Nitrogenase Synthesis in *Klebsiella pneumoniae*: Enhanced *nif* Expression without Accumulation of Guanosine 5'-Diphosphate 3'-Diphosphate

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Derepression of nitrogen fixation (*nif*) genes in *Klebsiella pneumoniae* following transfer from NH₄⁺-sufficiency to N-free medium was preceded by rapid expansion of the guanosine 5'-diphosphate 3'-diphosphate (ppGpp) pool. When derepressed in N-free medium supplemented with glutamine (600 μg ml⁻¹), expression from the *nifH* and *nifL* promoters, determined as β-galactosidase activity in *nif*: *lac* merodiploid strains, was stimulated 7-fold and nitrogenase activity 26-fold; ppGpp did not accumulate, remaining at the levels found in NH₄⁺-repressed populations. The relaxed mutant *K. pneumoniae* relA40, which accumulates only very low levels of ppGpp, showed partial derepression of nitrogenase activity in the presence of glutamine, thus ppGpp is unlikely to be an effector of *nif* expression. ATP and GTP levels were elevated under conditions where *nif* expression was enhanced, consistent with previous data suggesting that maintenance of ATP levels is a prerequisite for the expression of *nif* genes in *K. pneumoniae*.

INTRODUCTION

Following transfer from growth on NH₄⁺ to growth on N₂, anaerobic cultures of *Klebsiella pneumoniae* show the stringent response characteristic of N-starved organisms. Stringent control is well studied in *Escherichia coli* (Gallant, 1979), in which deprivation of a required amino acid abruptly curtails RNA accumulation, increases the rate of protein turnover and arrests net synthesis of glycolytic intermediates, lipid, phospholipid and peptidoglycan (Cashel, 1975; Gallant & Lazzarini, 1976). These metabolic changes are accompanied by the accumulation of two nucleotide polyphosphates: guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp) (Cashel & Gallant, 1969). ppGpp is known to have various regulatory effects central to the stringent response. Apart from its effects on the activities of metabolic enzymes (Gallant, 1979), it also inhibits transcription of stable RNA species (rRNA and tRNA), by affecting the conformation and specificity of RNA polymerase (Travers et al., 1981; Travers et al., 1982), and stimulates the transcription of a number of operons, e.g. *lac* (Gallant & Lazzarini, 1976), *his* and *trp* (Gallant, 1979).

The role of ppGpp as a unique effector molecule has been questioned. A lack of correlation between stringent response and an increase in the intracellular concentration of ppGpp has been reported (Hansen et al., 1975; Donini et al., 1978; Pao & Gallant, 1979; Pao & Dyess, 1981; Spadaro et al., 1981; Belitsky & Kari, 1982). However, this interpretation has been criticized by Ryals et al. (1982a, b) on the grounds that the methods used to measure RNA synthesis were not rigorous enough.

On starvation for fixed N, N₂-fixing bacteria respond with changes in ppGpp levels (Kleiner & Philips, 1981), a decrease in the intracellular level of amino acids (Drozd et al., 1972; Ohmori & Hattori, 1974; Kleiner, 1979; Friga et al., 1981) and the stabilization of mRNA (Kahn et al., 1982a). Under these conditions the nitrogen fixation genes (*nif*) are derepressed.

In *K. pneumoniae*, regulation of *nif* expression by fixed N is complex, occurring at two levels (for brief reviews see Merrick, 1982; Dixon et al., 1984; de Bruijn et al., 1984). The first involves a centralized *ntr* system which exerts a general control on nitrogen metabolism and is mediated by the *ntrA* (*glnF*), *ntrB* (*glnL*) and *ntrC* (*glnG*) gene products; the second involves a *nif* specific
regulation by the product of \(nifA\) (positive effector) and the product of \(nifL\) (negative effector). The \(nifLA\) promoter itself is regulated by the \(ntr\) system, and is the primary target for regulation of \(nif\) expression in response to available fixed N (Drummond et al., 1983).

Although a model for \(nif\) regulation has been developed, the underlying physiological basis is poorly understood. Studies with mutants unable to assimilate added \(NH_4^+\) into glutamine, or inhibition of this process by methionine sulfoximine (MSX), have shown that repression of \(nif\) by \(NH_4^+\) requires its assimilation into glutamine (Gordon & Brill, 1972).

As with various other operons, ppGpp has been implicated in the regulation of \(nif\) expression in \(K. pneumoniae\) for two reasons. Firstly, relaxed mutants unable to synthesize ppGpp do not derepress nitrogenase (Reisenberg et al., 1982). Secondly, in wild-type \(K. pneumoniae\) as in \(Clostridium pasteurianum, Azotobacter vinelandii\) and \(Rhodopseudomonas palustris\), the appearance of nitrogenase activity is preceded by expansion of ppGpp pools, and repression by added \(NH_4^+\) is accompanied by a rapid concomitant decrease in ppGpp levels (Kleiner & Philips, 1981; Zumft & Neumann, 1983). However, in \(K. pneumoniae\), provision of fixed nitrogen as amino acids does not always repress \(nif\) expression (Shanmugam & Morandi, 1976) and in particular, glutamine has a differential repressive effect on the \(nifL\) and \(nifH\) promoters (Merrick et al., 1982).

We have investigated the correlation between ppGpp levels and both the extent of transcription of \(nifH\) and \(nifL\) promoters, and the nitrogenase activity, in \(K. pneumoniae\) during derepression under both relaxed and stringent conditions, generated by the presence or absence of glutamine in the medium. Our data show that the levels of ppGpp do not correlate with the extent of \(nif\) derepression. A preliminary report of some of this work was given by Nair & Eady (1984). Also our results are consistent with a previous report (Jensen & Kennedy, 1982) that ATP has a role in the regulation of \(nif\) expression.

**METHODS**

**Organisms and growth conditions.** Three \(K. pneumoniae\) strains were used: the wild-type M5a1, UNF619(pMF183) [\(nif^- \Delta lac/nifH 2783::Mu(Ap lac)\)] and UNF619(pMF182) [\(nif^- \Delta lac/nifL 2782::Mu(Ap lac)\)]. The \(nif-lac\) fusion strains are both merodiploids carrying the \(lacZ\) gene (encoding \(\beta\)-galactosidase) fused in \((nifH)\), the structural gene for the Fe protein of nitrogenase, or \(nifL\), the \(nifL\) product being the negative effector of other operons in the \(nif\) regulon (Dixon et al., 1980). The relaxed mutant \(K. pneumoniae relA40\) was a gift from Dr Casba Kari (Institute of Genetics & Biochemistry, Szeged, Hungary).

Cultures in low phosphate medium were grown anaerobically sparged with \(N_2\) in NFDM (Cannon et al., 1974), modified by replacement of the phosphate buffer with 100 mM-MOPS pH 7.2, and supplemented with 0.2 mM-HPO\(_4^{2-}\) and 1.3 mM-KCl. \((NH_4)_2SO_4\) (15 mM) was added as N source to repress \(nif\) expression. At the end of exponential growth, organisms were harvested by centrifugation.

For derepression of nitrogenase, organisms were resuspended at approx. 200 \(\mu\)g protein ml\(^{-1}\) in 30 to 40 ml low phosphate medium, either N-free or containing glutamine (600 \(\mu\)g ml\(^{-1}\)), and incubated under \(N_2\) at 30 °C. These organisms were used as parallel unlabelled cultures for the measurement of nitrogenase activity. ATP levels, \(\beta\)-galactosidase activity and glutamine uptake as described below.

\[^{32}\text{P}\]Orthophosphate labelling of nucleotides. In order to equilibrate the nucleotide pools with added isotope before \(NH_4^+\) deprivation. 1 ml of repressed culture was harvested by centrifuging in an Eppendorf centrifuge, re-suspended in low phosphate medium containing 15 mM-(\(NH_4\))\(_2SO_4\) and carrier-free \[^{32}\text{P}\]orthophosphate [final sp. act. 1 Ci mmol\(^{-1}\), 200 \(\mu\)Ci ml\(^{-1}\) (1 Ci = 37 GBq)] and shaken for 120 to 150 min under \(N_2\). Cultures were then harvested rapidly and re-suspended in derepressing medium, with or without glutamine, as described above for the parallel cultures, except that the medium contained 32\(P\) (1 Ci mmol\(^{-1}\), 200 \(\mu\)Ci ml\(^{-1}\)).

For estimation of ppGpp and other nucleotides, samples (20 \(\mu\)l) were removed at intervals both before and after \(NH_4^+\) deprivation and rapidly mixed with an equal volume of 2 M ice-cold formic acid (Chaloner-Larson & Yamazaki, 1976). Extracts were analysed, either immediately or after storage at \(-20\) °C up to 3 d, by thin layer chromatography on polyethyleneimine cellulose plates (Polygram Cel 300, Machery Nagel, Düren, FRG). The solvent system was 1:5 M-KH\(_2\)PO\(_4\) for one-dimensional chromatography, and 1:5 M-\(LiCl\) in 2 M-HCOOH in the first dimension and 1:5 M-KH\(_2\)PO\(_4\) (pH 3.4) in the second dimension for two-dimensional chromatography (Gallant et al., 1976).

ppGpp, pppGpp, GTP and ATP were located by autoradiography, identified by co-migration of added authentic carrier nucleotides detected by quenching of fluorescence under UV light, and quantified by scintillation counting.

**Enzymic estimation of ATP.** In addition to the radiochemical method, ATP was also estimated from unlabelled parallel cultures by bioluminescence assay using the firefly luciferin–luciferase system. Culture samples (1 ml)
were withdrawn at intervals and mixed quickly into an equal volume of 10% TCA/4 mM-EDTA mixture with vigorous shaking under N₂. ATP was measured as described by Lundin et al. (1976).

Glutamine uptake assay. Glutamine uptake was measured by enzymic determination (Lund, 1974) of the residual glutamine in the supernatant after removal of the organisms by centrifugation.

Enzyme assays. Nitrogenase activity was assayed by C₂H₂ reduction, as described by Kahn et al. (1982b), and β-galactosidase activity as described by Dixon et al. (1980).

RESULTS

To investigate the putative correlation of ppGpp levels with the expression of nif genes, we imposed an abrupt physiological perturbation by NH₄⁺-deprivation. Under these conditions the onset of nif derepression in various strains is more reproducible (Dixon et al., 1980). In addition, as discussed by Gallant (1979), changes in the pool size of low-molecular-weight regulatory metabolites are more readily observed than would be the case in a gradual readjustment of metabolic processes following exhaustion of NH₄⁺ in the medium. To allow correlation of ppGpp levels with nif transcription and activity, merodiploid nif::lac fusions (Nif⁺) were used. Repressed suspensions of wild-type M5a1, a nifH::lacZ fusion and a nifL::lacZ fusion were derepressed in a low phosphate medium following NH₄⁺-deprivation shutdown as described in Methods.

Our experiments were designed to allow nif expression under both stringent and relaxed conditions. For cultures derepressing under relaxed conditions, glutamine (600 pg ml⁻¹) was included in the medium to relieve stringency. Samples of derepressing suspensions of a nifH::lacZ fusion strain or a nifL::lacZ fusion strain were removed at intervals for measurement of P-galactosidase activity and nucleotide levels. Derepression of nitrogenase activity was monitored by removal of gas samples for the analysis of C₂H₂ reduction. The derepression kinetics observed are shown in Fig. 1(a, b).

Following transfer from NH₄⁺ sufficiency to N-free medium (Fig. 1a) there was a rapid increase in the level of ppGpp, which peaked at 3360 pmol (mg protein)⁻¹ after approximately 45 min and then decreased to a high plateau level for the duration of the experiment. As previously observed by Dixon et al. (1980), onset of the transcription of nifH preceded the detection of nitrogenase activity by 1 h. Glutamine prevented the increase in ppGpp (Fig. 1b), which remained at the basal level of approximately 100 pmol (mg protein)⁻¹ detected in NH₄⁺-grown repressed cultures, but did not repress nif. On the contrary, the onset of nifH transcription and nitrogenase activity occurred 30 to 40 min earlier compared to N-starved cultures. Comparison of Fig. 1(a) and 1(b) shows that the presence of glutamine resulted in a 7-fold stimulation of nifH transcription and a 26-fold increase in nitrogenase activity. A similar pattern, with a 7-fold increase, was observed for nifL transcription (data not shown). Thus, in the presence of glutamine, where ppGpp did not accumulate, a higher level of nif expression and nitrogenase activity was observed. This was also observed with the relaxed mutant strain K. pneumoniae relA40, where the addition of glutamine stimulated nitrogenase activity 30-fold to give a specific activity of 2.2 nmol C₂H₂ min⁻¹ (mg protein)⁻¹ after 8 h derepression.

To determine whether the increased rate of transcription of nif genes was due to the stimulation of general protein synthesis, cultures were pulse-labelled with ¹⁴C-labelled amino acids to measure the rate of protein synthesis at different times of derepression. In the absence of glutamine the rate of protein synthesis declined initially and then remained at a steady level of 70% of the initial rate. The presence of glutamine resulted in a gradual stimulation of protein synthesis to a plateau level three- to fourfold higher than the rate of N-starved cultures. It is not clear whether the greater stimulation in the rate of nif expression compared with general protein synthesis is a specific effect. The concentration of glutamine in the medium decreased from 600 to 250 µg ml⁻¹ in the 3 h following removal of NH₄⁺, during which time nif derepression had occurred.

The levels of GTP and ATP were measured radiochemically in suspensions in the period following removal of NH₄⁺. The two nucleotides showed a similar pattern: in the absence of glutamine there was a gradual decrease with time (Fig. 2) and in the presence of glutamine there was a gradual increase to a level which, after 3 h, was twice that of N-starved cultures. Measurement
Fig. 1. Kinetics and ppGpp accumulation and nif derepression in *K. pneumoniae* following NH₄⁺-shiftdown. A *nifH::lacZ* merodiploid strain of *K. pneumoniae* grown in MOPS–NFDM containing NH₄⁺ was harvested and subjected to NH₄⁺-shiftdown at the time indicated by the break in the x-axis. Organisms were resuspended either in N-free medium (open symbols, a) or in medium supplemented with glutamine, 600 μg ml⁻¹ (filled symbols, b). Transcription from the *nifH* promoter was measured as β-galactosidase activity (□, ■), and nitrogenase activity from the rate of C₂H₂ reduction (○, ●). Cultures derepressed under similar conditions were labelled with ³²P and used for determination of ppGpp levels (△, ▲) as described in Methods.

Fig. 2. Effect of glutamine on the levels of ATP and GTP in *K. pneumoniae* following NH₄⁺-shiftdown. ATP (○, ●) and GTP (□, ■) were measured in a *nifH::lacZ* fusion strain of *K. pneumoniae* derepressed in medium containing ³²P in the presence (filled symbols) or absence (open symbols) of glutamine as described in the legend to Fig. 1.
of ATP levels by the luciferase assay system gave values consistent with those of the radiochemical technique (data not shown).

**DISCUSSION**

When nitrogen becomes limiting for growth, free-living \( N_2 \)-fixing bacteria derepress nitrogenase synthesis. In *K. pneumoniae* and *R. palustris* under these conditions ppGpp accumulates and a stringent response is observed (Reisenberg et al., 1982; Zumft & Neumann, 1983). During growth of *K. pneumoniae*, *A. vinelandii* and *C. pasteuriannum* on \( N_2 \), elevated levels of ppGpp which decrease rapidly when \( NH_3^+ \) is added have been reported (Kleiner & Philips, 1981).

The involvement of the stringent response in nif derepression has been studied in most detail in *K. pneumoniae*. Two mutants with a relaxed phenotype (RelA\(^{-}\)) and unable to accumulate ppGpp, do not derepress nitrogenase activity following exhaustion of a limiting concentration of \( NH_3^+ \) in the growth medium (Reisenberg et al., 1982). In one mutant the rate of synthesis of nitrogenase polypeptides showed a fivefold decrease compared with the wild-type strain. Since nitrogenase activity was restored by an \( F' \) relA\(^+\) plasmid from *E. coli*, it is apparent that derepression of nif under N-starved conditions requires a functional relA product, and a positive involvement of ppGpp in regulating nif expression has been proposed.

However, our data show that in *K. pneumoniae* there is no correlation between the levels of ppGpp and the extent of nif expression when organisms are derepressed in the presence of glutamine. Only low levels of ppGpp are to be expected under such conditions since the stringent response is triggered by the lack of charged tRNAs, a deficiency which would be corrected by the provision of glutamine, which occupies a central position in amino acid metabolism. The increase that we observed in the rate, both of general protein synthesis and of transcription from the nif\(H\) and nif\(L\) promoters, is consistent with release from amino acid starvation. This stimulation of nif transcription and nitrogenase activity occurs against the low background level of ppGpp found in \( NH_3^+ \)-grown cultures, where nif is repressed, and strongly suggests that ppGpp is not directly involved in nif regulation. This proposal finds some support in the finding that the nif promoter consensus sequence found in *K. pneumoniae* (Beynon et al., 1983) is conserved in the nif\(H\) promoter of *Rhizobium meliloti* (Ow et al., 1983; Better et al., 1983), an organism which fails to accumulate ppGpp on N-shiftdown (Belitsky & Kari, 1982). In view of the high degree of conservation of the promoter sequences, it would be surprising if the expression from these promoters involved interaction of ppGpp with RNA polymerase in *K. pneumoniae* and not *R. meliloti*.

Why then, is the relA product important in allowing nif expression under N-deficiency? Following the exhaustion or removal of \( NH_3^+ \), organisms are effectively N-starved before nif is derepressed. No net synthesis of protein can occur and nitrogenase synthesis must be at the expense of turnover of N reserves. ppGpp has a role in protein turnover since it activates proteases involved in these processes (Bridger & Paranchych, 1979). The inability of relaxed mutants to derepress nif can be rationalized if protein turnover is regarded as too slow to allow nitrogenase synthesis within the timescale of the experiments. ppGpp levels in these strains are apparently not sufficient to allow protein turnover. The low level of nif expression observed when the *K. pneumoniae* relA40 mutant is derepressed in the presence of aspartate and adenine (Reisenberg et al., 1982) could, at least partially, be a consequence of an enhanced rate of protein synthesis. Under these conditions, and when adenine was added alone, ATP levels were elevated 10- to 20-fold but nif expression required the presence of aspartate. Since the addition of aspartate alone did not result in nif derepression, the restoration of ATP levels, in the absence of a suitable N-source, is insufficient to allow derepression of nif. Our data indicate that the addition of glutamine alone is effective both in maintaining ATP levels and in stimulating general protein synthesis to a level where nif expression is observed in the relaxed strain.

The enhanced nif expression we observed in the presence of glutamine occurred under conditions where ATP and GTP pools were elevated. Relaxed strains have low ATP levels unless adenine is added (Reisenberg et al., 1982). Since ATP has been implicated as having a role in nif
expression in *K. pneumoniae* (Jensen & Kennedy, 1982), it would appear that there is a more significant correlation of *nif* expression with ATP or related nucleotides than there is with ppGpp.

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nif expression without ppGpp accumulation


