Overexpression and Rapid Purification of the orfE/rph Gene Product, RNase PH of Escherichia coli*

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The pyrE gene, encoding the pyrimidine biosynthetic enzyme orotate phosphoribosyltransferase, is the promoter-distal gene of the dicistronic orfE-pyrE operon. The promoter-proximal orfE gene, whose transcription and translation is important for regulation of the pyrE gene is the promoter-proximal gene orfE, whose transcription and translation is important for regulation of the pyrE gene product substantially, as well as the purification of the OrfE protein by ammonium sulfate precipitation and chromatography on phosphocellulose. The highly purified protein catalyzes the phosphorolytic cleavage of poly(A) at a rate of 1.6 pmol/min/mg and the formation of CDP from tRNA-CCCA-C, and orthophosphate at a rate equal to 0.14 pmol/min/mg, as characteristic for RNase PH. OrfE/RNase PH contains helix-turn-helix motifs resembling those in DNA-binding proteins, and it binds nonspecifically to DNA. On SDS gels, OrfE/RNase PH migrates as two distinct protein bands. This heterogeneity might be caused by post-translational modification other than proteolysis, or may be an artefactual artifact. The native protein is composed of two or more subunits.

the orfE gene is the promoter-proximal gene of the dicistronic pyrE operon located at 81.7 min on the Escherichia coli chromosome (Poulsen et al., 1983, 1984). Translation of the orfE cistron is involved in controlling transcriptional read-through past the intercistronic pyrE attenuator, but attenuator regulation could also be accomplished by a peptide formed from the fusion between the first few codons of lacZ and the last 14 codons of orfE, even after shifting the translational reading frame (Bonekamp et al., 1984, 1985, 1989; Poulsen and Jensen, 1987). This observation suggested that OrfE might have functions other than merely controlling the coupling between transcription and translation in the pyrE attenuator region. In an attempt to identify these functions we disrupted the orfE coding frame on the chromosome, but the mutants exhibited no distinct phenotypes under normal laboratory growth conditions (Poulsen et al., 1989).

Recently, Ost and Deutscher (1991) reported that the sequence of 21 N-terminal amino acids of a partially purified preparation of RNase PH matched with the N-terminal end of OrfE as deduced from the DNA sequence, and that orfE clones overproduced RNase PH. These observations allowed the authors to conclude that orfE is the structural gene for RNase PH, and they proposed that orfE should be renamed rph (Ost and Deutscher, 1991).

In this paper we report the construction of a plasmid vector overproducing OrfE/RNase PH at a level of about 15% of all cellular protein, as well as the purification of the protein in large quantities by a two-step purification procedure. In the accompanying paper, Kelly and Deutscher (1992) report some kinetic properties of the purified enzyme.

EXPERIMENTAL PROCEDURES

Materials—Cellulose powder (CF11), phosphocellulose (or cellulose phosphate, P11), and diethylaminoethyl cellulose (DE52) were from Whatman Biosystems Ltd. (Maidstone, United Kingdom), and Sephacryl S-300 was from Pharmacia (Uppsala, Sweden). Immobilon-P Transfer membranes were from Millipore Co. Sodium dodecyl sulfate (SDS)1 was from BDH (Poole, United Kingdom). Analytical grade standard chemicals were from Sigma or Merck (Darmstadt, Federal Republic of Germany). Restriction endonucleases, S1 nuclease, the Klenow fragment of DNA polymerase I, T4 DNA ligase, yeast alcohol dehydrogenase, and horse heart catalase were from Boehringer Mannheim (Federal Republic of Germany) and were used according to the manufacturer's instructions. Polyadenylic acid, high molecular weight calf thymus DNA, and bovine serum albumin were from Sigma. [32P]Orthophosphate (carrier-free) was from A.E.C. (Rissø, Denmark). E. coli orotate phosphoribosyltransferase was prepared as described by Poulsen et al. (1983). DNA-cellulose was prepared with calf thymus DNA according to Bautz and Dunn (1971). Polyethyleneimine-impregnated cellulose thin layer plates were made according to the procedure of Randerath and Randerath (1967).

Bacterial Strains and Plasmids—The E. coli strain NF1815 was a gift from N. Fül (University of Copenhagen) and is a recA1 derivative of MC1000. S06360 is NF1815 harboring the expression vector pJTA43. The "minicell" strain BD1854 was a gift from N. Fül (University of Copenhagen) and is recA1, but lacking the tet gene (Poulsen et al., 1984). The plasmid pPF is a CiaI deletion derivative of pPP4 containing the orfE/pyrE operon, but lacking the tet gene (Poulsen et al., 1984). The plasmid pPP4 is a CiaI deletion derivative of pPP1 containing the orfE/pyrE operon and the last 14 codons of orfE, even after shifting the translational reading frame (Bonekamp et al., 1984). Plasmid pPP4 was constructed from pPP1 by opening the unique PsulI site of pPP1 (Poulsen et al., 1983). Plasmid pPP4 was constructed from pPP1 by opening the unique PsulI site near the end of orfE, treating with S1 nuclease to remove the central 4 base pairs of the AatII site, and religating with T4 DNA ligase. The vector pUHE23-2 was a generous gift from H. Bujard (University of Heidelberg). Construction of the expression vector pJTA43 is described under "Results."

Media and Growth Procedures—The cells were grown with shaking
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at 37 °C in the (A+B) medium of Clark and Maalee (1967), supplemented with casamino acids (0.4%), glucose (0.4%), and thiamine (1 μg/ml). For plates, the medium was solidified with 1.5% agar (Difco). 1 L broth agar was prepared as described by Miller (1972). The analysis of [35S]methionine-labeled minicells was performed as described previously (Jensen et al., 1984).

Enzymes and Enzyme Assays—The assay of phosphorolytic cleavage of poly(A) is based on the procedure of Deutscher et al. (1988) and modified in the following way. The assay solution (100 μl) contained 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 60 mM NaCl, 10 mM [32P]orthophosphate (5 Ci/mol), and poly(A) at a concentration equivalent to 10 mM adenylyl residues as measured spectro-photometrically. The reaction, prewarmed at 37 °C, was started by addition of either crude extract or purified OrfE protein. At different time intervals, 10-μl aliquots were applied to a polyethyleneimine thin layer plate. After all samples were taken, the chromatogram was developed in methanol to the application point and then in 0.2 M potassium phosphate, pH 3.4, for about 16 cm. The plates were dried and subjected to autoradiography. The [3P]ADP spots were cut out and counted in a liquid scintillation apparatus (Packard Instrument Co.).

Assays of the production of [3H]CDP from rRNA-CCA-[3H]C₈ (100 μg/ml) and orthophosphate (15 mM) were performed by Karen Ost Kelly (The University of Connecticut, Farmington) as described previously (Deutscher et al., 1988; Ost and Deutscher, 1991).

Orotate phosphoribosyltransferase (EC 2.4.2.10) activity was determined spectrophotometrically as described by Poulsen et al. (1983). One unit is defined as the amount of enzyme that produces 1 μmol of orotidine 5′-monophosphate/min.

Molecular Mass of Native OrfE/RNase PH—The molecular mass of the native OrfE/RNase PH was estimated by loading 70 μg of the OrfE protein mixed with yeast alcohol dehydrogenase on three 5-20% sucrose gradients. After centrifugation for 24 h at 39,000 rpm in an SW41 rotor (Beckman) at 4 °C, the gradients were tapped from the bottom into the same equal sized fractions and assayed for alcohol dehydrogenase activity as described (Bühner and Sund, 1969) and for the poly(A) phosphorolytic activity associated with OrfE. In addition, the OrfE/RNase PH (3.4 mg) was passed through a Sephacryl S-300 column equilibrated with 25 mM Tris-HCl, pH 7.6, 2 mM EDTA and disrupted by sonication for 5 times for a 350-μl sample. The high molecular weight standards were bovine serum albumin (68,000), ovalbumin (44,000), egg-white lysozyme (14,300), RNase A (13,700), and RNase T₁ (13,000). The proteins of OrfE and bovine serum albumin were determined from the absorbance profile at 280 nm UV light. Moreover, RNase PH was identified by the poly(A) phosphorolytic activity. Catalase activity was measured by monitoring the disappearance of absorbance at 230 nm stemming from H₂O₂. Alcohol dehydrogenase and orotate phosphoribosyltransferase activities were measured as described above.

Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970), using 12.5% polyacrylamide slab gels (30 × 20 x 0.1 cm). The low molecular weight markers from Bio-Rad were included in each gel.

Isolation of Individual OrfE Bands—Samples containing purified OrfE protein (1.7, 3.4, and 8.6 μg) were run on an SDS gel and the proteins were transferred to an Immobilon™-P transfer membrane using the Semi-dry Electroblotter (JKA-BIOTECH, Denmark) as described (Matsudaia, 1990). The membrane was stained with Coomasie Blue and the three well-separated OrfE bands were cut out from each of the three lanes. The bands from the two samples with less protein were used to determine amino acid composition, while the bands from the sample with the most protein were used for limited tryptic and/or pancreatic sequencing.

Amino Acid Analysis and Sequencing—To determine the amino acid composition, aliquots of protein (in some cases bound to a piece of Immobilon™-P transfer membrane) were hydrolyzed for 24 h in 6 M HCl at 120 °C. After derivatization with phenylisothiocyanate, the phenylthiohydroxanlide-derivatives were analyzed and quantified by high performance liquid chromatography (Applied Biosystems). The N-terminal amino acid sequence was determined using an Applied Biosystems 477A sequenator.

RESULTS

Construction of an Expression Vector for Overproduction of the Two Proteins of the pyrE Operon of E. coli—The structural genes of the pyrE operon were isolated as a 1681-base pair DNA fragment after cutting of pPP1 (Poulsen et al., 1983) with AviI and PoulI. The restriction endonuclease AviI cuts between positions -3 and -4 relative to the transcription start site of the orfE promoter (Poulsen et al., 1984), while PoulI cuts 227 base pairs downstream from the stop codon of pyrE (Poulsen et al., 1983). To overproduce the two protein products of the operon, we constructed the plasmid pJT4A3 (Fig. 1) by inserting the 1681-base pair AviI-PoulI fragment into vector pUHE23-2 digested with EcoRI and HindIII and filled-in with the Klenow polymerase to create blunt ends. Thus, transcription of the orfE-pyrE operon was brought under control of the very strong T7 A1 promoter derivative (Fig. 1).

When introduced into minicells, plasmid pJT4A3 gave rise to synthesis of the same proteins as pPP1, which carries the native orfE-pyrE operon expressed from its own promoter (Fig. 2, lanes 1 and 3). Notably, the OrfE protein migrated as two distinct protein species, as seen previously (Poulsen et al., 1984). When pJT4A3 was harbored in NEF1815 (i.e. strain S06360), orotate phosphoribosyltransferase was overproduced nearly 1000-fold and the polyadenylate phosphorylase activity was increased 4-5-fold, relative to plasmid-free cells (Table 1). Analysis of crude extracts of S06360 by SDS-polyacrylamide gel electrophoresis showed orotate phosphoribosyltransferase and OrfE to be the most prominent of all protein bands (Fig. 3A, lanes A and B).

Purification of the OrfE Protein—The OrfE protein was purified from a 2-liter saturated culture of S06360 grown overnight with shaking at 37 °C. The resulting 13 g of wet cells were suspended in 30 ml of cold 100 mM Tris-HCl, pH 7.6, 2 mM EDTA and disrupted by sonication for 5 times for 1 min using a Branson sonifier. Subsequently, 7 μl of β-

![FIG. 1. Construction and structure of plasmid pJT4A3 expressing the orfE-pyrE operon at high levels. A 1681-base pair DNA fragment containing the orfE-pyrE operon minus the promoter was cut out from plasmid PPF1 (Poulsen et al., 1983) using the restriction endonucleases AviI and PoulI and cloned into the plasmid pUHE23-2 (obtained from H. Bujard) which was cleaved with EcoRI and HindIII and blunted with the Klenow fragment of DNA polymerase-1. A, structure of pJT4A3. P/O, promoter, operator region; kat, a λ-terminator transcription; bla, gene for β-lactamase; cat, gene for chloramphenicol acetyltransferase. B, EcoRI, truncated EcoRI site flanking the cloning region; HindIII, truncated HindIII site flanking the cloning region. B, the function between the T7 A1 promoter of plasmid pUHE23-2 and the start of orfE. The sequences in plain type represent vector DNA, and the sequences shown in bold italic type represent E. coli DNA. The two boxes indicate the -35 and the -10 regions of the strong T7 A1 early promoter transcribed by the E. coli RNA polymerase, while the two regions of dyad symmetry are two artificial lac-operator sites joined with the T7 promoter in pUHE23-2. The amino acid sequence below the nucleotide sequence is the N terminus of the OrfE protein.]
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Fig. 2. [35S]Methionine-labeled proteins synthesized in minicells under the direction of different plasmids. Lane 1, pJTA43 used for the overproduction of OrfE; lane 2, the vector plasmid pUHE23-2; lane 3, pPP1, in which the orfE-pyrE is expressed from the native promoter (Poulsen et al., 1983; 1984); lane 4, pFB102, encoding a truncated OrfE' protein (M, = 24,396); lane 5, pPP4, encoding the native OrfE protein (M, = 25,479) (Poulsen et al., 1984). Bla, the β-lactamase bands; CAT, chloramphenicol acetyltransferase; OPRT, orotate phosphoribosyltransferase, the pyrE gene product.

Table I

Overexpression of orotate phosphoribosyltransferase and polyadenylate phosphorylase activities from pJTA43

The cells were grown into stationary phase overnight.

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<th>NF1815</th>
<th>S06380</th>
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<tr>
<td>OPRTase</td>
<td>0.029</td>
<td>27.9</td>
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<tr>
<td>Poly(A) phosphorylase</td>
<td>0.016</td>
<td>0.074</td>
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mercaptoethanol was added to the extract. After removal of cell debris by low speed centrifugation (10,000 rpm for 15 min in a Sorvall SS-34 rotor), the extract was cleared at 34,000 rpm in an SW41 rotor (Beckman) for 90 min at 4 °C. The supernatant from the 100,000 × g spin was then brought to 10% saturation by addition of 2.6 g of solid (NH₄)₂SO₄ and stirred for 30 min. The resulting pellet, containing about two-thirds of the OrfE protein (see Fig. 3, lane 3), was collected by centrifugation for 15 min at 10,000 rpm in an SS-34 rotor (Sorvall). The protein was dissolved in 10 ml of 25 mM Tris-HCl, pH 7.6, 0.5 mM EDTA, 2 mM β-mercaptoethanol (buffer A) and loaded on a 0.8 cm² × 20-cm phosphocellulose column. The column was washed with 2 volumes of buffer A containing 0.25 M NaCl and eluted with a 300-ml linear gradient from 0.25 to 1.0 M NaCl. The pump rate was 20 ml/h and 10-ml fractions were collected. OrfE appeared in the eluate at about 0.6 M NaCl. The elution profile was analyzed by SDS-polyacrylamide gel electrophoresis and absorbance at 280 nm and is shown in Fig. 3, B and C. Fractions 12-17 were pooled, dialyzed against buffer A containing 50% glycerol, and stored at −20 °C. The yield after the purification was about 6 mg of OrfE protein/g wet cells.

As seen from Fig. 3A, lanes C and D, the 10% ammonium sulfate precipitation gave a considerable purification, and 60–70% of the OrfE protein was recovered. If the 10% ammonium sulfate precipitation was carried out using more dilute extracts, a larger fraction of OrfE was lost. Complete recovery of the protein could always be achieved by precipitation with >20% saturated ammonium sulfate. However, in that case, it was necessary to incorporate a chromatography step on DEAE-cellulose between the ammonium sulfate precipitation and the phosphocellulose column in order to obtain a pure protein. OrfE eluted from the DEAE-cellulose column at about 160 mM NaCl in buffer A (data not shown).

The amino acid composition of OrfE was determined after hydrolysis of a small sample of the protein in 6 M hydrochloric acid at 120 °C for 24 h. The amino acid content was unusual in the sense that very few of the aromatic amino acids, notably few phenylalanaines, were present. The analysis correlated well with the amino acid composition of the orfE protein product as predicted from the DNA sequence (Table II). The concentration of amino acids in the hydrolysates was used to estimate that a 1 mg/ml solution of OrfE had an absorption at 280 nm of about 0.43.

Heterogeneity—The OrfE protein preparation, eluted from
the phosphocellulose column, contained two major (and one minor) bands. These appeared closely spaced on an SDS gel in the molecular mass range of 30–33 kDa (Fig. 3). We considered this preparation pure since the orfE gene product also appeared heterogeneous when produced in minicells (Fig. 2).

The OrfE protein was subjected to four cycles of sequential Edman degradation. The only N-terminal sequence found was Met-Arg-Pro-Ala-, which corresponded to the predicted translation start site on the orfE mRNA (Poulsen et al., 1984). Moreover, the three individual protein bands were isolated by electrophoresis and blotting, as described under "Experimental Procedures." All the "bands" had the N-terminal amino acid sequence mentioned above, and they did not deviate significantly from each other in amino acid composition or in the yield of amino acids per N-terminal end. Furthermore, the heterogeneity of OrfE seemed not to be due to proteolytic processing at the C-terminal end or to result from translational read-through past the UGA stop codon ending the orfE gene. This conclusion was derived from an experiment where the central 4 base pairs in the unique AucI site 17 codons from the end of the orfE gene were removed. This resulted in a truncated OrfE' protein (Mr = 24,396), which was 10 amino acid residues shorter than the native OrfE. Upon analysis for translation products in minicells, the OrfE' protein still appeared as two major bands on an SDS gel (Fig. 2, lanes 4 and 5).

Since the different protein bands in the OrfE preparation seemed so identical with each other, it is possible that the heterogeneity might be due to a very reproducible electrophoretic artifact, seen over a 10-year period (Poulsen et al., 1984). However, since the relative distribution of protein between the bands was always the same, no matter whether small or large amounts were loaded on the gels, it is conceivable that OrfE/RNase PH contains a site for post- or co-translational modifications of the subunits in the oligomeric state of the protein.

**Molecular Weight of the Native Enzyme**—During ultracentrifugation in an SW41 rotor (Beckman) the native OrfE protein sedimented slightly slower than yeast alcohol dehydrogenase (s20,w = 7.61, Mr = 150,000 (Bühner and Sund, 1969)) through three 5–20% sucrose gradients (data not shown). According to Martin and Ames (1961) this sedimentation behavior corresponds with a sedimentation constant of Mr = 6.65 ± 0.15 and suggests a molecular mass of the native OrfE of about 120 kDa, if the protein was of globular shape. By gel filtration chromatography on a Sephacryl S-300 column, the OrfE protein eluted prior to horse heart catalase (data not shown), indicating a molecular mass over 200 kDa (Andrews, 1965). However, the observed high molecular mass may be due to aggregation, since we used very high concentrations of the OrfE protein for these analyses in order to detect it by SDS-gel electrophoresis. Kelly and Deutscher (1992) have shown that the apparent size of OrfE, detected by the RNase PH activity, becomes smaller and smaller as the protein concentration is reduced, and that RNase PH behaves as a dimer during gel filtration under the most diluted conditions.

**DNA Binding Properties of OrfE**—The OrfE protein contains regions of similarity with the helix-turn-helix motif found in many DNA-binding proteins (Pabo and Sauer, 1984; Wharton and Ptashne, 1986) (Fig. 4A). Furthermore, the strong binding to phosphocellulose is also frequently observed for nucleic acid-binding proteins. To test the potential DNA
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binding properties of OrfE, we mixed it with an equal quantity of orotate phosphoribosyltransferase and applied the mixture to a small column of (calf thymus) DNA-cellulose. In this experiment, the orotate phosphoribosyltransferase was unretained, while OrfE was adsorbed to the column and eluted only when the buffer contained more than 250 mM NaCl (Fig. 4B).

The binding of OrfE both to DNA-cellulose and phosphocellulose at pH 7.6 is not a simple electrostatic binding, since the protein does not adsorb to other negatively charged ion-exchange resins, like Mono-S from Pharmacia. Furthermore, OrfE is a neutral or slightly acidic protein, both as judged from the excess of acid over basic amino acids in the protein and from its behavior during two-dimensional polyacrylamide gel electrophoresis (O’Farrell, 1975) (not shown).

Phosphorolysis of Ribonucleic Acid Catalyzed by OrfE—When incubated with polyadenylate (10 mM, adenylate residues) and 10 mM [32P]orthophosphate, the OrfE preparation produced 32P-labeled ADP as a linear function of time. The rate was 1.5 μmol/min/mg protein, corresponding to the formation of 40 ADP molecules/min/molecule of OrfE. The polyadenylate phosphorolysis activity was superimposable with the OrfE protein peak in the elution profile from a phosphocellulose column, when an aliquot of the purified OrfE product was rechromatographed on a column of phosphocellulose (not shown).

The identity between the OrfE protein and the polyadenylate phosphorylase was also inferred by the fact that extracts of strain S63660, which harbors the expression vector pJTA43, contained 4–5 times more poly(A) phosphorolytic activity than extracts made from the corresponding plasmid-free strain, NF1815 (Table I). This increase is not anywhere near parallel with the observed increase in orotate phosphoribosyltransferase, because the phosphorolysis of poly(A) is also catalyzed by the traditional polynucleotide phosphorylase, but it is still significant.

The purified enzyme was also assayed for phosphorolytic activity toward tRNA-CCA-[3H]C, kindly performed by Karen Ost Kelly as previously described (Deutscher et al., 1988). These assays revealed the release of 1.4 μmol of [3H]CDP/min/mg of OrfE protein and showed that the ratio of poly(A) phosphorolysis over tRNA-CCA-C, phosphorolysis was 11 for the OrfE protein as found also for partially purified RNase PH, while the corresponding activity ratio is greater than 1400 for polynucleotide phosphorylase (Deutscher et al., 1988; Ost and Deutscher, 1991). More kinetic evidence for the identity between the OrfE protein and RNase PH is presented by Kelly and Deutscher (1992).

DISCUSSION

The orfE gene product was purified in large quantities by a simple two-step procedure from an extract of an E. coli strain that strongly overproduces the protein product. The purified OrfE catalyzed the phosphorylase of polyadenylate, and this activity was enhanced in crude extracts of strains carrying the expression vector pJTA43. These results support the conclusion of Ost and Deutscher (1991) that orfE is the structural gene for the phosphoribonucleotide RNase PH. These authors suggested that the orfE gene should be renamed rph. We support this proposition.

The described preparation of OrfE/RNase PH migrated as two major and one minor protein bands on SDS gels. The N-terminal analyses and C-terminal truncation studies made it unlikely that this heterogeneity arises by proteolytic processing of the primary translation product. Instead the heterogeneity might result from other types of post- or co-translation processing, but it could also be due to an electrophoretic artifact, since it was not seen by Kelly and Deutscher (1992) when they analyzed our preparation of OrfE/RNase PH.

It is remarkable that RNase PH contains the helix-turn-helix motif, normally seen in repressor or activator proteins, and that it binds to DNA. However, RNase PH should bind to tRNA precursors and, maybe, recognize double helical regions in these substrates. A similar helix-turn-helix motif was observed in the C-terminal domain of the ribosomal protein L7/L12, which is important for tRNA and elongation factor binding (Leonmarck and Liljas, 1987; Rice and Steitz, 1989; Terhorst et al., 1973); but also in that case the significance of the helix-turn-helix motif for RNA binding is unknown.

RNase PH was discovered due to the ability to remove nucleotides from the 3' ends of tRNA precursors (Deutscher et al., 1988). However, it is conceivable that the enzyme is also involved in mRNA degradation, in competition with polynucleotide phosphorylase and RNase II. This point may be relevant for the findings of McLaren et al. (1991), who noticed that stem-loop structures near the 3' ends of mRNAs in vitro did not protect the RNA chain against polynucleotide phosphorolysis and RNase II to the same extend as they appear to protect mRNAs in vivo. This observation was interpreted as indicative evidence for the existence of a stem-loop stabilizing protein in E. coli (McLaren et al., 1991), but it might also mean that ribonucleases other than polynucleotide phosphorolysase and RNase II are involved in the breakdown of such mRNAs in vivo. As the catalytic rate of RNase PH is lower than for polynucleotide phosphorylase and RNase II (Deutscher et al., 1988; Ost and Deutscher, 1991), and the affinity for hairpin structures may be higher (due to the helix-turn-helix motif?), RNase PH might selectively bind at stem-loop structures near the 3' ends and "protect" mRNAs from attack by the more aggressive ribonucleases.

The orfE/rph gene can be disrupted in E. coli without seriously affecting the growth under normal laboratory conditions (Poulsen et al., 1989), but the gene was shown to be essential if the cells lack several other RNases (Kelly and Deutscher, 1992). There has been a report stating that rph mutants exhibit filamentous growth and are inviable at 45 °C (Poulsen and Jensen, 1991), but these phenotypes depend on an unidentified mutation in the employed background. 2

The rph gene is co-transcribed in operon with the pyrE gene, which encodes a pyrimidine nucleotide biosynthetic enzyme. This genetic arrangement may be purely fortuitous and still be advantageous for the cell: by the evolution of the rph-pyrE operon the pyrE gene was equipped with a constitutively expressed promoter and obtained a gratuitous leader peptide. Moreover, the mRNA secondary structure, which probably would have to be present anyway in order to protect the rph mRNA against breakdown from the 3' end (Stern et al., 1984; Higgins et al., 1988; McLaren et al., 1991), has acquired the attenuator-function of regulating pyrE expression in response to the cellular supply of nucleotides and amino acids (Jensen et al., 1986).

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