Unusual *in vivo* turnover of transfer RNA in *Vibrio cholerae*

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Two lines of evidence suggest that, unlike in other organisms, the transfer RNAs of *Vibrio cholerae* undergo rapid turnover *in vivo*. Firstly, the tRNA content of *V. cholerae* cells treated with rifampicin (an inhibitor of initiation of RNA synthesis) decreased rapidly and continuously. Secondly, the newly synthesized tRNAs were rapidly degraded even under normal conditions of growth; the average half life of tRNA was 11.8 min. The degradation is mediated by an enzyme(s), present in *V. cholerae* cytoplasm, that apparently degrades tRNA completely. Rapid turnover is balanced by an enhanced rate of tRNA biogenesis, which was calculated to be 2.5 times higher than that in *Escherichia coli*.

Keywords: *Vibrio cholerae*, transfer RNA

INTRODUCTION

Intracellular RNA species can be classified into two groups, one with rapid turnover, such as messenger RNA, and the other which is quite stable and consists of ribosomal RNA and transfer RNA. Protection by ribosomal proteins can account for the stability of rRNA, while that of tRNAs is attributed to their compact three-dimensional structure. But under adverse conditions, such as starvation for essential nutrients like nitrogen (Ben-Hamida & Schlessinger, 1965), carbon (Nishi *et al.*, 1962), phosphate (Maruyama & Mizuno, 1965; Davis *et al.*, 1986) and magnesium (McCarthy, 1962; Natori *et al.*, 1966), treatment with EDTA and rifampicin (Yuan & Shen, 1975), or growth at elevated temperatures (Nozawa *et al.*, 1967), ribosomes and rRNAs in *Escherichia coli* become susceptible to degradation. tRNA, however, remains essentially stable even under these conditions (Maruyama & Mizuno, 1965; McCarthy, 1962; Natori *et al.*, 1966; Yuan & Shen, 1975; Davis *et al.*, 1986; Nozawa *et al.*, 1967). Even after exposure to drastic conditions, like ultra-violet irradiation or productive infection by bacteriophage lambda, which leads to cell death, tRNA molecules remain intact (King *et al.*, 1986). This is also true for other prokaryotes, like *Aerobacter aerogenes* (Kennell & Kotoulas, 1967) and *Pseudomonas aeruginosa* (Hou *et al.*, 1966).

The compact three-dimensional structure of tRNA notwithstanding, the conserved CCA sequence at the 3'-terminus of tRNA undergoes limited turnover in *E. coli* independent of the rest of the RNA chain (Deutscher *et al.*, 1977). The defective tRNA so generated is quickly

repaired by the enzyme tRNA nucleotidyltransferase (Deutscher & Evans, 1977). Mutants lacking this enzyme accumulate defective tRNAs which are subsequently degraded by RNase D (Zhang & Deutscher, 1988). Total yield of tRNA from such mutants is considerably lower than that from wild-type cells (Deutscher *et al.*, 1977).

Vibrio cholerae, a Gram-negative bacterium, is the causative agent of the disease cholera. Recently, we have examined various aspects of tRNA of this organism and its phages. Cholera phage ϕ 149 and phage ϵ_4 have been shown to induce synthesis of phage-coded tRNA molecules upon infection. These tRNAs have been characterized (Chattopadhyay & Ghosh, 1988a; Mandal & Ghosh, 1988), the tRNA genes have been localized on the phage genome (Chattopadhyay & Ghosh, 1988b) and the gene clusters have been cloned (Chattopadhyay & Ghosh, 1989). It was observed that the yields of tRNA from *V. cholerae* cells were always lower than those obtained from *E. coli*. We have conducted a systematic investigation of this phenomenon and report here that, unlike in other organisms, tRNAs of *V. cholerae* undergo rapid turnover *in vivo*. The process is mediated by a cytoplasmic nuclease(s) that can degrade intact tRNA completely.

METHODS

Bacterial strains. All *V. cholerae* strains were obtained from the National Institute of Cholera and Enteric Diseases, Calcutta. Cultures were maintained lyophilized or frozen at -70°C in nutrient broth. For routine use, strains were maintained on nutrient agar slants and subcultured every two weeks. Every month strains were passaged through animals to restore

toxinogenicity as described earlier (Roy *et al.*, 1982a). *E. coli* A19 (*metB1 rna*) was obtained from Professor Murray Deutscher of UCON Health Centre, Farmington, USA. *E. coli* C-600 (*thi-1 thr-1 leu-6 lacY1 tonA21 supE44*) was used as RNase I⁺ strain.

Media and growth conditions. Cells were grown at 37 °C with shaking (180 r.p.m.) in nutrient broth [Bactopeptone (Difco) 10 g, Lablemco powder (Oxoid) 10 g and sodium chloride 5 g per litre of distilled water, pH 7.5] to an OD₅₄₀ of 1.0, harvested and washed with 0.9% sodium chloride. Cells were stored frozen at -70 °C prior to use for either enzyme or tRNA isolation. For isolation of [³²P]tRNA, cells were grown in low-phosphate nutrient broth in the presence of carrier-free [³²P]orthophosphoric acid as already described (Ghosh & Guhathakurata, 1983).

Isolation of tRNA and diesterase-treated tRNA. Labelled and unlabelled tRNAs were isolated by the method of Ghosh & Guhathakurata (1983). To prepare defective tRNA, [³²P]tRNA was treated with snake venom phosphodiesterase to remove about 3–4 residues from the 3'-termini (Ghosh & Deutscher, 1978).

Assay of the nuclease activity. Nuclease activity was assayed in a final volume of 0.1 ml containing 20 mM Tris/HCl, pH 7.5, 1 mM MgCl₂ and 10 µg [³²P]tRNA or diesterase-treated tRNA. After incubation at 37 °C for 15 min, the reaction was stopped by addition of 0.3 ml carrier yeast RNA (1 mg ml⁻¹) and 0.4 ml 20% (w/v) trichloroacetic acid. After 30 min on ice, the mixture was centrifuged at 10000 g for 5 min. Radioactivity in 0.4 ml of the supernatant was measured in 10 ml Bray's scintillation fluid [2,5-diphenyloxazole (PPO) 4 g, (1,4 bis[2-(5-phenyloxazolyl)]-benzene; 2,2'-p-phenylene-bis[5-phenyloxazole] (POPOP) 0.2 g, naphthalene 60 g, methanol 100 ml, ethylene glycol 20 ml, dioxan 880 ml].

Polyacrylamide gel electrophoresis of tRNA. Electrophoresis of [³²P]tRNA was carried out on polyacrylamide gel slabs (40 cm × 20 cm × 0.1 cm) containing 10% (w/v) acrylamide, 0.5% N,N'-methylene bisacrylamide and 0.1% ammonium persulfate, as described by Peacock & Dingman (1968). Electrophoresis was carried out at 15 V cm⁻¹ for 8 h. Running buffer contained 89 mM Tris base, 89 mM boric acid, pH 8.2, and 2.5 mM EDTA. After electrophoresis, the gels were fixed in solution containing 10% (v/v) acetic acid and 10% (v/v) methanol, dried and autoradiographed.

Preparation of the cell-free extract and periplasmic supernatant. For routine assays, cells were grown to an OD₅₄₀ of 1.0 and harvested by centrifugation. After washing with saline, cells were resuspended in 0.1 vol. extraction buffer containing 20 mM Tris/HCl, pH 7.5, 1 mM MgCl₂, 1 mM DTT, 0.1 mM phenylmethylsulphonyl fluoride and 10% (v/v) glycerol. Aliquots of 5 ml suspension were sonicated with five 15 s pulses. This gave complete lysis: the OD₅₄₀ of the suspension decreased by about 95% for all the strains examined. The extract was then centrifuged at 100000 g for 1 h. The supernatant (S100) was directly used for the appropriate assay. Periplasmic supernatant was prepared as described by Roy *et al.* (1982b). All procedures were carried out at 4 °C.

All metabolic experiments were repeated five times and the data presented are means of these sets.

RESULTS AND DISCUSSION

Yield of tRNA from various strains of *V. cholerae*

It was observed in this laboratory that the yield of tRNA from *V. cholerae* is lower than that from *E. coli* cells (see Introduction). To confirm this observation, tRNA was

isolated from *V. cholerae* strains of various bio- and serotypes and with different levels of toxinogenicity grown to an OD₅₄₀ of 1.0. The yields were compared with that from *E. coli* cells as obtained by us and others (Zhang & Deutscher, 1988). *E. coli* cells were also grown under identical conditions. The yields of tRNA from all strains of *V. cholerae* tested were about 30% lower than those obtained from *E. coli* (Table 1). All *V. cholerae* strains tested, as well as the *E. coli* strains used, had similar growth rates, with a generation time of about 24 min.

Degradation of intact tRNA by cell-free extract from *V. cholerae*

The presumption from the aforementioned studies is that the tRNA may be degraded *in vivo* in *V. cholerae* cells, or the rate of tRNA biogenesis is very low in this organism. Considering the inherent stability of tRNA, it may be suggested that the 3'-ends of tRNA in *V. cholerae* undergo a very rapid turnover *in vivo* by RNase T-like (Deutscher *et al.*, 1985) activity. This would generate a high population of defective tRNA molecules which would be quickly degraded by RNase D-like activity present in the cytoplasm (Zhang & Deutscher, 1988). However, the possibility of the presence of an enzyme that degrades intact tRNA also cannot be ruled out.

To test these possibilities, extracts of *V. cholerae* 154 were incubated with tRNA from *E. coli* A19, which is known to contain intact tRNA molecules (Deutscher *et al.*, 1977), and with tRNA from *V. cholerae* cells. As can be seen in Table 2, both the tRNAs were degraded efficiently (about 25%) by the *V. cholerae* extract. Complete degradation could be achieved by using 10-fold concentrated extract (data not shown). This strongly suggests that *V. cholerae* cells contain an enzyme that can degrade intact tRNAs very efficiently. Extract from *E. coli* A19, which is known to lack such an activity (Ghosh & Deutscher, 1978) did not degrade the above tRNAs. This indicates that tRNAs from *V. cholerae* are not defective and are, therefore, not amenable to degradation by the RNase D that is present in

Table 1. Yield of tRNA from different strains of *E. coli* and *V. cholerae*

Strain	tRNA yield [mg (g wet wt) ⁻¹]	Relative yield (%)
<i>E. coli</i> A19	2.0	100
<i>E. coli</i> C-600	2.0	100
<i>V. cholerae</i> 569B*	1.5	75
<i>V. cholerae</i> 154†	1.45	72
<i>V. cholerae</i> Mak 757‡	1.42	71
<i>V. cholerae</i> EW6§	1.45	72

* Classical biotype; serotype Inaba; highly toxinogenic.

† Classical biotype; serotype Ogawa; mildly toxinogenic.

‡ El Tor biotype; serotype Ogawa; mildly toxinogenic.

§ El Tor biotype; serotype Ogawa; non-toxinogenic.

Table 2. Degradation of tRNA by extracts of *V. cholerae* and *E. coli* A19

³²P-labelled intact or diesterase-treated tRNAs from *V. cholerae* (VC) or *E. coli* (EC) were incubated with cell-free extracts (equivalent to 50 µg protein) from these cells at 37 °C for 15 min under standard assay conditions. Acid-soluble radioactivity was determined as described in Methods. The degradation was linear during the entire incubation time for each experiment.

Substrate	Percentage degradation by extracts from:	
	<i>V. cholerae</i>	<i>E. coli</i>
Intact tRNA (VC)	20	1.8
Defective tRNA (VC)	23.2	6.3
Intact tRNA (EC)	22.8	0.78
Defective tRNA (EC)	24.5	7.6

Fig. 1. tRNA content of rifampicin-treated *V. cholerae* (●) and *E. coli* A19 (□) cells. Cells were grown to an OD₅₄₀ of 0.2. Rifampicin (20 µg ml⁻¹) was then added to each flask. After varying periods of incubation at 37 °C, samples were removed for tRNA isolation. An untreated *V. cholerae* culture served as control (○). The bars represent SD.

E. coli. Defective tRNAs produced by treatment with snake venom phosphodiesterase are degraded by *E. coli* cells (Ghosh & Deutscher, 1978), and more rapidly by *V. cholerae* cells. Periplasmic supernatant of *V. cholerae* cells failed to hydrolyse any of the above tRNAs to an appreciable extent, whereas [³²P]tRNA or [³H]poly(A) was easily digested. This indicates that although the periplasm of *V. cholerae* contains a ribonuclease, the activity cannot degrade intact tRNA molecules (T. Gangopadhyay & R. K. Ghosh, unpublished observation). The enzyme(s) that degrades tRNA must therefore be located in the cytoplasm. It is important to mention that it is not yet known whether the enzyme is specific for tRNA; establishing this must await purification of the enzyme. Use of *E. coli* C600 instead of *E. coli* A19 gave identical results. Hence, in all subsequent experiments *E. coli* C600 was used.

Degradation of tRNA in rifampicin-treated cells

To ascertain the contribution of the putative tRNA-degrading activity to the observed lower yield of tRNA, we made use of the antibiotic rifampicin, which is known to abolish tRNA synthesis. Incubation of *V. cholerae* cells with this antibiotic for various lengths of time and determination of the tRNA content under each of these conditions should give information about the *in vivo* role of the degrading enzyme(s). *V. cholerae* 154 cells grown to mid-exponential phase were incubated for 2 h in the presence of rifampicin. Samples were withdrawn at various times for isolation of tRNA. As can be seen in Fig. 1, the amount of tRNA in *V. cholerae* cells decreased continually over a period of 2 h. The amount of tRNA recovered from *E. coli* cells treated identically remained unchanged for 4 h, a phenomenon which has also been reported by others (Yuan & Shen, 1975; Nozawa *et al.*, 1967; Kennell & Kotoulas, 1967; Hou *et al.*, 1966; Deutscher *et al.*, 1977). Cells of both the strains did not lyse at the concentration of rifampicin (20 µg ml⁻¹) used. Untreated *V. cholerae* cells also maintained a steady-state level of tRNA throughout the period of incubation. This strongly suggests that the steady-state level (about 1.45 mg g⁻¹) of *V. cholerae* tRNA is a balance between net synthesis and breakdown; when synthesis is arrested, the tRNA level decreases due to the breakdown of tRNAs by the putative enzyme(s).

Fate of newly synthesized tRNA in *V. cholerae* cells

It may be argued that rifampicin treatment has an adverse effect on the metabolic environment such that tRNAs become accessible to various nucleases. In normal cells, these molecules may be separated from the enzyme(s) that degrade(s) tRNAs. We thus examined the fate of newly synthesized tRNA molecules in *V. cholerae* cells under normal growth conditions.

V. cholerae 154 cells were grown to mid-exponential phase in low-phosphate medium. The cells were labelled with [³²P]orthophosphoric acid for 30 min, then washed and transferred to prewarmed high-phosphate medium without [³²P]orthophosphoric acid and incubated as usual. The [³²P]tRNA of these cells was then chased for 30 min. Around 80% of the label in *V. cholerae* tRNA disappeared during the 30 min chase while that of *E. coli* tRNA remained unchanged under identical conditions (Fig. 2a). These results strongly suggest that tRNA molecules in *V. cholerae* undergo very rapid turnover: the average half life of tRNA was 11.8 min. The rate of tRNA degradation was calculated to be 0.0827 mg min⁻¹ (g wet wt)⁻¹ [rate = 1.45 - 1.45 × 2^{-1/11.8}, where tRNA per unit biomass = 1.45 and half life of tRNA = 11.8 min]. From the logarithmic plot (Fig. 2a, inset) the decay rate constant was found to be 0.0589 min⁻¹. That the observed degradation was solely due to tRNA was confirmed by running aliquots of tRNA after 0 min and 30 min chase on polyacrylamide gel followed by autoradiography. As expected, the bands due to tRNA almost disappeared in 30 min for *V. cholerae* cells (Fig. 2b). The specific activity of tRNA [c.p.m. (mg tRNA)⁻¹] decreased from 7–8 × 10⁷

Fig. 2. (a) Fate of newly synthesized tRNA in *V. cholerae* (○) and *E. coli* C600 (●) cells. Cells were grown to an OD_{540} of 0.2 in 25 ml low-phosphate medium. Each flask then received 1 mCi (37 MBq) carrier-free ^{32}P orthophosphate. After 30 min incubation at 37 °C, cells were harvested and transferred to 25 ml nutrient broth (high phosphate). After varying periods of incubation at 37 °C, samples were withdrawn for tRNA isolation. The total amount of ^{32}P tRNA present in each of these isolates was determined. The bars represent SD. The inset is a logarithmic plot against time. (b) Autoradiograph of newly synthesized tRNA in samples from 0 min and 30 min chase of the experiment in (a). Total tRNA preparation from 25 ml culture was dissolved in a final volume of 1 ml, and 10 μl used for each time point. Each set contained equal amounts of A_{260} material.

to $5\text{--}7 \times 10^6$ in 30 min. As there was almost equivalent disappearance of all bands it may be concluded that the putative enzyme(s) do(es) not discriminate between various tRNA species. tRNA from *E. coli* cells treated identically remained unchanged as expected (Fig. 2a, b).

The results presented above indicate that tRNA in *V. cholerae* undergoes very rapid turnover *in vivo* – a phenomenon so far unknown in any other prokaryote. The mechanism of rapid *in vivo* degradation of tRNA in *V. cholerae* is very difficult to speculate upon at this stage; its elucidation must await purification of the putative degrading activities.

The observed rapid turnover of tRNAs in *V. cholerae* cells would call for an enhanced rate of tRNA biogenesis to maintain the steady-state intracellular level of

$1.45 \text{ mg (g wet wt)}^{-1}$. In the absence of any degradation this steady-state level would have required a synthesis rate of $0.0423 \text{ mg min}^{-1} \text{ (g wet wt)}^{-1}$ [rate = $1.45 \times 2^{1/24} - 1.45$] for a generation time of 24 min. Compensating for the observed rate of decay [$0.0827 \text{ mg min}^{-1} \text{ (g wet wt)}^{-1}$ of the cell; Fig. 2a] the actual rate of tRNA synthesis in *V. cholerae* would be $0.125 \text{ mg min}^{-1} \text{ (g wet wt)}^{-1}$. The rate of tRNA biogenesis in *E. coli* cells grown under identical conditions would be $0.0584 \text{ mg min}^{-1} \text{ (g wet wt)}^{-1}$ [rate = $2 \times 2^{1/24} - 2$; where tRNA yield per biomass was $2 \text{ mg (g wet wt)}^{-1}$ and generation time was 24 min]. Thus, *V. cholerae* cells apparently synthesize tRNA at a rate about 2.5 times that of *E. coli* cells.

The rapid degradation of tRNA and its enhanced rate of synthesis raise a couple of interesting points. First, why the cell should adopt such an uneconomic process is difficult to understand. Isolation and analysis of mutants deficient in this enzyme(s) should throw some light on its significance. Second, rapid synthesis implies a high rate of transcription of tRNA gene clusters as well as the presence of an elevated level of tRNA-processing activity in *V. cholerae* cells.

V. cholerae is the host for a large number of lytic phages. Like T_4 and T_5 phages of *E. coli*, two of the cholera phages, namely phages $\phi 149$ and e_4 , induce synthesis of phage-coded tRNA molecules upon infection (Chattopadhyay & Ghosh, 1988a; Mandal & Ghosh, 1988). With respect to the *E. coli* phages it was suggested that phage-coded tRNAs are not essential for phage development as phage mutants lacking tRNA genes are viable (Wilson *et al.*, 1972). However, in view of the rapid turnover of host tRNA in *V. cholerae*, the tRNA genes of cholera phages may play a very significant role in phage development.

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