Metabolic Control of *Klebsiella pneumoniae* mRNA Degradation by the Availability of Fixed Nitrogen

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The chemical and functional stability of *Klebsiella pneumoniae* mRNAs was dependent on the nitrogen status of the organism. During exponential growth on N_2 or NH_4^+ as N-source the half-lives of mRNAs were 10 and 4 min, respectively. When NH_4^+ -grown cells were starved of NH_4^+ for 3 h, under which conditions the nitrogen fixation (*nif*) genes were derepressed, the half-life of mRNAs increased to 20 min. Addition of NH_4^+ to such N-starved suspensions rapidly destabilized mRNAs with a resultant half-life of 9 min. This destabilization occurred even in the presence of transcription inhibitors, which indicated metabolic control of mRNA degradation. Under most conditions studied the functional decay of *nif*-specific mRNAs paralleled the decay of bulk mRNAs.

INTRODUCTION

Prokaryotic messenger RNAs are generally unstable with half-lives ranging from 1.5 to 5 min (Schlessinger, 1972). This contrasts with the known stability of various eukaryotic mRNAs and is believed to facilitate the rapid adaptation of bacteria to abrupt changes in their environment. The occurrence of more stable mRNAs in prokaryotes has nevertheless been reported (Pollock, 1963; Lindahl & Forchhammer, 1969; Hirashima & Inouye, 1973; Ikemura *et al.*, 1979), particularly under conditions of energy source shift down (Jacobson & Jen-Jacobson, 1980) or amino acid starvation (Forchhammer & Kjeldgaard, 1967; Leal, 1976; Sanchez de Rivas & Mendez, 1977). In *Escherichia coli* mRNA half-life remains constant at growth rates between 0.6 and 2.5 doublings h^{-1} (Pato & von Meyenburg, 1970). Here we report that in the closely related bacterium *Klebsiella pneumoniae*, mRNA half-life changed dramatically between growth rates of 0.23 and 0.63 doublings h^{-1} .

When nitrogen becomes growth limiting for free-living N_2 -fixing bacteria, nitrogenase is derepressed (Brill, 1979). N-starvation causes a severe metabolic stress that the cell must overcome in order to synthesize nitrogenase and resume growth. Important physiological changes are therefore expected to occur. We and others have observed a stringent response of *K. pneumoniae* under N-starvation (Kleiner & Phillips, 1980; Riesenburger & Kari, 1981; D. Kahn, M. Hawkins & R. R. Eady, unpublished observations). Under these conditions there is a rise of the ppGpp pool and a decrease in the rate of stable RNA synthesis. Here we report that during N-starvation, mRNAs in *K. pneumoniae* were unusually stable, and present data indicating that mRNA degradation was under metabolic control. The effect of N-status on the stability of mRNAs exhibited by non-growing suspensions was also shown by exponentially growing cells. N_2 -grown cultures of *K. pneumoniae* possessed more stable mRNAs than NH_4^+ -grown cultures. The *nifHDK* and *nifJ* mRNAs exhibited a similar behaviour to bulk mRNAs when *K. pneumoniae* was shifted from N-starvation to N-sufficiency. A preliminary report of some of this work was given by Eady *et al.* (1981).

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METHODS

Bacterial growth. *Klebsiella pneumoniae* strain M5a1 was grown anaerobically on ammonium (as $1.5 \text{ g } (\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$) or on N_2 in N-free medium (NFD) (Cannon *et al.*, 1974) at 30°C . N-starved suspensions were prepared by collecting an NH_4^+ -grown stationary culture by centrifugation, resuspending it in NFD plus sodium aspartate (0.1 g l^{-1}) under argon, and incubating it at 30°C with shaking in a flask with a rubber closure; mRNA stability experiments were performed 3 h later, at which time *nif* derepression was in the steady state. All the cultures contained between 0.1 and $0.2 \text{ g protein l}^{-1}$.

Measurement of mRNA stability. (a) *Chemical decay.* RNAs were labelled *in vivo* by supplementing cultures with $2 \mu\text{M}$ - $[^3\text{H}]$ uridine ($5\text{--}10 \mu\text{Ci ml}^{-1}$). After a suitable time interval ($1\text{--}7$ min depending on conditions) proflavine ($50 \mu\text{g ml}^{-1}$) was added to stop further transcription and 0.5 ml samples were removed at intervals for determination of tritiated RNA as described by Bremer & Yuan (1968). For analysis, unstable RNA was obtained by subtracting the final plateau value corresponding to stable RNA. A semi-log replot of unstable RNA as a function of time gave the chemical half-life of unstable RNA. The efficiency of proflavine as a transcription inhibitor was verified in a control experiment.

(b) *Functional decay.* Rifampicin (0.2 g l^{-1}) was added to the culture to stop the initiation of transcription and 1 ml samples were removed at intervals and pulse-labelled for 2 min at 30°C with $2.5 \mu\text{Ci}$ (92.5 kBq) ^{14}C -labelled amino acids (specific activity $1.85 \text{ GBq milliatom}^{-1}$) (CFB104, Amersham). Protein synthesis was stopped with chloramphenicol (0.1 g l^{-1}) and samples were kept on ice; cells were collected by centrifugation, resuspended in 0.1 ml ORTEC sample buffer (Cannon, 1980) and left for 4 min in a boiling water bath. Debris was sedimented by centrifugation and $10 \mu\text{l}$ portions from the supernatants assayed for radioactive TCA-precipitable material. This measured the rate of protein synthesis. A semi-log plot of the rate of protein synthesis versus time gave the functional half-life of mRNA. The efficiency of rifampicin as a transcription inhibitor was verified in a control experiment.

(c) *Functional decay of *nif* specific mRNAs.* ^{14}C pulse-labelled proteins were prepared as described above and analysed for *nif*-specific polypeptides by SDS-PAGE (Laemmli, 1970). The gel was dried, autoradiographed and the film scanned (wavelength 440 nm , slit 0.1 mm). The peaks corresponding to *nif*-specific polypeptides were integrated, allowing quantification of their rates of synthesis (Merrick *et al.*, 1978; Elmerich *et al.*, 1978; Roberts *et al.*, 1978). A semi-log plot of their rates of synthesis versus time gave the functional half-lives of *nif* mRNAs.

RESULTS

Influence of N-status on the chemical stability of K. pneumoniae mRNAs

During exponential NH_4^+ -supported growth (growth rate, $\mu = 0.63 \text{ h}^{-1}$), mRNAs turned over rapidly, with a half-life of $4 \pm 1 \text{ min}$ (Fig. 1a, d). If an NH_4^+ -grown culture was N-starved, mRNAs proved much more stable. After 3 h of N-starvation, mRNAs turned over with a half-life of $20 \pm 2 \text{ min}$ (Fig. 1b, e). Control cell suspensions treated similarly, but not subjected to N-starvation, showed the usual short half-life of mRNAs characteristic of NH_4^+ -grown cultures (data not shown). During exponential N_2 -supported growth ($\mu = 0.23 \text{ h}^{-1}$), mRNA stability was intermediate (half-life $10 \pm 1 \text{ min}$) between that observed during conditions of N-starvation and in NH_4^+ -supported growth (Fig. 1c, f).

Influence of N-status on the functional stability of K. pneumoniae mRNAs

The functional half-lives of mRNAs of N_2 -grown, NH_4^+ -grown and N-starved cultures were obtained as described above (Fig. 2). They were in good agreement with the corresponding chemical half-lives (Table 1).

Influence of added NH_4^+ on mRNA stability in N-deprived cultures

Since we observed significant differences in the stability of mRNAs in N-starved suspensions and NH_4^+ -grown cultures of *K. pneumoniae*, the question arose as to how the organism would respond to a change of N-status. To investigate this we labelled an N-starved suspension for 5 min with $[^3\text{H}]$ uridine and then added NH_4^+ together with proflavine to stop further transcription. Samples were removed at intervals for measurement of tritiated RNA (Fig. 3). The half-life of mRNAs was then $9 \pm 1 \text{ min}$.

Similar experiments using pulse-labelling of proteins with ^{14}C -labelled amino acids after the addition of rifampicin and NH_4^+ yielded a functional half-life of $11 \pm 1 \text{ min}$ (Fig. 4b). Thus,

Fig. 1. Chemical stability of mRNAs in *K. pneumoniae* under conditions of different N-status. Bacteria were grown anaerobically at 30 °C with either N₂ or NH₄⁺ as N-source as indicated. N-starved suspensions were prepared by harvesting an NH₄⁺-grown stationary culture as described in Methods, and RNA stability was determined after 3 h of N-starvation. RNA was labelled *in vivo* with 2 µM-[³H]uridine. At the end of the labelling period proflavine was added to stop transcription and samples were removed at intervals for estimation of ³H-labelled RNA as described by Bremer & Yuan (1968). (a) NH₄⁺-grown culture; (b) N-starved organisms; (c) N₂-grown culture; curves (d), (e) and (f) are the respective semi-logarithmic plots of the unstable RNA under these conditions.

addition of NH₄⁺ to an N-starved suspension of *K. pneumoniae* resulted in a rapid acceleration of mRNA degradation. This occurred in the absence of *de novo* RNA synthesis. Hence, mRNA degradation was controlled by the metabolic status of *K. pneumoniae* as determined by the availability of fixed nitrogen.

Functional stability of *nif* mRNAs during nitrogen starvation

In *K. pneumoniae* the *nifHDKY* operon codes for the nitrogenase polypeptides and the *nifY* product of unknown function (Klipp & Pühler, 1981). The *nifJ* gene encodes a protein of molecular weight 120 000 (Merrick *et al.*, 1978; Elmerich *et al.*, 1978; Roberts *et al.*, 1978). The polypeptides encoded by *nifH*, *nifD*, *nifK* and *nifJ* are readily detected on electrophoretograms. Protein pulse-labelling followed by SDS-PAGE and autoradiography allows quantification of the coding capacity for the polypeptides encoded by these genes (Fig. 4a). After the addition of rifampicin, the rates of synthesis of these polypeptides decayed with the half-lives shown in Table 1. During N-starvation, the *nifHDK* and *nifJ* mRNAs turned over at the same rate as bulk mRNAs with functional half-lives near 23 min. Upon addition of NH₄⁺, with or without rifampicin, these mRNAs decayed at an accelerated rate with a half-life of 5–12 min (Fig. 4b and Table 1). Thus addition of NH₄⁺ to suspensions of *K. pneumoniae* during *nif* derepression

Fig. 2.

Fig. 3.

Fig. 2. Functional stability of mRNAs in *K. pneumoniae* under conditions of different N-status. Initiation of transcription was inhibited by the addition of rifampicin, and at various times samples were removed and pulse-labelled for 2 min with a mixture of ^{14}C -labelled amino acids. TCA-precipitable radioactivity was determined as described in Methods. Semi-logarithmic plots of the rate of protein synthesis in NH_4^+ -grown cultures ($\blacktriangle$), N-starved organisms ($\circ$) and N_2 -grown cultures ($\bullet$) were used to estimate the functional half-life of bulk mRNA.

Fig. 3. Effect of added NH_4^+ on the stability of mRNA in N-starved *K. pneumoniae*. After 3 h N-starvation of a suspension of *K. pneumoniae*, $(\text{NH}_4)_2\text{SO}_4$ (1.5 mg ml^{-1}) was added together with proflavine or rifampicin, and the chemical ($\bullet$) and functional ($\blacksquare$) stabilities of bulk mRNAs determined as described in the legends of Fig. 1 and Fig. 2. The half-lives for mRNA stability so obtained are compared with untreated N-starved suspensions in Table 1.

Table 1. *Half-lives of mRNAs from K. pneumoniae under various physiological conditions*

K. pneumoniae strain M5a1 was grown or derepressed for *nif* genes as described in the legend to Fig. 1. Chemical and functional half-lives of bulk mRNA were measured from the decay curves shown in Figs 1–3 and 4(b). Functional decay of *nif*-specific mRNAs was measured using rifampicin as described in the legend to Fig. 4.

Condition	Chemical half-life (min) Bulk mRNA	Functional half-life (min)			
		Bulk mRNA	<i>nifH</i>	<i>nifDK</i>	<i>nifJ</i>
NH_4^+ -growth	4 ± 1	5 ± 1	—	—	—
N-starved (3 h)	20 ± 2	23 ± 2	21 ± 2	25 ± 2	25 ± 2
NH_4^+ added after 3 h N-starvation	9 ± 1	11 ± 1	11 ± 2	12 ± 2	5 ± 1
N_2 -growth	10 ± 1	12 ± 1	—	—	—

stopped *nifHDK* and *nifJ* transcription and accelerated the degradation of *nifHDK* and *nifJ* mRNAs, even in the absence of *de novo* RNA synthesis.

DISCUSSION

The stability of *K. pneumoniae* mRNAs was inversely related to the availability of combined nitrogen, and mRNA degradation rates rapidly responded to a change of N-status, independently of transcription. Induction of a new operon was not required to increase the rate of mRNA degradation after a shift from nitrogen-starvation to nitrogen-sufficiency. Messenger RNA degradation was therefore controlled by the metabolic status of *K. pneumoniae*. This result

Fig. 4(a)

Fig. 4(b)

Fig. 4. Functional decay of *nif*-specific mRNAs following the addition of NH_4^+ and rifampicin to N-starved *K. pneumoniae*. A culture of *K. pneumoniae* was derepressed for *nif* as described in Methods. After 3 h N-deprivation $(\text{NH}_4)_2\text{SO}_4$ and rifampicin were added and the rate of protein synthesis determined from pulse-labelling with ^{14}C -labelled amino acids as described in Methods. Samples were electrophoresed and the gels autoradiographed to allow identification of the *nifHDK* and *J* products (a). The intensity of these peaks was determined by microdensitometry and the peaks corresponding to Kp1 (■), Kp2 (□) and *J* (△) integrated. Semi-logarithmic plots (b) of these data were used to determine the functional half-life of these *nif* mRNAs.

agrees with the hypothesis that macromolecular syntheses and degradation are under metabolic control, that is, are regulated by the concentration of some specialized metabolites (Tomkins, 1975; Danchin, 1979). The half-life of *K. pneumoniae* mRNA changed dramatically between growth rates of 0.23 and 0.63 doublings h^{-1} . This result conflicts with an earlier concept that mRNA turnover does not change with growth rate (Pato & von Mayenberg, 1970). However, these authors had noticed a possible trend towards longer half-lives at growth rates below 0.6 doublings h^{-1} , a trend which we confirm in the case of *K. pneumoniae*.

Amino acid starvation elicits a stringent response of *K. pneumoniae* with a rise in ppGpp concentration (Riesenburger & Kari, 1981; D. Kahn, M. Hawkins & R. R. Eady, unpublished observations). The level of ppGpp is significantly higher in N_2 -grown cells than in NH_4^+ -grown cells (Kleiner & Phillips, 1980). Levels of ppGpp, like mRNA stability, depend on the N-status of *K. pneumoniae*. This does not necessarily imply that ppGpp controls the rate of mRNA degradation, since other parameters including energy charge and the ADP/ATP ratio show significant differences between N_2 - and NH_4^+ -supported growth (Upchurch & Mortenson, 1980; Marriot *et al.*, 1981). The function of ppGpp in stringent control itself is presently questioned (Spadaro *et al.*, 1981). Therefore this topic requires further investigation.

This study bears particular relevance to the understanding of *nif* derepression in *K. pneumoniae*. Although all *nif* mRNAs have been initiated 20 min after NH_4^+ deprivation, nitrogenase activity does not appear until 60 min later (Collmer & Lamborg, 1976). Nitrogenase is a complex enzyme that consists of two components, the Mo-Fe protein (Kp1) and the Fe protein (Kp2) (Eady & Smith, 1979). Nitrogenase polypeptides are not active *per se*, but are activated in processes involving several genes including *nifB*, *nifN* and *nifE* for the synthesis or the insertion of the Fe-Mo cofactor of Kp1, and *nifM* for the activation of Kp2 (Roberts *et al.*, 1978). Moreover, a *nif*-specific electron transport chain is necessary for nitrogenase activity in

vivo (Hill & Kavanagh, 1980). The complete system is apparently built up in a slow fashion. In this context, one understands the need for more stable mRNAs if nitrogenase is to be expressed and N-starvation overcome.

The stability of *nifHDK* and *nifJ* mRNAs paralleled the stability of bulk mRNAs. Thus, their degradation was accelerated upon a shift from N-starvation to N-sufficiency. Functionally, *nifJ* mRNA appeared to be degraded faster (half-life 5 ± 1 min) than *nifHDK* mRNA in the presence of NH_4^+ (half-life 11–12 min). Our experiments could not rule out the possibility of some translational control of *nif* expression after the addition of NH_4^+ .

While this paper was in preparation, two reports provided data on the unusual stability of *nif* mRNAs. The first report (Houmard & Bogusz, 1981) dealt with the functional stability of *nifHDK*, *nifJ*, *nifU* and *nifF* mRNAs during growth of *K. pneumoniae* on N_2 . Although these authors did not quantify the stability of *nif* mRNAs, they showed that in their conditions (growth on N_2) the *nif* mRNAs studied are more stable than bulk mRNAs. We did not measure the functional stability of *nif* mRNAs under their conditions. In our conditions, however (N-starvation without growth), the *nif* mRNAs studied were as stable as bulk mRNAs. This discrepancy is not understood, but since we have shown that the stability of mRNAs depends on the N-status of the culture, these differences may have arisen from the use of cultures with different N-status, or from the use of a relatively long pulse-labelling time (15 min) by these workers. The second paper (Kaluza & Hennecke, 1981) reported both chemical and functional half-lives of *nifHDK* mRNA. They determined the stability of this mRNA directly by hybridization to the plasmid pSA30 which carried *K. pneumoniae nifH*, *D*, and *K* genes (Cannon *et al.*, 1979). The data they obtained using NH_4^+ -limited batch cultures in the early stages of *nif* derepression are in good agreement with the data reported here. In this report we present further evidence extending their findings to *nifJ* mRNA and bulk mRNAs.

This work and a previous paper (Kahn *et al.*, 1982) allow us to propose a new interpretation of the data of Collmer & Lamborg (1976). These authors compared the timings of chloramphenicol and rifampicin inhibition of nitrogenase derepression to evaluate the length of the last *nif* operon expressed after ammonium starvation. They deduced it to be 20000 nucleotides long. Since no *nif* operon exceeds 6000 nucleotides (Riedel *et al.*, 1979), 'transcriptional mapping' fails in the case of *nif*. One reason is that chloramphenicol inhibits the activation of Kp1 (Kahn *et al.*, 1982), the rate-limiting process for the synthesis of active nitrogenase during early derepression (R. R. Eady, unpublished results). Moreover, *nif* specific products involved in the activation of nitrogenase have to build up before nitrogenase activity can appear. Thus, one should not use 'transcriptional mapping' whenever polypeptides under study are not active *per se* and undergo some activation process. Collmer & Lamborg (1976) also demonstrated that NH_4^+ curtails nitrogenase synthesis faster than rifampicin. They postulated a new target for the regulation of *nif* by NH_4^+ at a locus other than the operator. Here we show that NH_4^+ accelerates the degradation of mRNAs, including *nif* mRNAs. Since prolonged *nif*-specific syntheses are required before nitrogenase activity can appear, premature repression of *nif* by NH_4^+ followed by rapid mRNA degradation will prevent the appearance of nitrogenase activity. This accounts for the rapid arrest of nitrogenase synthesis after the addition of NH_4^+ compared with rifampicin. In the case of *nifHDK*, expression is regulated solely by transcriptional control (Kaluza & Hennecke, 1981).

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