Volume 216, number 1, 133-139 FEB 04718 May 1987

The prephenate dehydrogenase component of the bifunctional T-protein in enteric bacteria can utilize L-arogenate

Suhail Ahmad and Roy A. Jensen

Department of Microbiology and Cell Science, McCarty Hall, IFAS, University of Florida, Gainesville, FL 32611, USA

Received 3 February 1987; revised version received 19 March 1987

The prephenate dehydrogenase component of the bifunctional T-protein (chorismate mutase:prephenate dehydrogenase) has been shown to utilize L-arogenate, a common precursor of phenylalanine and tyrosine in nature, as a substrate. Partially purified T-protein from *Klebsiella pneumoniae* and from *Escherichia coli* strains K12, B, C and W was used to demonstrate the utilization of L-arogenate as an alternative substrate for prephenate in the presence of nicotinamide adenine dinucleotide as cofactor. The formation of L-tyrosine from L-arogenate by the T-protein dehydrogenase was confirmed by high-performance liquid chromatography. As expected of a common catalytic site, dehydrogenase activity with either prephenate or L-arogenate was highly sensitive to inhibition by L-tyrosine.

Tyrosine synthesis; T-protein; Enteric bacteria; Regulatory enzyme

1. INTRODUCTION

Escherichia coli and *Klebsiella pneumoniae*, closely related enteric bacteria, possess a pair of bifunctional proteins that compete for chorismate as initial substrate molecules in reactions leading to biosynthesis of aromatic amino acids. Fig.1 illustrates that the P-protein (chorismate mutase-P: prephenate dehydratase) channels chorismate to L-phenylalanine, while the T-protein (chorismate mutase-T: prephenate dehydrogenase) channels chorismate to L-tyrosine at a metabolic branch point [1,2]. Each bifunctional protein has been purified to homogeneity from *E. coli* and *K. pneumoniae* [3-5]. These proteins were postulated to have evolved by fusion of genes specifying cyclohexadienyl dehydrogenase and cyclohexadienyl dehydratase with one of two gene duplicates specifying chorismate mutase [2]. Cyclohexadienyl dehydrogenase and cyclohexadienyl dehydratase (often referred to as arogenate dehydrogenase and arogenate dehydratase in earlier papers) are able to accept either prephenate or L-arogenate as substrates. Within the Superfamily-B cluster, a phylogenetic assemblage comprising one of three major groups of Gram-negative bacteria (includes enteric genera, *Oceanospirillum, Xanthomonas*, fluorescent pseudomands and *Acinetobacter*) all lineages possess the P-protein and most retain a cyclohexadienyl dehydratase as well [6]. In contrast, the T-protein evolved very recently within Superfamily B, being present only within the enteric lineage [2]. Superfamily-B organisms possess either cyclohexadienyl dehydrogenase (e.g. *Acinetobacter*, fluorescent pseudomonads and *Xanthomonas*) or the T-protein, but not both [6].

L-Arogenate has become recognized as a widely utilized precursor of tyrosine and/or phenylalanine in nature [7]. However, the exclusive formation of prephenate within the two channeling systems of *E. coli* and *K. pneumoniae* essentially excludes prephenate as a substrate for one or more

Correspondence address: R.A. Jensen, Department of Microbiology and Cell Science, 1059 McCarty Hall, IFAS, University of Florida, Gainesville, FL 32611, USA.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

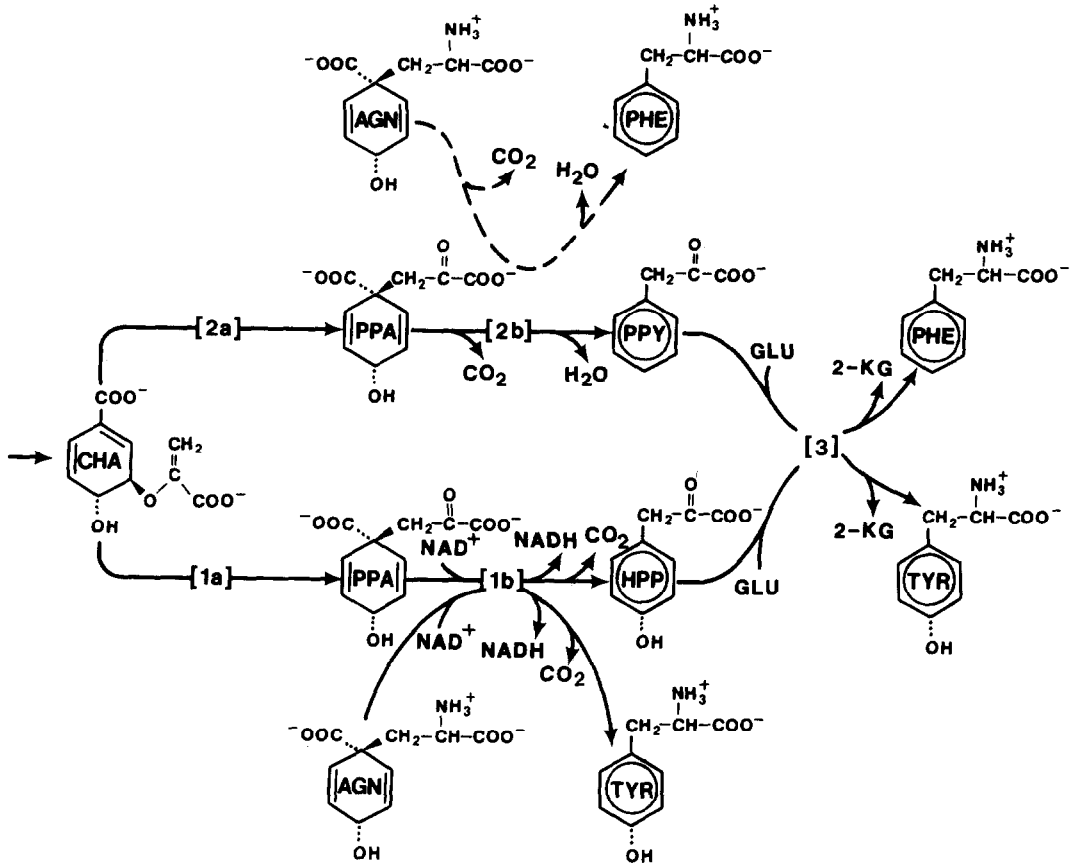

Fig.1. Operation of two bifunctional proteins in biosynthesis of phenylalanine (PHE) and tyrosine (TYR) in enteric bacteria. Chorismate (CHA) is competed for as substrate by both the P-protein (enzymes [2a] and [2b]) and the T-protein (enzymes [1a] and [1b]). Phenylpyruvate (PPY) and 4-hydroxyphenylpyruvate (HPP) are transaminated to PHE and TYR, respectively, in the presence of glutamate (GLU). The catalytic activities of the P-protein are chorismate mutase-P [2a] and prephenate dehydratase [2b]; the catalytic activities of the T-protein are chorismate mutase-T [1a] and prephenate dehydrogenase [1b]. The reaction of arogenate (AGN) dehydrogenase which is catalyzed by the [1b] component of the T-protein is shown at the bottom. The section of arogenate dehydratase which is not catalyzed by component [2b] of the P-protein is shown (dashed lines) at the top. Enzymes able to utilize both PPA and AGN as cyclohexadienyl substrates have been referred to as cyclohexadienyl dehydratases or as cyclohexadienyl dehydrogenases. Thus, the T-protein is a cyclohexadienyl dehydrogenase, but the P-protein is not a cyclohexadienyl dehydratase.

aminotransferases able to form L-arogenate from prephenate in vivo. Hence, the competing channeling systems essentially preclude the endogenous formation of L-arogenate. If abilities of the P-protein and T-protein to use L-arogenate have lost selective value, then enzymatic ability to use L-arogenate as substrate might diminish with evolutionary time. Thus, this might be expected to be more pronounced for the P-protein than for the T-protein because the P-protein gene originated much earlier.

We have indeed found that the P-protein dehydratase is specific for prephenate, whereas the T-protein dehydrogenase still functions as a cyclohexadienyl dehydrogenase. The T-protein dehydrogenase is actually quite similar to the cyclohexadienyl dehydrogenases present in Superfamily-B organisms that lack the evolved T-protein [8,9]. Partially purified T-protein from *E. coli* and *K. pneumoniae* was used to demonstrate the utilization of L-arogenate as an alternative substrate for prephenate. Other generic members

belonging to the enteric lineage also possess T-proteins able to utilize L-arogenate as substrate.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and culture conditions*

E. coli K12 ATCC 10798, *E. coli* B ATCC 23226, *E. coli* C ATCC 13706, *E. coli* W ATCC 9637 and *K. pneumoniae* ATCC 25304 were obtained from the American Type Culture Collection (Rockville, MD). The organisms were grown as described [10].

2.2. *Preparation of cell extracts and enzyme assays*

Crude cell-free extracts (free of small molecules) were prepared as in [10].

Chorismate mutase was assayed by the method of Cotton and Gibson [1]. Prephenate dehydrogenase and arogenate dehydrogenase activities were assayed by following the continuous formation of reduced nicotinamide adenine dinucleotide (NADH) using a spectrophotofluorometer as described by Patel et al. [11].

To confirm the formation of L-tyrosine in the arogenate dehydrogenase reaction, the enzyme was assayed as described below followed by high-performance liquid chromatographic (HPLC) detection of the tyrosine formed as outlined by Lindroth and Mopper [12]. The reaction mixture in a final volume of 225 μl contained 50 mM K phosphate (pH 7.0) containing 1 mM dithiothreitol (DTT), 0.5 mM NAD, 1.25 mM K arogenate and a suitable amount of enzyme protein. After incubation at 37°C for 20 min, the contents were transferred to ice for HPLC analysis. A 40 μl aliquot was derivatized with 200 μl OPA reagent [54 mg *o*-phthalaldehyde dissolved in 1 ml methanol, followed by the addition of 9.0 ml of 0.4 M Na-borate buffer (pH 9.5) and 200 μl of 2-mercaptoethanol]. After 90 s, the derivatized sample was injected into a 20 μl loop and swept onto a C_{18} column (Alltech Associates, Deerfield, IL) (4.6 × 250 mm) equilibrated and eluted with methanol/20 mM Na-phosphate buffer (pH 6.8) (45:55, v/v) at a flow rate of 1 ml/min. Controls in which only enzyme was incubated, only substrates were incubated, or in which enzyme was added to the other components at zero time (just before derivatization) were also included.

Protein in the crude extract was estimated by the method of Bradford [13] with bovine serum albumin as the standard.

2.3. *DE52 column chromatography*

Approx. 100 mg crude extract protein were applied to a DEAE-cellulose (DE52) column (1.5 × 20.0 cm) equilibrated in 10 mM K-phosphate buffer (pH 7.0) containing 1 mM DTT. The column was washed with two bed volumes of the equilibrating buffer, and then the bound proteins were eluted with 300 ml of a linear gradient of KCl (0.0–0.4 M) contained in the equilibration buffer. Fractions of 2.2 ml were collected.

2.4. *Biochemicals and chemicals*

Amino acids, NAD, NADP, DTT, OPA, bovine serum albumin and Sephadex G-25 were obtained from Sigma (St. Louis, MO). DE52 was purchased from Whatman (Clifton, NJ). Prephenate was prepared as the barium salt from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* [14] and was converted to the potassium salt before use. Chorismate was isolated from the accumulation medium of a triple auxotroph of *K. pneumoniae* 62-1 and purified as the free acid [15]. L-Arogenate was prepared from a triple auxotroph of *Neurospora crassa* as in [16]. All other chemicals were standard reagent grade.

3. RESULTS AND DISCUSSION

The elution profiles of the enzyme activities participating in the biosynthesis of tyrosine from *E. coli* K12 (top panel), *E. coli* B (middle panel), and *E. coli* W (bottom panel) following DE52 chromatography of the crude extracts are shown in fig.2. Two peaks of chorismate mutase activity eluted in each case. The leading peak of chorismate mutase activity (chorismate mutase-P) co-eluted with the prephenate dehydratase activity, thus identifying the location of the bifunctional P-protein (not shown). The trailing activity peak of chorismate mutase (chorismate mutase-T) co-eluted with the prephenate dehydrogenase activity, thus marking the position of the bifunctional T-protein in each case. All column fractions exhibiting prephenate dehydrogenase activity also showed activity when arogenate was used as an alternative substrate. Thus, arogenate dehydro-

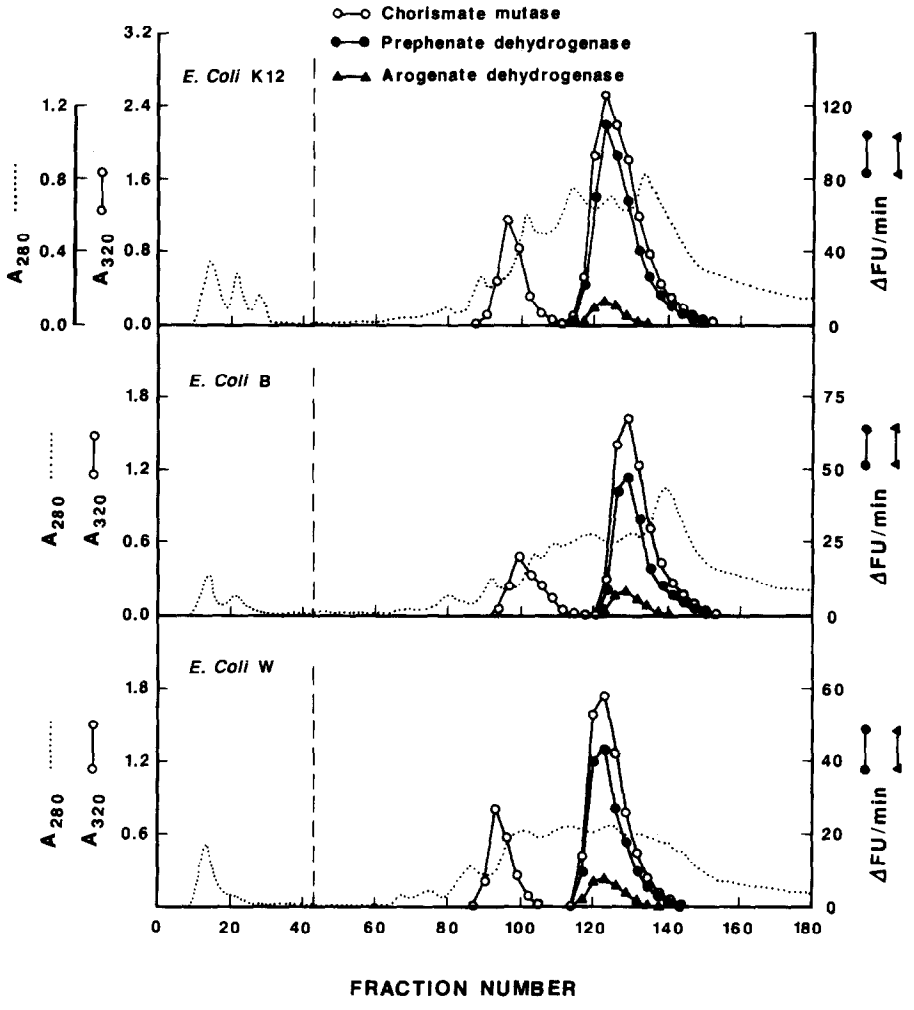

Fig.2. Elution profiles of chorismate mutase, prephenate dehydrogenase and arogenate dehydrogenase following DE52 column chromatography of the crude extracts prepared from *E. coli* K12 (top panel), *E. coli* B (middle panel) and *E. coli* W (bottom panel). The DE52 column chromatography was performed as described in section 2. The vertical dashed lines indicate the onset point of gradient elution. Chorismate mutase activity is expressed as phenylpyruvate absorbance in base at 320 nm. The dehydrogenase activities are expressed as change in fluorescence units per minute (ΔFU/min). The distribution of proteins (as monitored by A_{280}) is shown by dashed lines.

genase, prephenate dehydrogenase, and chorismate mutase-T activities all shared a common elution profile (fig.2). Both the prephenate dehydrogenase and arogenate dehydrogenase activities functioned only with NAD as cofactor, NADP failing to satisfy the cofactor requirement. Arogenate dehydrogenase activity was not detected in other column fractions, with either NAD or NADP present. Similar results were obtained with *E. coli* C (not shown).

The elution profiles of enzymes from *K. pneumoniae* that were obtained following DE52 chromatography of crude extracts are shown in fig.3. Two peaks of chorismate mutase activity eluted, one of which co-eluted with prephenate dehydratase activity and the other of which co-eluted with prephenate dehydrogenase activity, as has been reported [1]. However, the fractions exhibiting prephenate dehydrogenase activity also showed activity when L-arogenate was utilized as

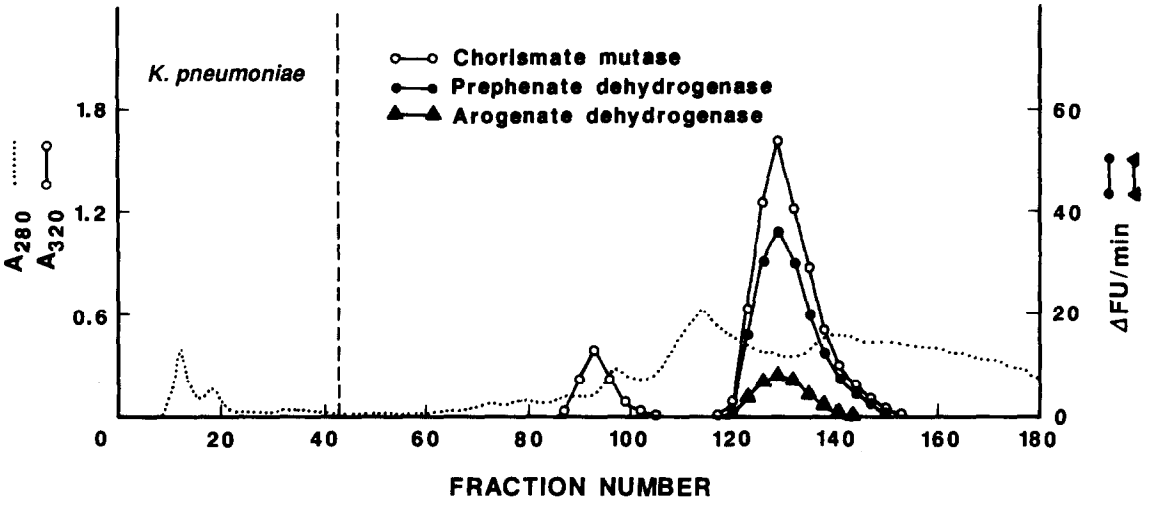

Fig.3. Elution profiles of chorismate mutase, prephenate dehydrogenase and arogenate dehydrogenase following DE52 column chromatography of the crude extract prepared from *K. pneumoniae*. The DE52 column chromatography was performed as described in section 2. The symbols used are the same as in fig.2.

an alternative substrate, and the arogenate dehydrogenase activity followed the same profile as the prephenate dehydrogenase activity (fig.3). Dehydrogenase activity with either substrate was reactive only with NAD, NADP failing to satisfy the cofactor requirement.

The sensitivity of the T-protein activities to inhibition by the aromatic amino acids was studied. Both of the dehydrogenase activities were sensitive to inhibition by L-tyrosine (table 1) in contrast to the chorismate mutase component of the T-protein (not shown) from both *E. coli* and *K. pneumoniae*. Other aromatic amino acids, L-phenylalanine and L-tryptophan, did not affect the T-protein activities.

Finally, to confirm the identity of L-arogenate as the dehydrogenase substrate (i.e. to exclude an impurity which might be present in the arogenate preparation), we carried out experiments to prove that L-tyrosine was the product of the reaction. This was achieved by separating the reaction mixture components, after the arogenate dehydrogenase reaction, on an HPLC column as described in Section 2. The appearance of a peak corresponding to L-tyrosine following arogenate dehydrogenase catalysis (fig.4) and its absence in control samples confirmed the formation of L-tyrosine in the arogenate dehydrogenase reaction. L-Tyrosine formed enzymatically exhibited the same specific retention time as an authentic sample of L-tyrosine that was run under the conditions of HPLC specified.

Other members of the purple sulfur bacteria (Superfamily B) that lack the T-protein possess a multi-specific aromatic-pathway dehydrogenase which appears to be a single cyclohexadienyl dehydrogenase [8,9]. This monofunctional (i.e. no chorismate mutase) dehydrogenase inevitably exhibits preference for prephenate, with K_m values for prephenate and L-arogenate varying by an order of magnitude [8,9,17]. It seems likely in view of the similar enzymological properties observed that an ancestral gene for cyclohexadienyl dehydrogenase was the progenitor of the dehydrogenase catalytic component of the T-protein.

In contrast to the similarity of the T-protein dehydrogenase of enteric bacteria and cyclohexadienyl dehydrogenase within the remainder of Superfamily-B prokaryotes, the P-protein dehydratase lacks the substrate ambiguity of cyclohexadienyl dehydratase. This could mean that the P-protein dehydratase evolved independently of cyclohexadienyl dehydratase, a possibility that is consistent with the joint presence of the P-protein and cyclohexadienyl dehydratase in most Superfamily-B organisms. A second possibility [2] is that the P-protein and T-protein evolutionary

Table 1

Feedback inhibition of aromatic-pathway dehydrogenase activities by L-tyrosine

Organism	% inhibition	
	Prephenate dehydrogenase	Arogenate dehydrogenase
E. coli K12	93	100
E. coli B	97	100
E. coli C	95	100
e. coli W	98	100
K. pneumoniae	93	100

The enzyme activities were recovered from DE52 column fractions (refer to column profiles shown in figs.1,2). L-Tyrosine was used at a final concentration of 0.5 mM. The sensitivity of the dehydrogenases to L-tyrosine was studied at prephenate concentrations of 0.1 mM, while L-arogenate substrate concentrations for arogenate dehydrogenase were 1.0 mM.

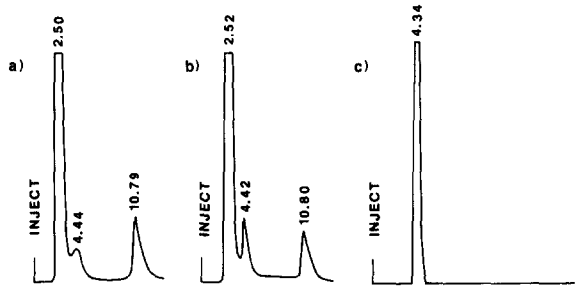

Fig.4. Separation and identification of L-tyrosine as the reaction product of the reaction catalyzed by arogenate dehydrogenase from *E. coli* K12. The enzyme was recovered from DE52 column fractions (refer to column profile in fig.1, top panel). The enzyme assay and the HPLC analysis were performed as described in section 2. The numbers represent retention time (in min) of L-arogenate, L-tyrosine and L-phenylalanine (present as a contaminant in L-arogenate preparations), respectively. (a) Control reaction mixture in which only enzyme was incubated and the substrates were added later (identical to data obtained from other controls where only substrates were incubated and the enzymes was included later, or where both the substrates and the enzyme were mixed at zero time just before derivatization and injection. (b) Complete reaction mixture showing the appearance of a new peak. (c) Identification of the new peak as L-tyrosine by comparing it with the retention time of the authentic L-tyrosine.

scenarios of gene fusion are essentially analogous, but that the relatively recent origin of the T-protein has not allowed enough evolutionary time for the casual loss of substrate recognition for L-arogenate at the dehydrogenase catalytic center. If the latter is so and the P-protein dehydratase and cyclohexadienyl dehydratase originated from a common ancestral gene, an event of gene duplication for the cistron encoding the dehydratase must have occurred (e.g. a transposon mechanism accomplishing the postulated gene-fusion event [18]) to account for the joint presence of P-protein dehydratase and cyclohexadienyl dehydratase in most Superfamily-B organisms.

Although the case has been made (see section 1) that neither the P-protein dehydratase nor the T-protein dehydrogenase is likely to encounter L-arogenate molecules of endogenous origin, the possible availability of L-arogenate from the exogenous environment could provide selective value for continued substrate recognition of L-arogenate by T-protein dehydrogenase. This could also account for maintenance of the broadly specific cyclohexadienyl dehydratase in most Superfamily-B bacteria, at the same time allowing the narrowed specificity of the co-existing P-protein dehydratase to proceed.

ACKNOWLEDGEMENT

This investigation was supported by grant PCM 8376275 from the National Science Foundation.

REFERENCES

[1] Cotton, R.G.H. and Gibson, F. (1965) Biochim. Biophys. Acta 100, 76–88.
[2] Ahmad, S. and Jensen, R.A. (1986) Trends Biochem. Sci. 11, 108–112.
[3] Davidson, B.E., Blackburn, E.H. and Dopheide, T.A.A. (1972) J. Biol. Chem. 247, 4441–4446.
[4] Hudson, G.S., Wong, V. and Davidson, B.E. (1984) Biochemistry 23, 6240–6249.
[5] Koch, G.L.E., Shaw, D.C. and Gibson, F. (1970) Biochim. Biophys. Acta 212, 375–386.
[6] Jensen, R.A. (1985) Mol. Biol. Evol. 2, 92–108.
[7] Jensen, R. and Fischer, R. (1987) Methods Enzymol. 142, 472–478.
[8] Ahmad, S. and Jensen, R.A. (1987) Arch. Microbiol. 147, in press.

[9] Byng, G.S., Berry, A. and Jensen, R.A. (1985) Arch. Microbiol. 143, 122–129.
[10] Ahmad, S., Johnson, J.L. and Jensen, R.A. (1987) J. Mol. Evol., in press.
[11] Patel, N., Pierson, D.L. and Jensen, R.A. (1977) J. Biol. Chem. 252, 5839–5846.
[12] Lindroth, P. and Mopper, K. (1979) Anal. Biochem. 51, 1667–1674.
[13] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[14] Dayan, J. and Sprinson, D.B. (1970) Methods Enzymol. 17A, 559–561.
[15] Gibson, F. (1968) Biochem. Prep. 12, 94–97.
[16] Zamir, L.O., Jensen, R.A., Arison, B.H., Douglas, A.W., Albers-Schonberg, G. and Bowen, J.R. (1980) J. Am. Chem. Soc. 102, 4499–4504.
[17] Byng, G.S., Berry, A. and Jensen, R.A. (1986) Arch. Microbiol. 144, 222–227.
[18] Ahmad, S. and Jensen, R.A. (1987) Orig. Life, in press.