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Proteins associated with RNase E in a multicomponent ribonucleolytic complex

(DnaK/enolase/RNA helicase)

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ABSTRACT **The *Escherichia coli* endoribonuclease RNase E is essential for RNA processing and degradation. Earlier work provided evidence that RNase E exists intracellularly as part of a multicomponent complex and that one of the components of this complex is a 3′-to-5′ exoribonuclease, polynucleotide phosphorylase (EC 2.7.7.8). To isolate and identify other components of the RNase E complex, FLAG-epitope-tagged RNase E (FLAG–Rne) fusion protein was purified on a monoclonal antibody-conjugated agarose column. The FLAG–Rne fusion protein, eluted by competition with the synthetic FLAG peptide, was found to be associated with other proteins. N-terminal sequencing of these proteins revealed the presence in the RNase E complex not only of polynucleotide phosphorylase but also of DnaK, RNA helicase, and enolase (EC 4.2.1.11). Another protein associated only with epitope-tagged temperature-sensitive (Rne-3071) mutant RNase E but not with the wild-type enzyme is GroEL. The FLAG–Rne complex has RNase E activity *in vivo* and *in vitro*. The relative amount of proteins associated with wild-type and Rne-3071 expressed at an elevated temperature differed.**

RNase E is an *Escherichia coli* endoribonuclease whose function is essential for RNA processing (1) and degradation (2, 3). It is a large enzyme consisting of 1061 amino acids (GenBank accession no. L23942). Its calculated molecular mass is 118 kDa, but it migrates as a 180-kDa protein in SDS/polyacrylamide gels (4). Recently, it has been shown that the N-terminal half [498 amino acids (5)] of the protein is responsible for the endonucleolytic activity.

A series of observations suggest that RNase E is complexed with one or more other proteins in *E. coli*. Roy and Apirion (6) found that during some steps of RNase E purification, the specific activity decreased and that this decrease could be reverted by adding less purified material to the purified fraction. Additionally, RNase E was found to cosediment with a high molecular weight cell membrane fraction (7). Recently, it has been shown that RNase E forms a large complex *in vivo* with polynucleotide phosphorylase, a 3′ exoribonuclease, and other proteins (8, 9) and that polynucleotide phosphorylase interacts functionally with RNase E to affect its activity (10). Additionally, GroEL, a chaperonin and heat shock protein, has been reported to copurify with an RNase E-like activity (11).

Here we describe the identification of, in addition to polynucleotide phosphorylase, several other proteins from an RNase E-containing multicomponent complex purified from *E. coli* by antibody-agarose column chromatography of the epitope-tagged Rne protein. Moreover, we show that the stoichiometry of proteins in the complex is altered by a temperature-sensitive mutation in RNase E.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The following *E. coli* strains were used: HB101; N3431 (*lacZ43, relA, spoT1, thi1, rne-3071*) carrying a temperature-sensitive mutation in *rne* gene and the isogenic *rne*$^+$ strain N3433 (12); N3438 (*rne-3071, recA, his, trp, rpsL, lac*). Plasmid pGP1–2 contains the T7 RNA polymerase gene under the control of a temperature-sensitive bacteriophage λ repressor (13). pGM102 (obtained from George Mackie, University of British Columbia, Vancouver), contains the full-length *rne* gene (14). pF:TBP-11d, which was obtained from Cheng-Ming Chiang (The Rockefeller University, New York), contains a DNA segment encoding the FLAG epitope and a gene for a human transcription factor (15) 3′ to the T7 promoter. Construction of pRE196: a 562-bp DNA was prepared by PCR amplification of N3433 chromosomal DNA by using the oligonucleotide primers 5′-GCGCATATGAAAAGAATGTTA and 5′-CGGAAGCTTAAATCCCATTGC. This DNA was cut with *Nde* I and *Hin*dIII (this fragment contains the beginning of the *rne* gene from the translation start codon to the unique *Hin*dIII site) and then inserted into the *Nde* I- and *Hin*dIII-treated pF:TBP-11d plasmid by replacing the human gene. The resulting plasmid was cleaved with *Hin*dIII and ligated to a 3-kb *Hin*dIII fragment derived from pGM102 to create pRE196. pRE205 contains the *rne* gene carrying the *rne-3071* mutation and was constructed as described for pRE196, except that the template DNA used for PCR was prepared from N3431. The *rne-3071* mutation causes a Leu → Phe change at position 68 (16), which was confirmed in pRE205 by DNA sequence analysis. Because both pRE196 and pRE205 expressed a protein of the correct size that had RNase E activity, their entire nucleotide sequences were not determined.

Expression and Purification of Rne Fusion Protein. Rne was expressed in HB101 containing pGP1–2 and pRE196 (or pRE205) by the method of Tabor and Richardson (13). Briefly, cells containing the two plasmids were grown exponentially at 30°C in 500 ml of LB medium (17) in the presence of ampicillin (50 μg/ml) and kanamycin (50 μg/ml). The average doubling time of cells was 2 h at 30°C; this long doubling time may be due to partial expression of plasmid-encoded RNase E (see *Results*) in the absence of induction of T7 RNA polymerase. At an OD_{600} of 0.6–1.0 unit, rifampicin was added to 100 μg/ml (we obtained similar results without the addition of rifampicin), and overexpression of Rne was induced by a temperature shift to 42°C for 25 min, and we did not use a longer period of induction since it resulted in a reduction of the intracellular concentration of the enzyme, possibly because of its ability to autoregulate its own synthesis (18, 19). In addition, we did not observe overexpression of either enzyme using the two plasmid system when strains were

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stored at room temperature or at 4°C for several days prior to use, due in some instances to loss of the *rne* plasmid, as has been observed (20); therefore, strains carrying *rne* plasmids were stored at −70°C until used. After induction the temperature was shifted to 37°C for an additional 30 min and cells were harvested by centrifugation at 4°C.

Cell lysates were prepared as described by Carpousis *et al.* (9) with some modifications. Cells were resuspended in 10 ml of lysozyme/EDTA buffer [50 mM Tris·HCl, pH 7.5/100 mM NaCl/5% (vol/vol) glycerol/3 mM EDTA/1 mM dithiothreitol/lysozyme (1.5 mg/ml) (Sigma)/1 mM phenylmethylsulfonyl fluoride] and incubated on ice for 30 min. Cells were sonicated (four 20-sec sonic bursts with intervening periods of 2 min) in a Vibra Cell sonicator (Sonics & Materials, Danbury, CT) with the micro tip. DNase/Triton buffer [5 ml; 50 mM Tris·HCl, pH 7.5/100 mM NaCl/5% glycerol/1 mM dithiothreitol/30 mM magnesium acetate/3% (vol/vol) Triton X-100/1 mM phenylmethylsulfonyl fluoride/DNase I (20 μg/ml)] was added, the suspension was incubated for 30 min, and 3.75 ml of 5 M NH_4Cl was added slowly with stirring. The lysate was incubated for another 30 min and cell debris was removed by centrifugation at 10,000 × *g* for 15 min. The supernatant was precipitated with ammonium sulfate (40% saturation). One-half of the pellet was resuspended in 10 ml of TBS buffer (50 mM Tris·HCl/150 mM NaCl, pH 7.4); the other part was dissolved in 2 ml of 50 mM Tris·HCl, pH 8.8/50 mM NaCl/5 mM $CaCl_2$ containing 1000 units of micrococcal nuclease (Boehringer Mannheim), incubated for 11 h at 4°C, and diluted by addition of 8 ml of 140 mM NaCl/125 mM Tris·HCl (pH 6.8 to a final pH 7.4). Corresponding fusion proteins from both preparations were independently purified on an anti-FLAG M2 affinity gel (Eastman Kodak) column according to the manufacturer's recommendation by using 1 ml of a FLAG oligopeptide (Eastman Kodak) solution (1 mg/ml) for elution. By using the same procedures, we prepared control samples from cell extracts lacking the FLAG fusion protein.

Detection of RNA Copurified with Fusion Rne Protein. The eluted FLAG–Rne complex (100 μl) was extracted sequentially by phenol, phenol/chloroform, and chloroform, mixed with 50 μl of 3 M sodium acetate, and ethanol-precipitated. The pellet was suspended in 20 μl of TE buffer and 4 μl of 0.25% bromophenol blue/0.25% xylene cyanol/30% glycerol was added. The prepared solution (8 μl) was analyzed by electrophoresis in 0.8% agarose gel.

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out as described by Laemmli (21). For protein separation, 8% resolving gels containing 0.1% SDS were used. Gels were stained by Coomassie brilliant blue R250 or by silver with the Silver Stain Plus kit (Bio-Rad) to visualize protein bands.

Molar ratios were calculated from silver-stained gels by using a Molecular Dynamics computing densitometer.

Western Blot Analysis. The ECL Western blot detection kit (Amersham) was used. Anti-FLAG (Eastman Kodak), anti-GroEL (StressGen Biotechnologies, Vancouver), and anti-DnaK (StressGen, Biotechnologies) monoclonal antibodies were used according to the vendor's instructions.

Protein Sequencing. Proteins were separated by SDS/PAGE, transferred to poly(vinylidene difluoride) membranes (Immobilon, Millipore), and stained with Coomassie brilliant blue R250; we did not use acetic acid for the staining and destaining solutions. Bands were excised and then peptides were sequenced by using an Applied Biosystems model 475A protein sequencer (IMB Core Facility).

***In Vitro* RNase E Assay.** Preparation of cell extracts and assay conditions were as described (22, 23). RNA I prepared by *in vitro* transcription (24) was used as substrate. The cleavage products were separated on 5–12% polyacrylamide gels containing 7 M urea.

RESULTS AND DISCUSSION

Multiple Proteins Copurify with Rne. FLAG epitope tagging is a widely used technique for affinity purification of proteins expressed in bacteria. The antibody-resin-bound factors (FLAG-epitope-tagged polypeptide-associated complex) can be eluted by competition with the synthetic FLAG peptide under mild conditions in regular protein buffers (15).

To identify proteins complexed with RNase E, the plasmids pRE196 and pRE205, which express the FLAG–Rne or FLAG–Rne (*rne-3071*) fusion proteins, respectively, were constructed. Fig. 1*A* presents the nucleotide and amino acid sequences in part of the FLAG–Rne fusion protein. Fig. 1*B* shows a Coomassie blue-stained Western blot of proteins eluted from the affinity column by competition with the FLAG peptide (250 μg/ml) and separated by SDS/PAGE. It can be seen that multiple other proteins are associated with both the wild-type Rne and Rne encoded by the gene carrying the *rne-3071* mutation. These FLAG–Rne-associated proteins were not removed by buffer containing high salt (750 mM NaCl), detergents (1% Triton X-100/1% Tween-20), or 0.5 M urea. In addition, in a control experiment, we detected some proteins bound to the column from a host cell extract that lacked the FLAG fusion protein, but the mobility of these proteins was different from the mobility of proteins associated with the FLAG–Rne fusion protein (Fig. 1*D*). In addition to the 180-kDa fusion protein, Western blot analysis (Fig. 1*C*) showed other faint bands recognized by the anti-FLAG monoclonal antibody. These proteins may be FLAG-tagged proteolytic products, as RNase E is highly sensitive to proteolysis (9).

Identification of Proteins That Copurify with Rne. The proteins that copurified with the fusion protein were identified by N-terminal sequencing (Table 1) or/and Western blot analysis. Polynucleotide phosphorylase, which is autocontrolled posttranscriptionally (25) and was identified as a component of the RNase E complex (8, 9), forms a major band in the preparation isolated from cells expressing Rne from pRE196 (Fig. 1*B*, band 5). In addition to polynucleotide phosphorylase, DnaK (26), *rhlB* RNA helicase (27), and enolase (M. Klein and R. Freudl, data deposited in GenBank, accession no. X82400) were found in this complex (Fig. 1*B*, bands 6, 8, and 9). The N-terminal ends of proteins isolated from bands 2, 3, and 4 could not be sequenced, possibly because of N-terminal blockage. Band 7, which migrates as a 60-kDa protein shown by Western blot analysis to be associated with the temperature-sensitive mutant RNase E, Rne-3071, but not with the wild-type Rne protein under the conditions we used in these experiments, is GroEL (Fig. 1*C*), a chaperonin-mediating protein folding and assembly (28, 29). We do not know whether the selective binding of GroEL to the mutant enzyme reflects greater affinity of the chaperonin for the mutant RNase E or a failure of GroEL to be released from an abnormally folded gene product.

The average molar ratio of proteins in the preparation made by expressing pRE196 was estimated from silver-stained gels as follows: Rne, 1; polynucleotide phosphorylase, 0.9; RNA helicase, 0.93; enolase, 1.8. In the temperature-sensitive enzyme preparation, these values were as follows, respectively: 1, 0.4, 0.6, and 0.83. The standard deviation observed for these values in two experiments was about 20%. However, staining by Coomassie brilliant blue R250, showed a much higher relative amount of polynucleotide phosphorylase (Fig. 1*B*). While the relative amount of these proteins, as determined by different staining reagents (Coomassie blue and silver) varied slightly, the amount of polynucleotide phosphorylase, RNA helicase, and enolase associated with the wild-type enzyme was reproducibly much higher than the amount of these proteins associated with the temperature-sensitive Rne protein (Fig. 1*B*). This difference may reflect a lower rate of synthesis of these proteins in the presence of the temperature-sensitive

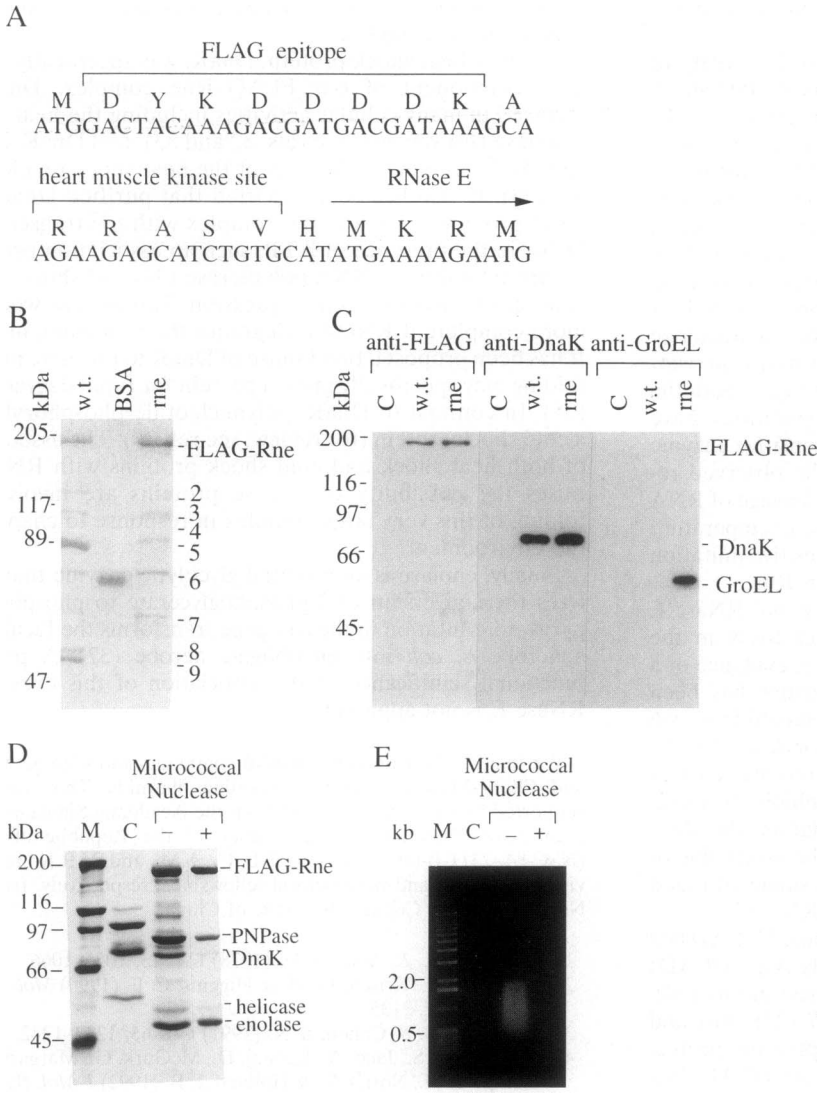

FIG. 1. Isolation of proteins from the RNase E complex. (*A*) Part of the plasmid construct used for expression of the fusion protein is shown. Wild-type and mutant Rne as well as a control sample were prepared and separated. Proteins were separated by electrophoresis in SDS/polyacrylamide gels and transferred to poly(vinylidene difluoride) membranes. (*B*) One membrane was stained with Coomassie brilliant blue. This blot was used for sequencing (band numbers are indicated and are the same as in Table 1). (*C*) The other membranes were visualized by immunodetection using anti-FLAG, anti-DnaK, and anti-GroEL monoclonal antibodies and an Amersham ECL detection kit. Proteins bound to the anti-FLAG gel column from cell extract lacking overexpressed Rne (lane C) or from cell extracts containing overexpressed wild-type Rne before (−) and after (+) micrococcal nuclease treatment were either compared by electrophoresis in SDS/polyacrylamide gels and staining with Coomassie brilliant blue (*D*) or extracted by phenol, precipitated by ethanol, suspended in TE buffer, and analyzed by gel electrophoresis in 0.8% agarose gel (*E*). Designations are as in *D*. w.t., FLAG-epitope-tagged wild-type Rne (pRE196); rne, FLAG-epitope-tagged mutant Rne (*rne-3071*) (pRE205); BSA, bovine serum albumin; C, control. Molecular masses of protein (kDa) and DNA (kb) standards (lanes M) are shown.

enzyme, or alternatively, the mutant gene product may bind less polynucleotide phosphorylase, RNA helicase, and enolase. Carpousis *et al.* (9) have shown that heat inactivation of the mutant RNase E dramatically affects the stability of the Rne complex; the different distribution of the associated proteins in the temperature-sensitive complex may explain its instability. While the results obtained indicate the relative amounts of components associated with Rne when this enzyme is overexpressed, these values may not reflect the normal stoichiometry, as the cellular concentration of individual associated proteins may be limiting during overexpression of Rne.

In addition to containing Rne and other copurified proteins, the fraction eluted from anti-FLAG gel column with the FLAG oligopeptide contained cellular RNA (Fig. 1*E*) that, however, was not found in a corresponding fraction from a control experiment lacking the FLAG–Rne peptide (Fig. 1*E*, lane C). To determine whether the formation of the complex is dependent on this RNA, we prepared affinity-purified proteins from cell extract treated with micrococcal nuclease to digest the RNA component. As seen in Fig. 1*D*, complete digestion of copurified cellular RNA did not eliminate binding of PNPase, DnaK, *rhlB* helicase, or enolase to RNase E,

Table 1. Proteins identified by N-terminal sequencing

Band	N-terminal sequence	Identity (GenBank accession no.)	Ref.
5	MLNPIVRKFQ	100% with *E. coli* polynucleotide phosphorylase* (J02638)	38
6	MKIIGIDLGT	100% in a 9-amino acid overlap with *E. coli* DnaK† (P04475)	26
8	SRTHLTEQKF	100% with *E. coli rh1B* RNA helicase-like protein‡ (X56310)	27
9	SKIVKIIG-E	100% in an 8-amino acid overlap with *E. coli* enolase (X82400)	Klein and Freudl (deposited in GenBank)

Data bank search was done by using the program manual for the Wisconsin Package, version 8, Genetic Computer Group (Madison, WI).
*Similar identity was found with *Photorhabdus luminescens* polynucleotide phosphorylase.
†Similar identity was found with six other DnaK proteins from different species.
‡Ninety percent identity was found in a 10-amino acid overlap with *Salmonella typhimurium* RNA helicase.

although it had a slight affect on the stoichiometry of the associated proteins.

The FLAG–Rne Fusion Protein Has RNase E Activity *in Vivo* and *in Vitro*. The temperature-sensitive *rne* mutant strain N3438 (*rne-3071, recA*) carrying pRE196 can grow at 43°C; however, the same host strain remains temperature-sensitive when carrying pRE205. The observed complementation of the ts host mutation by a plasmid containing the wild-type *rne* gene suggested that the FLAG–Rne fusion protein has RNase E activity and, furthermore, that RNase E is expressed in pRE196 from a cryptic promoter; N3438 does not contain pGP1–2, which encodes the T7 RNA polymerase required for transcription from the T7 promoter. To further characterize the FLAG–Rne and FLAG–Rne mutant proteins, *in vitro* cleavage of RNA I was performed. As seen in Fig. 2, both the wild-type and the mutant FLAG–Rne preparations have RNase E activity, which for the temperature-sensitive enzyme was inactivated by preincubation at 43°C. The observed reduced activity of and the absence of a second cleavage of RNA I by the mutant Rne preparation at the permissive temperature may be due to an effect of the temperature-sensitive mutation on the distribution of proteins associated with Rne.

Carpousis *et al.* (9) suggested the possibility that RNase E and polynucleotide phosphorylase act cooperatively in the processing and degradation of RNA, and direct evidence of a functional interaction between the two enzymes has been shown by the decrease in RNase E activity observed in *E. coli* mutants defective in polynucleotide phosphorylase (10). In addition, it is known that RNase E cleaves short stretches of single-stranded RNA and that stem–loops inhibit its action (24). We speculate that during RNA degradation, the Rne-associated RNA helicase may participate in the unwinding of some double-stranded RNA regions into single-stranded RNA, allowing cleavage of these regions by RNase E.

The RNA helicase-like proteins have been called DEAD box proteins because of a conserved Asp-Glu-Ala-Asp (DEAD) sequence motif, among several other conserved motifs (30). There are at least five such genes in *E. coli* (27). Iost and Dreyfus (31) have shown that DEAD box proteins protect mRNA from endonucleases, although not all DEAD box proteins tested had this effect. They suggested that overexpressed DEAD box proteins hinder RNase E action by binding directly to its cleavage sites. An alternative explanation may be that too much or too little (as in case of the temperature-sensitive RNase E) RNA helicase can alter the RNase E activity of the complex.

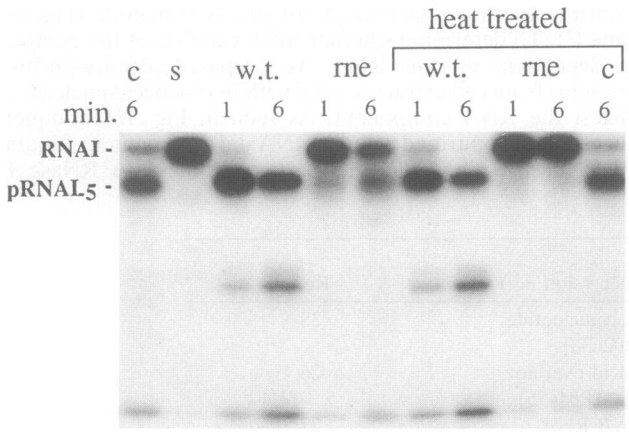

FIG. 2. *In vitro* cleavage of RNA I by the FLAG–Rne (w.t.) and FLAG–Rne (*rne-3071*) (rne) preparations. c, Crude cell extract from N3433 made as described (22); s, substrate (RNA I) alone. To determine heat sensitivity, Rne preparations were preincubated at 43°C for 20 min (heat treatment). Reaction mixtures were incubated at 30°C for 1 or 6 min as indicated. The preparation of RNA I was described (24). pRNAI-5 is 5 nt shorter at the 5′ end of RNA I and is the cleavage product generated by RNase E (23).

Another heat shock protein, DnaK, was specifically found as a component of our FLAG–Rne complex. DnaK is involved in many cellular activities including the heat shock response (for reviews, see refs. 32 and 33), and DnaK, DnaJ, and GrpE are key modulators of the heat shock regulon via σ^{32} (33). It also has been reported that purified DnaK and DnaJ proteins form a stable complex with σ^{32} transcription factor in the presence of ATP; such an association prevents σ^{32} from binding to RNA polymerase (34) and shuts off the heat-shock-mediated gene expression. This process would be more complete if RNase E degrades the remaining mRNA. It has been proposed that failure of DnaK to promote protein folding may specifically mark a protein for rapid degradation (35). In contrast to DnaK, polynucleotide phosphorylase is a cold shock protein (for review, see ref. 36). The association of both heat shock and cold shock proteins with RNase E raises the possibility that these proteins are involved in folding of this very large complex in response to changes in the environment.

Finally, enolase is an essential glycolytic enzyme that catalyzes the conversion of 2-phosphoglycerate to phospho*enol*-pyruvate. Mutation in the *eno* gene transforms the facultative anaerobe *E. coli* into an obligate aerobe (37). A possible functional significance of the association of this enzyme to RNase E is not apparent.

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