 min on the *E. coli* linkage map (5). Hazelbauer and Adler (26) detected a ribose-binding activity, released from *E. coli* by osmotic shock, that had properties that were identical to those of the ribose chemoreceptor. This activity was subsequently identified as the ribose-binding protein (17, 49), and a similar protein was found in *Salmonella typhimurium* (1). Characterization of presumed frameshift mutants defective for ribose utilization and their phenotypic revertants in *S. typhimurium* (2) and *E. coli* (17) revealed a strong correlation between absence of the ribose-binding protein and defective ribose taxis and high-affinity transport. However, because the mutations had not been defined genetically, it was not possible from these studies to conclude definitely that the ribose-binding protein itself was essential for taxis or transport or to determine the relationship between *rbsP* and the binding protein.

To learn more about the *rbs* system, we isolated additional mutants and obtained cloned fragments containing the *rbs* region. Characterization of those mutants and clones has allowed identification of several *rbs* genes, their roles in ribose utilization, and their respective protein products.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this work are all derivatives of *E. coli* K-12. Bacterial strains and plasmids are listed in Table 1.

Chemicals. All of the restriction enzymes and T4 DNA ligase were obtained from Takara Shuzo Co. Ltd., Kyoto, Japan. L-[^{35}S]methionine (≥1,000 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass., and [^{14}C]ribose (60 mCi/mmol) was from Amersham, England. All sugars used were the D form.

Media. LB medium, tryptone broth, and M9 minimal salts medium were prepared as described by Miller (37). Sugars (0.2%), glycerol (0.4%), sodium succinate (0.4%), thiamine

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid[a]	Sex	Genotype,[b] comment, or reference
Bacteria		
W3110	F^-	Prototroph (4)
B14	F^+	*rpsL* (24)
EJ500	F^-	W3110 *cfs* (31)
UH114	F^+	B14 *rbs-101*::Tn*5*
UH889	F^-	*minA minB rpsL mgl*(?) *rbs-102*::Tn*10 recA*
AI372	F^-	*his trp*(Am) *lac*(Am) *tyr*(Am) *tonA*(Am) *tsx*(Am) *btuB sup-126 uncA401 ilv*::Tn*10*
Plasmids		
pBR322		bla^+ tet^+ (6)
pACYC184		tet^+ cat^+ (9)
pUH25		ColE1 hybrid plasmid (10) carrying *rbs* genes
pUH27		ColE1 hybrid plasmid (10) carrying *rbs* genes
pUH28		ColE1 hybrid plasmid (10) carrying *rbs* genes
F′*lac*::Tn*10*		*lacZ260*::Tn*10* $proA^+$ $proB^+$

[a] Other bacterial strains and plasmids constructed in this study are described in the text.

[b] Genetic symbols for bacteria are those of Bachmann (5). In addition, the abbreviation *cfs* (constitutive for flagella synthesis) is used.

(10 μM), and amino acids (1 mM) were added to minimal medium when required. Solid media for plates contained 1.5% agar. MacConkey plates contained 4% MacConkey agar base (Difco Laboratories, Detroit, Mich.) and 1% sugar. Chemotaxis swarm plates were prepared as described previously (24). Antibiotics were supplemented in agar plates at the following concentrations when required: kanamycin at 40 μg/ml, tetracycline at 15 μg/ml, streptomycin at 100 μg/ml, ampicillin at 25 μg/ml, and chloramphemicol at 25 μg/ml.

Genetic procedures. Conventional genetic procedures were as described by Miller (37). Construction of *recA* strains was performed as described by Harayama and Iino (23). A *cys*::Tn*5* or *cys*::Tn*10* mutation in the *cysC-J* region was introduced into strains by P1*kc*-mediated transduction, and the resulting transductants were crossed with KL16-99 (Hfr type KL16 *recA1*). Cys^+ transconjugants were isolated, and the Rec^- phenotype was determined by testing for sensitivity to UV light. Preparation of competent cells of *E. coli* by $CaCl_2$ treatment and transformation with plasmid DNA was carried out by the method of Dargent and Ehrlich (11).

Transposon-insertion mutagenesis with Tn*5* or Tn*10* with bacteriophage λ*km* (*c*I857 *b*221 *rex*::Tn*5*) or λ*tc* (N^+ *c*I857 *b*221 *O*am29 *c*III::Tn*10*) has been described previously (24). Strains containing the *rbs-101*::Tn*5*, *rbs-102*::Tn*10*, or *rbs-103* mutation were originally isolated as clones unable to make a chemotactic ring on a minimal swarm plate containing ribose. Other mutants were isolated as white colonies on MacConkey plates containing ribose. Tn*1000* (formerly called γδ) insertion mutagenesis of pBR322 and pACYC184 derivatives was performed as described by Sancar and Rupp (43) by mobilization of these plasmids with F′*lac*::Tn*10*. Mobilization always occurred by Tn*1000*-, not Tn*10*-mediated cointegrate formation.

Manipulation of DNA. Techniques for the preparation of plasmid DNA from a cleared lysate by CsCl-ethidium bromide centrifugation were outlined previously (22). For pACYC184 derivatives, spectinomycin at 25 μg/ml was used to amplify plasmids. Plasmid DNA was used after phenol extraction and ethanol precipitation. The small-scale preparation of plasmid DNA described by Kado and Liu (33) was also used in some cases.

The reaction buffers used for digestion of DNA were as described by Maniatis et al. (36), except that 100 μg of bovine serum albumin per ml was included in the buffer. For double digestions, the restriction enzyme active in the lower ionic strength buffer was used first. For partial digestion, appropriate dilutions of the restriction enzyme were used, and the reaction was carried out at 37°C for 5 or 10 min. The reaction was stopped by treatment at 65°C for 10 min. Ligation of DNA with T4 DNA ligase was carried out in ligation buffer (66 mM Tris [pH 7.6]–6.6 mM $MgCl_2$–10 mM dithiothreitol–1 mM ATP) at 16°C for 2 to 12 h.

Analysis of DNA was performed by electrophoresis in vertical gels which contained 0.7% agarose (Seakem) in Tris-acetate buffer (40 mM Tris [pH 8.0]–20 mM sodium acetate–2 mM EDTA). *Hin*dIII-digested fragments of λ*c*I857*S*7 were used as molecular weight standards (47).

Chemotaxis swarm assay. Swarm assays were carried out by the method of Ordal and Adler (39). Colonies were picked up from a plate with toothpicks and spotted on a tryptone swarm plate and three minimal salts plates containing 0.1 mM ribose, galactose, or maltose. The plates were examined after 6 to 9 h (tryptone) or after 16 to 24 h (minimal) at 37°C.

Transport assay. Cells grown in M9 minimal medium containing 0.4% glycerol, 0.2% ribose, and a supplement of trace elements (46) were washed four times with 10 mM KPO_4 (pH 6.9) at 0°C and suspended to an optical density at 660 nm of 0.3 (about 3×10^8 cells per ml) in 0.1 M KPO_4 (pH 7.2). A 100-μl sample of chloramphenicol (400 μg/ml) was added to 800 μl of the cell suspension, and the mixture was brought to 30°C. A 100-μl sample of [^{14}C]ribose (1 μM final concentration) was added at 0 time. At 15 or 30 s, 800 μl of the mixture was filtered through 0.45-μm pore cellulose filters (Millipore Corp., Bedford, Mass.) on a 1225 Sampling Manifold (Millipore Corp.) and washed with 5 ml of 10 mM Tris (pH 7.3)–0.5 mM $MgCl_2$–0.15 M NaCl at 0°C. The filters were dried, and the radioactivity was determined by liquid scintillation counting. Values for nonspecific binding of ribose on the cells and on the filter were estimated by using cells treated with 10% formaldehyde.

Ribokinase assay. An assay with DEAE-cellulose paper was carried out essentially as described by Newsholme et al. (38). Cells grown in M9 minimal medium containing 0.4% glycerol, 0.2% ribose, and a mixture of 20 amino acids (1 mM) were washed four times with distilled water and suspended in water at an optical density at 560 nm of 20. The suspension cooled in an ice-salt bath was sonicated for six 10-s intervals with a 60-s pause between each sonication. Cell extracts were dialyzed against 0.1 M Tris (pH 8.0)–10 mM $MgCl_2$ at 4°C. A 10-μl sample of an extract was added to 90 μl of reaction solution to yield 0.1 M Tris (pH 8.0), 10 mM $MgCl_2$, 10 mM ATP, and 0.1 mM [^{14}C]ribose. After 30 min at 37°C, an equal volume of 95% ethanol was added to stop the reaction. Samples (40 μl) of each reaction mixture were spotted on DEAE-cellulose paper (Whatman) and washed with 50 ml of distilled water. Filters were dried, and radioactivity was determined by liquid scintillation counting. Proteins in cell extracts were determined by the method of Bradford (8) with bovine serum albumin as the standard.

Preparation of minicells. Minicells were isolated by the method of Frazer and Curtiss (16) with a stationary-phase culture grown in LB medium containing 0.4% ribose. Analy-

TABLE 2. Mapping of *rbs* mutation by a three-point transductional cross

Donor	Recipient	Selected marker	Unselected markers		No. of transductants (208 total)
			rbs	*uncA*[a]	
UH114	AI372	*ilv*$^+$	+	+	2
(*rbs-101*::Tn*5*)	(*ilv*::Tn*10*		+	−	6
	uncA)		−	+	112
			−	−	88

[a] The test was growth on a minimal salts plate containing sodium succinate.

sis of proteins synthesized in minicells by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and autoradiography was performed as described previously (15).

Immunoprecipitation. Immunoprecipitation was carried out as described by Randall (41). About 2.5 × 10^8 cells grown in tryptone broth containing 0.2% ribose were solubilized directly in 0.5% SDS. Immunoprecipitates were analyzed by SDS-gel electrophoresis. Antiserum to ribose-binding protein from *S. typhimurium* was the kind gift of C. Furlong (University of Washington, Seattle). *Salmonella* sp. anti-ribose-binding protein serum is known to cross-react with ribose-binding protein in *E. coli* (49).

RESULTS

Isolation and mapping of *rbs* mutants. After transposon insertion mutagenesis of *E. coli* K-12, we isolated eight independent mutants specifically defective in utilization or transport of ribose as reflected in their inability to grow and form a tactic ring on a chemotactic swarm plate containing minimal salts and 0.1 mM ribose. Tactic ring formation occurs only when the bacteria take up the ribose, metabolize the sugar, and respond tactically to the gradient created by metabolism at the point of inoculation (39). Motile mutant cells defective solely in tactic response spred from the point of inoculation without forming a distinct ring (24), whereas mutants defective in high-affinity uptake or metabolism exhibit little growth and thus no spreading. The mutants considered in this study did not form tactic rings on ribose swarm plates, but formed normal rings on plates containing galactose, maltose, serine, or aspartate, indicating that the general chemotactic system was functional, and that ribose metabolism or high-affinity uptake (or both) was likely to be defective. Two of the mutations were not the result of transposon insertions, but rather were the result of spontaneous mutations in the parent strains. All of the mutations were cotransducible with *ilv* at a frequency of 80 to 95%, consistent with a common location at 84 min on the *E. coli* linkage map, the same position at which *rbsP* and *rbsK* has been previously mapped (3, 48). A three-point cross with P1*kc*-mediated transduction of *rbs-101*::Tn*5* showed 95% linkage to *ilv* and 55% linkage to *uncA* (Table 2). The occurrence of Ilv$^+$ Rbs$^+$ Unc$^+$ recombinants as the lowest frequency class implies a gene order *uncA rbs ilv*.

Phenotypic characterization. A set of isogenic *rbs* mutant strains was created by P1*kc*-mediated transduction of each mutation into EJ500, a derivative of W3110. One insertion, *rbs-101*::Tn*5*, is somewhat unstable since we found derivatives of the original mutant strain that carried deletions of DNA beginning at the insertion site (see below). The set of *rbs* mutants including the deletion strains was examined for a number of ribose-related phenotypic properties (Table 3). All of the mutations except *rbs-101*::Tn*5* essentially eliminated transport of ribose at 1 μM, indicating that those mutations affected components of the high-affinity transport system for ribose. The *rbs-101*::Tn*5* insertion eliminated ribokinase activity, but did not affect ribose uptake; thus the insertion was a candidate for a specific *rbsK* mutation. All mutations that affected transport activity, with the exception of *rbs-103*, also eliminated ribokinase activity as well as the production of ribose-binding protein. As discussed below, this observation is consistent with a polar effect of mutations in the gene for ribose-binding protein or in genes further upstream in the operon on the promotor-distal *rbsK* gene. The *rbs-103* mutation affected the ribose-binding protein and eliminated high-affinity uptake of ribose, but had no affect on the growth of colonies at high (13 mM) ribose concentrations or on ribokinase activity. No ribose-binding protein was released from *rbs-103* mutant cells by the standard osmotic shock procedure. However, immune precipitation of whole cell extracts with an anti-ribose-binding protein serum revealed a protein with a molecular weight about 2,000 higher than the normal ribose protein. Work to be described elsewhere demonstrates that *rbs-103* is a mutation in the gene for the ribose-binding protein, which we shall call *rbsB*,

TABLE 3. Phenotypes of *rbs* mutations

Strain[a]	Mutation	Growth on ribose[b]	Ribokinase activity (pmol/30 min per μg of protein)	Transport activity[c] (pmol/min per μg of protein)	Presence of ribose-binding protein[d]
EJ500	None	+	76[e]	11	+
AI271	*rbs-101*::Tn*5*	−	0.1	15	+
AI101	*rbs-101*Δ1::Tn*5*	−	0.1	0.2	−
AI182	*rbs-101*Δ2::Tn*5*	−	0.1	0.1	−
AI102	*rbs-102*::Tn*10*	−	0.1	0.2	−
AI104	*rbs-103*	+	55	0.1	+[f]
AI103	*rbs-104*	−	5.4	0.1	−
AI296	*rbs-105*::Tn*10*	−	5.4	0.3	−
AI297	*rbs-106*::Tn*10*	−	5.0	0.2	−
AI298	*rbs-107*::Tn*10*	−	4.0	0.3	−
AI299	*rbs-108*::Tn*10*	−	2.2	0.2	−

[a] All mutant strains are derivatives of EJ500.
[b] Minimal salts plus 13 mM ribose; all mutants grew normally on minimal salts plus glucose.
[c] Initial rate of uptake from 1 μM ribose solution.
[d] Determined by analysis of immune precipitates from lysed cells by SDS-polyacrylamide gel electrophoresis.
[e] Uninduced (glycerol-grown) cells exhibited an activity of 7.5 pmol/30 min per μg of protein.
[f] The apparent molecular weight was 2000 higher than that of ribose-binding protein from the wild-type strain.

that results in defective export and proteolytic processing of the precursor form of that protein.

Cloning the *rbs* region. We isolated ColE1 hybrid plasmids that complemented the inability of an *rbs-102*::Tn*10* mutant to grow on ribose from a pooled collection of the plasmids constructed by Clarke and Carbon (10). Three different plasmids of 17.6, 31.7, and 15.4 kilobase pairs (kb) were designated pUH25, pUH27, and pUH28, respectively. All three contained a 1.5- and a 3.7-kb *Hin*dIII fragment of cloned DNA (data not shown) and thus appeared to include a common region of the *E. coli* chromosome. Physical maps of pUH25 and pUH28 were constructed with a variety of restriction endonucleases (Fig. 1). Since the two chromosomal fragments differed in length and orientation relative to the ColE1 vector, they represent independent clones. The transposon insertion *rbs-101*::Tn*5* was transferred from the chromosome to pUH25 by reciprocal recombination, and the position of Tn*5* on the resulting plasmid, pAI28, was determined by mapping with restriction endonucleases to be within the region common to both *rbs* clones (Fig. 1). In two initial attempts to isolate a pUH25 derivative carrying *rbs-101*::Tn*5*, we found that the recombined plasmids carried deletions of chromosomal DNA contiguous to the Tn*5* insertion site. pAI3 had a 7.5-kb deletion, and pAI17 had a 4.5-kb deletion, each also carried the Tn*5* insertion, so we designated the two mutations *rbs-101*Δ1::Tn*5* and *rbs-101*Δ2::Tn*5*, respectively (Fig. 1).

Fragments of pUH25 were subcloned into pBR322 (Fig. 1). pAI6 contained an *Hin*dIII fragment resulting from incomplete digestion of pUH25. Deletion of the *Bam*HI fragment of pAI6 created pAI13, and deletion of the *Sma*I fragment of pAI13 yielded pAI16. pAI5 carried the 3.7-kb *Hin*dIII fragment of pUH25. pAI13 was not stably maintained in the host cells. From a strain containing a chromosomal Tn*5* insertion, a stable derivative, pAI15, was isolated and shown to carry an insertion in the 1-kb *Sma*I fragment of pAI13. The insertion is likely to be IS*50* translocated from the Tn*5* on the chromosome since it was 1.5 kb in length and contained one *Hin*dIII and one *Pst*I site (32). Each of the plasmids derived from pUH25 was tested for the ability to complement *rbs* mutations (Table 4). Strains carrying an *rbs* mutation, a *recA* mutation, and an *rbs* plasmid were tested for the ability to ferment ribose and for the ability to grow and form a tactic ring on a ribose swarm plate. As shown in Table 3, the ability to metabolize ribose was correlated with active ribokinase, but did not require high-affinity uptake of ribose, whereas growth and tactic ring formation on a swarm plate containing 0.1 mM ribose required both ribokinase and high-affinity uptake activity. Plasmid pAI13 and the larger

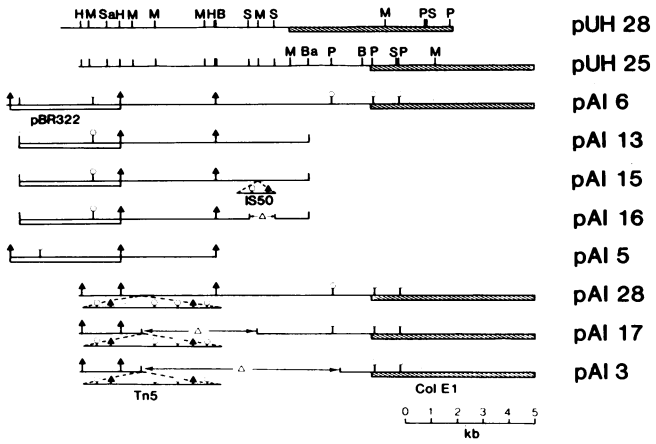

FIG. 1. Linearized physical maps of plasmids carrying *rbs* genes. Symbols: single line, cloned chromosomal DNA or insertion sequences Tn*5* (32) or IS*50*; double line, pBR322 DNA; cross-hatched regions, ColE1 DNA (14). Restriction endonuclease sites are labeled as follows: H, △, or ▲, *Hin*dIII; M, *Mlu*I; Sa, *Sal*I; B, *Bgl*II; S, *Sma*I, P or ○, *Pst*I; Ba, *Bam*HI.

plasmids from which it was derived complemented all of the *rbs* mutations examined in both tests (Table 4). Plasmid pAI5, containing the 3.7-kb *Hin*dIII fragment, complemented the ribose utilization defect of all mutants, indicating that the ribokinase gene, *rbsK*, was included in the fragment. The same plasmid complemented the defect in tactic ring formation for a group of mutations including the specific ribose-binding protein mutation, *rbs-103*, indicating that the gene for the binding protein *rbsB* was also on the *Hin*dIII fragment. However, the defect in ring formation of a second group of mutations was not corrected by pAI5, pAI15, or pAI16, but only the larger, unaltered plasmids like pAI13. When ability to take up 1 μM ribose was determined directly for plasmid-containing mutants carrying *rbs-101*Δ1::Tn*5* or *rbs-102*::Tn*10*, complementation of defective uptake corresponded exactly to the complementation of tactic ring formation shown in Table 4. Thus we conclude that a gene required for high-affinity transport of ribose, which we shall call *rbsA*, is present on a segment of DNA included in the larger plasmids, but not on pAI5, and the gene overlaps the *Sma*I fragment deleted in pAI16.

The location of *rbsB* and *rbsK* within pAI5 was defined by using derivatives of the plasmid that had acquired insertions of Tn*1000* or deletions of DNA after digestion in vitro with a specific restriction enzyme (Fig. 2). The plasmids were

TABLE 4. Complementation of *rbs* mutations by hybrid plasmids

Strain[a]	*rbs* mutation	Plasmid[b]						
		pUH25	pAI6	pAI13	pAI15	pAI16	pAI5	None
AI174	*rbs-101*Δ1::Tn*5*	+/+	+/+	+/+	+/−	+/−	+/−	−/−
AI221	*rbs-101*Δ2::Tn*5*	+/+	+/+	+/+	+/−	+/−	+/−	−/−
AI176	*rbs-104*	+/+	+/+	+/+	+/−	+/−	+/−	−/−
AI312	*rbs-105*::Tn*10*	NT	+/+	+/+	+/−	+/−	+/−	−/−
AI313	*rbs-106*::Tn*10*	NT	+/+	+/+	+/−	+/−	+/−	−/−
AI359	*rbs-101*::Tn*5*	NT	NT	NT	NT	NT	+/+	−/−
AI207	*rbs-102*::Tn*10*	+/+	+/+	+/+	+/+	+/+	+/+	−/−
AI320	*rbs-108*::Tn*10*	NT	NT	NT	NT	NT	+/+	−/−
AI208	*rbs-103*	NT	NT	NT	NT	NT	+/+	+/−

[a] The strains are all *recA* derivatives of the strains shown in Table 3. In addition, AI174, AI176, and AI208 also carry *rpsL*.

[b] The first symbol (+ or −) indicates ability to metabolize ribose on a MacConkey indicator plate; the second symbol (+ or −) indicates ability to form a chemotactic ring on a swarm plate containing 0.1 mM ribose (see the text). NT, Not tested.

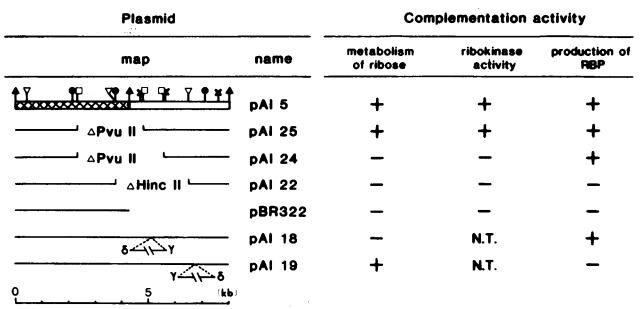

FIG. 2. Complementation of Rbs defects by *rbs* plasmids. The plasmids shown were introduced into an *recA rbs-101*::Tn*5* (kinase assay) or an *recA rbs-102*::Tn*10* host, and the resulting strains were tested for metabolism of ribose on MacConkey indicator plates, ribokinase activity, and production of ribose-binding protein (RBP) with an anti-ribose-binding protein serum as in Fig. 4. Ribokinase activity in the strains carrying pAI5 or pAI25 was 120 and 194 pmol/30 min per μg of protein, respectively (compared with a wild-type level of 76; Table 3), whereas the other strains tested contained no detectable activity. N.T., not tested. Symbols: ▲, *Hin*dIII; ▽, *Hin*cII; ●, *Acc*I; □, *Pvu*II; ×, *Mlu*I. Orientation of Tn*1000* inserted in pAI18 and pAI19 is indicated by the γ and δ ends of the transposon (20). The size of Tn*1000* is not to scale.

introduced into *recA* strains carrying *rbs-101*::Tn*5* (kinase negative) or *rbs-102*::Tn*10* (kinase negative, binding protein negative), and the phenotypes of the transformants were determined (Fig. 2). We concluded that the sequence of *rbsK* includes some of the DNA between the two *Pvu*II sites on the insert and that *rbs*B is to the right of *rbsK* on the map in Fig. 2 and includes the *Hin*cII site.

Polypeptide products of *rbs* genes. Analysis of proteins synthesized in minicells containing pUH25, pUH28, or pAI6 revealed five common polypeptides, with apparent molecular weights of 50,000, 34,000, 31,500, 29,000, and 27,000, produced from the cloned DNA (Fig. 3). All but the 50-kilodalton (kdal) protein were also coded for by the 3.7-kb *Hin*dIII fragment (Fig. 4). The 29- and 31.5-kdal proteins were shown to be, respectively, the ribose-binding protein and the precursor form of that protein by the following criteria. (i) The 29-kdal polypeptide comigrated in SDS-polyacrylamide gel electrophoresis with authentic ribose-binding protein. (ii) Both proteins were precipitated by antiserum to the ribose protein (Fig. 4). (iii) The proteins exhibited similar patterns of peptides, consistent with a precursor-product relationship, after limited proteolysis with V8 protease from *Staphylococcus aureus* (data not shown). (iv) As observed for other precursor forms (21), the 31.5-kdal polypeptide was seen as a distinct species in minicells and accumulated when synthesis proceeded in minicells uncoupled by 50 μM carbonyl cyanide-*m*-chlorophenyl hydrazone (data not shown).

The 34-kdal protein was identified as ribokinase. The protein was synthesized in minicells carrying kinase-positive plasmids, but not in those carrying deletion plasmids that were kinase negative (compare Fig. 2 and 5). Presence of the *rbs-101*::Tn*5* insertion in pAI28, a derivative of pUH25, also eliminated synthesis of the 34-kdal protein in minicells (data not shown).

Synthesis of the 50-kdal protein correlated with the presence of *rbsA*, the gene required for high-affinity ribose transport that was present on the complete *rbs*-plasmids pUH25, pUH28, pAI6, and pAI13, but absent on pAI5 or pAI16 (Fig. 3 and 5). The gene coding for the 27-kdal protein was localized at the right-hand end of the *Hin*dIII fragment (Fig. 2) since the protein was synthesized in minicells containing all *rbs* plasmids including pAI22, which carries only the 1.5-kb *Hin*cII-*Hin*dIII fragment.

Reversion analysis of transposon insertion mutants. All *rbs*::Tn*10* insertions drastically reduced ribokinase activity, high-affinity transport, and the production of ribose-binding protein, whereas the *rbs-101*::Tn*5* insertion eliminated ribokinase activity without affecting the other features (Table 3). These phenotypes suggested that the Tn*10* insertions were reducing, but not eliminating, ribokinase production by polarity on the promotor distal *rbsK* gene. Derivatives of *rbs-102*::Tn*10* selected for growth on minimal plates containing 13 mM ribose regained normal ribokinase activity, but not the ability to synthesize ribose-binding protein or to grow and make a chemotactic ring on a swarm plate containing 0.1 mM ribose. Some of these pseudorevertants still carried a tetracycline resistance marker linked to *ilv*, implying that at least part of the original Tn*10* insertion was still in place (Table 5). The phenotype of these pseudorevertants implies that the *rbs-102*::Tn*10* insertion is in the gene for ribose-binding protein. In contrast, revertants of *rbs-101*::Tn*5* able to grow normally on 13 mM ribose occurred only rarely and were all sensitive to kanamycin, indicating loss of the Tn*5* insertion (Table 5). This observation suggests that the insertion is in the structural gene for ribokinase and that the revertants represent reformation of an active *rbsK* gene upon precise excision of the inserted Tn*5*.

Reversion analysis was also performed for the other *rbs* transposon mutations. Ribose-positive derivatives of an *rbs-108*::Tn*10* strain occurred at a frequency even higher than

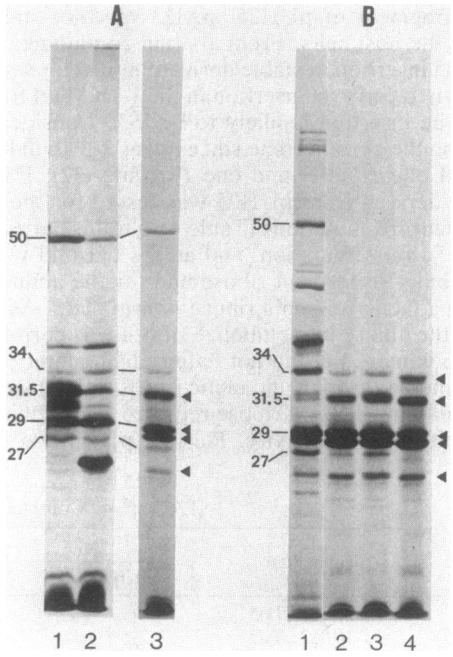

FIG. 3. Proteins synthesized in minicells containing *rbs* plasmids. The figures are autoradiograms of SDS-polyacrylamide (12%) gels containing [^{35}S]methionine-labeled proteins. Products coded for by *rbs* genes are indicated by numbers (in kdal). Products coded for by pBR322 are indicated by arrows. (A) Proteins synthesized in minicells from UH889 carrying ColE1 hybrid plasmids pUH25 (lane 1), pUH28 (lane 2), and pAI6, which also carries pBR322 (Fig. 1) (lane 3). (B) Proteins synthesized in minicells carrying pBR322 hybrid plasmids pAI13 (lane 1), pAI16 (lane 2), pAI5 (lane 3), and the vector with no insert (lane 4).

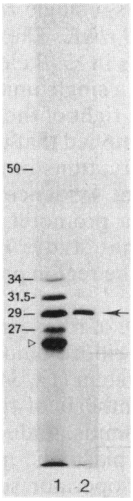

FIG. 4. Immunoprecipitation of ribose-binding protein and precursor ribose-binding protein from minicells containing an *rbs* plasmid. Procedures and symbols are as described in the legend to Fig. 3, except that the gel contained 10% acrylamide. The arrow indicates the position of authentic ribose-binding protein. The plasmid pAI11 consists of the 3.7-kb *Hin*dIII insert of pAI5 cloned into pACYC184. This plasmid was used for experiments in which synthesis of RBP was monitored since the β-lactamase produced by the pBR322 vector of pAI5 co-migrated electrophoretically with RBP (Fig. 3 and 5). ▷, Chloramphenicol acetyltransferase produced by pACYC184. The sample in lane 1 was minicells containing pAI11, and the sample in lane 2 contained proteins precipitated from solubilized minicells by anti-ribose-binding protein serum.

that observed for *rbs-102*::Tn*10* pseudorevertants, implying that the ribose-negative phenotype of *rbs-108*::Tn*10* mutants was also due to a polar effect of the insertion on *rbsK*. However, ribose-positive derivatives of strains carrying *rbs-105*::Tn*10* or *rbs-106*::Tn*10* insertions apparently located in the *rbsA* region (Table 4) were not found. Thus, the frequency of alterations that relieve the polarity of Tn*10* insertions may vary with the specific insertion.

Complementation of *rbs* point mutations. The complementation pattern of eight independent *rbs* mutations induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine supports the notion of a single ribokinase gene, preceded by the ribose-binding protein gene. Mutations induced on pAI5 were identified by the inability of the mutant plasmids to complement *rbs-102*::Tn*10*, carried on the chromosome of a host strain, in the ribose swarm plate test. Five of these mutations were identified as ribokinase defects since they conferred a ribose-negative phenotype, did not complement *rbs-101*::Tn*5*, and did complement *rbs-103*. All five mutations plus *rbs-101*::Tn*5* fall in the same complementation group, implying that there is only a single ribokinase gene. The three other mutations appeared to be in the ribose-binding protein gene since they all allowed an *rbs-102*::Tn*10* tester strain to metabolize ribose, but not to take up 1 μM ribose, and complemented mutations in the ribokinase group, but not the ribose-binding protein mutation, *rbs-103*.

DISCUSSION

Early studies of ribose utilization by *E. coli* K-12 (3) suggested that two closely linked genes were involved; *rbsK* was required for ribokinase activity, and *rbsP* defined a ribose-specific transport system. However, reexamination of the two mutants used in those studies revealed that neither is a simple mutant defective specifically in ribokinase activity or transport. AT715, the original mutant used to define *rbsP* (3), is ribose negative as well as transport defective. Ribokinase activity in the cells grown with or without ribose is only 10% of the level in the fully induced wild-type parent as documented in the original study (3) and confirmed in this laboratory. We suggest that the ribose-negative phenotype of the *rbsP1* mutant is the result of the ribokinase defect and not the transport defect. This notion is supported by the observation that the ribose-negative phenotype of *rbsP1* was complemented by pAI25, but not by pAI24. The complementing plasmid contains the entire *rbsK* sequence and two transport genes, whereas the noncomplementing plasmid contains the same transport genes, but only a segment of *rbsK*. Groarke et al. (19) also used *rbs* plasmids to complement the ribose-negative phenotype of *rbsP1*, and the pattern observed can now be seen to indicate a requirement for *rbsK*, but not for any transport gene. As shown in the initial study (3) and confirmed in this laboratory, the mutant strain AA100, which is lacking ribokinase activity and served to define the *rbsK* locus, is also significantly defective in ribose transport. This is not the case for strains carrying *rbs-101*::Tn*5* (Table 3), so we suspect that AA100 contains either a polar mutation or double mutations that affect both *rbsK* and a gene for high-affinity ribose transport.

The work described here provides a substantial amount of new information about the *rbs* region which is summarized in Fig. 6. The *rbsK* product is a 34-kdal polypeptide that is likely to be the ribokinase itself. We found no evidence for an alternative possibility that it is a control protein necessary for synthesis of the enzyme. Immediately adjacent to *rbsK* is the gene coding for the ribose-binding protein. We suggest that this gene be named *rbsB* for ribose-binding protein. The 50-kdal protein is coded for by a gene that extends to the right of the *Hin*dIII site at 3.7 kb (Fig. 6) and may overlap that restriction site. The gene is required for high-affinity transport of ribose, and thus we expect that the 50-kdal protein is a component of that transport system. We suggest that the gene be named *rbsA*. The 27-kdal protein is synthesized from a gene located between *rbsB* and the *Hin*dIII site at 3.7 kb (Fig. 6). There are no data which provide information about the function of the 27-kdal protein, but the position of the gene in the midst of *rbs* genes implies that the product has a role related to ribose, most likely as a component of the high-affinity transport system. Thus we suggest the provisional name *rbsC*.

TABLE 5. Reversion analysis of transposon insertion mutants

Mutation	Reversion frequency[a]	Ribokinase activity[b]		Ribose tactic ring		Drug sensitivity[c]
		+	−	+	−	
rbs-101::Tn*5*	8×10^{-9}	3/3	0/3	6/6	0/6	6 Km^s/6
rbs-102::Tn*10*	1×10^{-7}	4/4[d]	0/4	0/20	20/20	17 Tc^s/20 3 Tc^r/20[e]

[a] Selection for growth on a minimal salts plate containing 13 mM ribose.

[b] +, Activity similar to that in a wild-type strain (Table 3).

[c] Km^s, Sensitive to kanamycin; Tc^s, sensitive to tetracycline; Tc^r, resistant to tetracycline.

[d] The four revertants tested include the three tetracycline-resistant strains. These same revertants were tested for presence of ribose-binding protein by immune precipitation as in Table 3. No binding protein was detected.

[e] Tetracycline resistance was 99% (100 colonies examined) transductionally linked to *ilv* for all three tetracycline-resistant revertants.

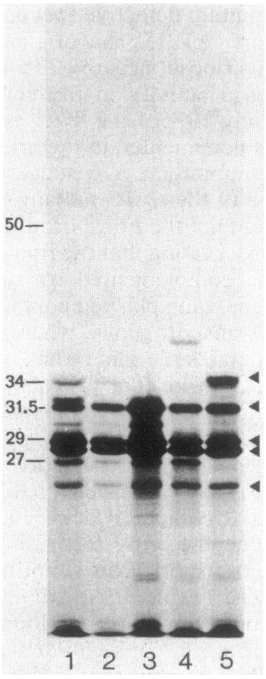

FIG. 5. Proteins synthesized in minicells containing *rbs* plasmids carrying deletions in the *Hin*dIII fragment. Procedures and symbols are as described in the legend to Fig. 3. Lanes: 1, proteins synthesized in minicells carrying the pBR322 hybrid plasmid, pAI5, which contains the intact *Hin*dIII fragment (Fig. 2); 2, pAI25, which has a *Pvu*II deletion; 3, pAI24, which has a larger *Pvu*II deletion; 4, pAI22, which has a *Hin*cII deletion; and 5, the vector alone. The filled arrowheads on the right mark the position of pBR322 proteins. The uppermost band of the pBR322 pattern is the product of the *tet* gene, which is not synthesized in the hybrid plasmids, all of which contain the insert in that gene. The *tet* product migrates just above the 34-kdal ribokinase. The 29-kdal ribose-binding protein and its 31.5-kdal precursor form are obscured by the β-lactamase protein of pBR322 and its precursor form, respectively, but immune precipitation experiments (Fig. 2) demonstrate that ribose-binding protein and its precursor are present in lanes 1 through 3 but not in lanes 4 and 5.

It appears that there is a single *rbs* operon transcribed in the order *rbsA rbsC rbsB rbsK*. The characterization of polar effects of Tn*10* insertions in *rbsB* clearly indicated transcription of *rbsB* and *rbsK* as a single unit in that order. The effect of Tn*10* insertions to the right of the *Hin*dIII site at 3.7 kb on the expression of *rbsK* implied that there was a single operon for all four genes. Observations in the accompanying paper (35) provide independent evidence for an *rbs* operon that includes *rbsK* and has a promoter in the region where we have placed *rbsA*. In that study, *rbsA* and *rbsC* were not distinguished, and thus the region promotor proximal to *rbsB* was referred to as *rbsT*.

Growth in the presence of ribose induces the production of ribokinase (3) (Table 3), ribose-binding protein (34), and the high-affinity transport system (17, 34). Thus ribose would be expected to increase synthesis of the Rbs proteins in minicells containing *rbs* plasmids. Induction by ribose was not observed for any *rbs* plasmids, perhaps because of an unnatural ratio between operator sequences and regulatory protein that occurred in the minicells. Evidence for negative control of the *rbs* operon and for apparent titration of repressor by excess operator is provided in the accompanying paper (35). The level of synthesis of the products of *rbsC*, *rbsB*, and *rbsK* was comparable in minicells containing plasmids carrying the entire *rbs* region or just the *Hin*dIII fragment. If there is only a single *rbs* promotor in front of *rbsA*, then transcription of *rbs* genes on the smaller plasmids must initiate from a promotor on the vector.

For Fig. 6, the precise position and size of *rbsB* is that defined by the nucleotide sequence of the gene (19). The size of the other *rbs* genes was estimated from the apparent molecular weights of their respective products, and the genes were placed on the restriction map in accord with the observations reported in this study. The position of *rbsK* could be determined rather precisely since there are *rbsK*::Tn*1000* insertions near each end of the gene. The *rbsC* gene occupies most of the DNA between *rbsB* and the *Hin*dIII site at 3.7 kb, indicating that it is contiguous to *rbsB*. Our data do not define a precise location for *rbsA*. In Fig. 6 it was positioned to include the IS*50* insertion site and, arbitrarily, to be adjacent to *rbsC*. Thus, our data leave open the

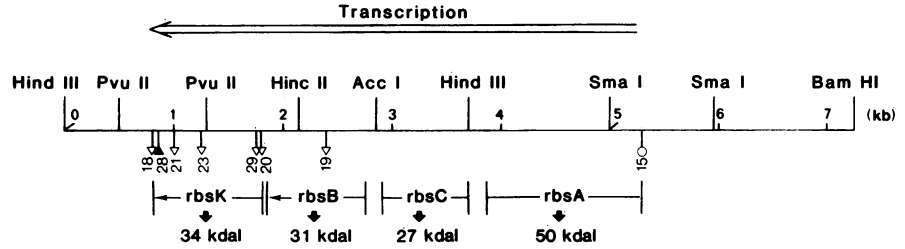

FIG. 6. Map of the *rbs* region. See the text for detailed discussion. The length of DNA shown is the chromosomal fragment cloned in pAI13. The segment to the right of the *Hin*dIII site at 3.7 kb was not examined for *Acc*I, *Pvu*II, or *Hin*cII sites. The position of the *rbs-101*::Tn*5* insertion in pAI28 (Fig. 1) is labeled 28, the position of the IS*50* insertion in pAI15 (Fig. 1) is labeled 15, and the position of the Tn*1000* insertions in pAI18 and pAI19 (Fig. 2) are labeled 18 and 19, respectively. The positions of additional Tn*1000* insertions obtained in hybrid pACYC184 plasmids carrying the *Hin*dIII fragment (pAI11, Fig. 4) are numbered. All of these insertions destroy the ability of the plasmid to complement a defect in ribose metabolism as tested in Fig. 2. Plasmids carrying insertion 21 or 23 were found to code for ribose-binding protein, but not for the 34-kdal ribokinase in minicells. Other insertions were not tested. rbsB was positioned precisely by using the nucleotide sequence (19). The sizes of the other genes were calculated by using the apparent molecular weight of the respective products as determined by SDS-polyacrylamide gel electrophoresis and the average value of units of molecular weight per amino acid residue observed for the *rbsB* product (19). *rbsK* was drawn to include the sites of all Tn*1000* insertions that conferred a specific kinase-negative phenotype. This required a gene of 1 kb, which is slightly larger than the length calculated by using the apparent molecular weight of ribokinase. The apparent molecular weight of the *rbsC* product varied with the percent acrylamide in the gel. The 27-kdal value is a representative value, which could differ from the actual value by as much as 3-kdal. *rbsC* was placed between *rbsB* and the *Hin*dIII site with one end at that restriction site. The *rbsA* product migrates in gel electrophoresis at almost the same rate as the *mglA* product (21). Arbitrarily, *rbsA* was placed with the right end just including the site of the RbsA IS*50* insertion. Our data indicate only that *rbsA* includes the insertion site and is situated between *rbsC* and the *Mlu*I site at 6.5 kb.

possibility that an undetected *rbs* gene is present on the *Hin*dIII-*Bam*HI fragment. This gene could be located either between *rbsA* and the *Bam*HI site or between *rbsC* and *rbsA* if the latter gene were closer to the *Bam*HI site than shown in Fig. 6. There is a precedent for an undetected gene in an operon of a binding protein-mediated transport system. The *hisM* gene in *S. typhimurium* was identified only when the nucleotide sequence of the relevant operon was determined (28). It is unlikely that there is an additional ribose transport gene on the promotor-distal side of *rbsK* since deletion of DNA to the left of the *Pvu*II site at 0.5 kb (Fig. 6) did not affect *rbs* complementation activity and the *rbs-101*::Tn*5* insertion affected only ribokinase activity.

In this study we found that genes for a high-affinity transport system for ribose are located in an operon at 84 min on the linkage map of *E. coli*, where the original *rbsP* mutation had been mapped. Strains that we know are defective specifically in the high-affinity transport system are phenotypically ribose positive where the sugar is provided at high (13 mM) concentrations, demonstrating that *E. coli* K-12 possesses a second, low-affinity transport system (or systems) for ribose, distinct from the high-affinity system that involves the periplasmic ribose-binding protein. Thus the only mutations that confer a ribose-negative phenotype are those that are in *rbsK* or affect *rbsK* expression. An early study in *E. coli* K-12 strain χ289 (12) provided evidence for two distinct ribose transport systems. In contrast, mutants of *E. coli* K-12 (17) and *Salmonella typhimurium* (2) lacking ribose-binding protein were isolated by searching for ribose-negative colonies. However, frameshift mutagens were used in both these studies, and thus it is likely that the ribose-negative phenotype of the mutants isolated resulted from polar effects of frameshift mutations on *rbsK*. If that were the case, selection for ribose-positive revertants would yield, as for *rbs-102*:::Tn*10*, many strains with the polarity relieved, but the initial mutation still intact. This is precisely what was observed in the *Salmonella* study (2), where 173 of 176 spontaneous ribose-positive revertants of a particular mutant remained binding protein negative. In the study of *E. coli* frameshift mutants (17), ribose-positive revertants regained the binding protein with a much higher frequency, perhaps because the frameshift mutagen was used to generate revertants.

Both this study and an earlier report (18) provided evidence for a larger precursor form of ribose-binding protein. Recently, the existence of an N-terminal extension on the protein has been confirmed by determination of the nucleotide sequence of *rbsB* (19; C. Furlong and A. Iida, unpublished data). The absence of high-affinity transport of ribose in strains carrying the nonpolar Tn*1000* insertion in *rbsB* provides definitive evidence for the participation of ribose-binding protein in that system, confirming earlier conclusions (17) based on phenotypes of polar mutations. Three membrane-associated components, in addition to the binding protein(s), have been found for the histidine (28), maltose (40, 44), and leucine (D. L. Oxender, personal communication) transport systems. Analogous organization of the Rbs system would imply that there is, in fact, an undetected *rbs* transport gene. Alternatively, the 50-kdal RbsA protein, which is significantly larger than components of the other systems, might perform functions that otherwise would be carried out by two separate polypeptide chains. Examination of the sequence of the *his* transport operon in *S. typhimurium* (28) reveals that alternatives of one large or two small transport proteins could be created by a simple mutational event. A single base insertion in the termination codon of *hisQ* could result in fusion of the *hisQ* and *hisM* coding sequences, creating a 51-kdal product comprising the HisQ and HisM proteins connected by a bridge of two amino acids. In this context it is interesting that mutations in *hisQ* and *hisM* constitute a common complementation class (28).

The Rbs transport system is unusual among binding protein-dependent systems in that a gene involved in metabolism is included in the operon. In other features, the Rbs system resembles the galactose (Mgl) system (21, 42) more than other binding protein-dependent systems. Both the Rbs and Mgl systems include a 50-kdal component (the MglA and RbsA proteins are found at almost the same position on SDS-polyacrylamide gels) and may have only two membrane-associated transport proteins. The amino acid sequences of the ribose- and galactose-binding proteins exhibit greater homology than that observed for any other pair, with the exception of binding proteins that share common membrane-associated components (19). Finally, both the ribose- and galactose-binding proteins function as chemoreceptors by interacting with a common transmembrane transducer, the Trg protein (27). Further characterization of the Rbs and Mgl systems may reveal features critical for their common functions in transport and chemotaxis.

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