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 NH_4^+ -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 ($600\ \mu\text{g ml}^{-1}$), 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 NH_4^+ -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 NH_4^+ to growth on N_2 , 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, N_2 -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 *Rhodospseudomonas 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*⁺ Δ *lac*/*nifH* 2783::Mud(Aplac)] and UNF619(pMF182) [*nif*⁺ Δ *lac*/*nifL* 2782::Mud(Aplac)]. The *nif-lac* fusion strains are both merodiploids carrying the *lacZ* gene (encoding β -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. $(\text{NH}_4)_2\text{SO}_4$ (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 μg protein ml^{-1} in 30 to 40 ml low phosphate medium, either N-free or containing glutamine (600 μg 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, β -galactosidase activity and glutamine uptake as described below.

[^{32}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, resuspended in low phosphate medium containing 15 mM- $(\text{NH}_4)_2\text{SO}_4$ and carrier-free [^{32}P]orthophosphate [final sp. act. 1 Ci mmol^{-1} , 200 μCi ml^{-1} (1 Ci = 37 GBq)] and shaken for 120 to 150 min under N_2 . Cultures were then harvested rapidly and resuspended 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 μCi ml^{-1}).

For estimation of ppGpp and other nucleotides, samples (20 μl) 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, Macherey Nagel, Düren, FRG). The solvent system was 1.5 M- KH_2PO_4 for one-dimensional chromatography, and 1.5 M-LiCl in 2 M- HCOOH in the first dimension and 1.5 M- KH_2PO_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 N_2 . 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 C_2H_2 reduction, as described by Kahn *et al.* (1982*b*), 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 NH_4^+ -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 NH_4^+ 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 NH_4^+ -deprivation shiftdown 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 ($600 \mu g\ ml^{-1}$) 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 β -galactosidase activity and nucleotide levels. Derepression of nitrogenase activity was monitored by removal of gas samples for the analysis of C_2H_2 reduction. The derepression kinetics observed are shown in Fig. 1(*a*, *b*).

Following transfer from NH_4^+ sufficiency to N-free medium (Fig. 1*a*) 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. 1*b*), which remained at the basal level of approximately $100\ pmol\ (mg\ protein)^{-1}$ detected in NH_4^+ -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 $2.2\ nmol\ C_2H_4\ min^{-1}\ (mg\ protein)^{-1}$ 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 ^{14}C -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 $250\ \mu g\ ml^{-1}$ in the 3 h following removal of NH_4^+ , during which time *nif* derepression had occurred.

The levels of GTP and ATP were measured radiochemically in suspensions in the period following removal of NH_4^+ . 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 NH_4^+ -shiftdown. A *nifH::lacZ* merodiploid strain of *K. pneumoniae* grown in MOPS-NFDM containing NH_4^+ was harvested and subjected to NH_4^+ -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, $600 \mu\text{g ml}^{-1}$ (filled symbols, *b*). Transcription from the *nifH* promoter was measured as β -galactosidase activity ($\square$, $\blacksquare$), and nitrogenase activity from the rate of C_2H_2 reduction ($\circ$, $\bullet$). Cultures derepressed under similar conditions were labelled with ^{32}P and used for determination of ppGpp levels ($\triangle$, $\blacktriangle$) as described in Methods.

Fig. 2. Effect of glutamine on the levels of ATP and GTP in *K. pneumoniae* following NH_4^+ -shiftdown. ATP ($\circ$, $\bullet$) and GTP ($\square$, $\blacksquare$) were measured in a *nifH::lacZ* fusion strain of *K. pneumoniae* derepressed in medium containing ^{32}P 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. pasteurianum* on N_2 , elevated levels of ppGpp which decrease rapidly when NH_4^+ 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_4^+ 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 *nifH* and *nifL* 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_4^+ -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 *nifH* 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_4^+ , 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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REFERENCES

- BELITSKY, B. & KARI, C. (1982). Absence of accumulation of ppGpp during amino acid starvation in *Rhizobium meliloti*. *Journal of Biological Chemistry* **257**, 677-679.
- BETTER, M., LEWIS, B., CORBIN, D., DITTA, G. & HELINSKI, D. R. (1983). Structural relationships among *Rhizobium meliloti* symbiotic promoters. *Cell* **35**, 479-485.
- BEYNON, J., CANNON, M., BUCHANAN-WOLLASTON, V. & CANNON, F. (1983). The *nif* promoters of *Klebsiella pneumoniae* have a characteristic primary structure. *Cell* **34**, 665-671.
- BRIDGER, W. A. & PARANCHYCH, W. (1979). Guanosine tetraphosphate: coordinator of metabolic response to nitrogen starvation in bacteria. *Trends in Biochemical Sciences* **4**, 176-179.
- DE BRUIJN, F. J., SUNDARESAN, V., SZETO, W., OW, D. W. & AUSUBEL, F. M. (1984). Regulation of the nitrogen fixation (*nif*) genes of *Klebsiella pneumoniae* and *Rhizobium meliloti*: role of nitrogen regulation (*ntr*) genes. In *Advances in Nitrogen Fixation Research*, pp. 627-633. Edited by C. Veeger & W. E. Newton. The Hague: Nijhoff/Junk; Wageningen: Pudoc.
- CANNON, F. C., DIXON, R. A., POSTGATE, J. R. & PRIMROSE, S. B. (1974). Chromosomal integration of nitrogen fixing genes in *Escherichia coli*. *Journal of General Microbiology* **80**, 227-239.
- CASHEL, M. (1975). Regulation of bacterial ppGpp and pppGpp. *Annual Review of Microbiology* **29**, 301-318.
- CASHEL, M. & GALLANT, J. (1969). Two compounds implicated in the function of the RC gene of *E. coli*. *Nature, London* **221**, 838-841.
- CHALONER-LARSON, G. & YAMAZAKI, H. (1976). Synthesis of guanosine 5'-triphosphate, 3'-diphosphate in a *spoT* strain of *Escherichia coli*. *Canadian Journal of Biochemistry* **54**, 935-940.
- DIXON, R., EADY, R., ESPIN, G., HILL, S., ICCARINO, M., KAHN, D. & MERRICK, M. (1980). Analysis of regulation of *Klebsiella pneumoniae* nitrogen fixation (*nif*) gene cluster with gene fusions. *Nature, London* **286**, 128-132.
- DIXON, R. A., ALVAREZ-MORALES, A., CLEMENTS, J., DRUMMOND, M. & MERRICK, M. (1984). Transcriptional control of the *nif* regulon in *Klebsiella pneumoniae*. In *Advances in Nitrogen Fixation Research*, pp. 635-642. Edited by C. Veeger & W. E. Newton. The Hague: Nijhoff/Junk; Wageningen: Pudoc.
- DONINI, P., SANTONASTASO, V., ROCHE, J. & COZZONE, A. J. (1978). The relationship between guanosine tetraphosphate, polysomes and RNA synthesis in amino acid starved *Escherichia coli*. *Molecular Biology Reports* **4**, 15-29.
- DROZD, J. W., TUBB, R. S. & POSTGATE, J. R. (1972). A chemostat study of the effect of fixed nitrogen sources on nitrogen fixation, membranes and free amino acids in *Azotobacter chroococcum*. *Journal of General Microbiology* **73**, 221-232.
- DRUMMOND, M., CLEMENTS, J., MERRICK, M. & DIXON, R. (1983). Positive control and autogenous regulation of the *nifLA* promoter in *Klebsiella pneumoniae*. *Nature, London* **301**, 302-307.
- FRIGA, G. M., BROBELY, G. & FARKAS, G. L. (1981). Accumulation of guanosine tetraphosphate (ppGpp) under nitrogen starvation in *Anacystis nidulans*, a cyanobacterium. *Archives of Microbiology* **129**, 341-343.
- GALLANT, J. A. (1979). Stringent control in *E. coli*. *Annual Review of Genetics* **13**, 393-415.
- GALLANT, J. A. & LAZZARINI, R. A. (1976). The regulation of ribosomal RNA synthesis and degradation in bacteria. In *Protein Synthesis: a Series of Advances*, pp. 309-359. Edited by E. McConkey. New York: Dekker.
- GALLANT, J., SHELL, L. & BITTNER, R. (1976). A novel nucleotide implicated in the response of *E. coli* to energy source downshift. *Cell* **7**, 75-84.
- GORDON, J. K. & BRILL, W. J. (1972). Mutants that produce nitrogenase in the presence of ammonia. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 3501-3503.
- HANSEN, M. T., PATO, M. L., MOLIN, S., FIIL, N. & VON MEYENBERG, K. (1975). Simple downshift and resulting lack of correlation between ppGpp pool size and ribonucleic acid accumulation. *Journal of Bacteriology* **122**, 585-591.
- JENSEN, J. S. & KENNEDY, C. K. (1982). Pleiotropic effect of *his* gene mutations on nitrogen fixation in *Klebsiella pneumoniae*. *EMBO Journal* **1**, 197-204.
- KAHN, D., HAWKINS, M. & EADY, R. R. (1982a). Metabolic control of *Klebsiella pneumoniae* mRNA degradation by the availability of fixed nitrogen. *Journal of General Microbiology* **128**, 3011-3018.
- KAHN, D., HAWKINS, M. & EADY, R. R. (1982b). Nitrogen fixation in *Klebsiella pneumoniae*: nitrogenase levels and the effect of added molybdate on the nitrogenase derepressed under molybdenum deprivation. *Journal of General Microbiology* **128**, 779-787.
- KLEINER, D. (1979). Regulation of ammonium uptake and metabolism by nitrogen fixing bacteria. III. *Clostridium pasteurianum*. *Archives of Microbiology* **120**, 263-270.
- KLEINER, D. & PHILIPS, S. (1981). Relative levels of guanosine 5'-diphosphate, 3'-diphosphate (ppGpp) in some N₂ fixing bacteria during derepression and repression of nitrogenase. *Archives of Microbiology* **128**, 341-342.

- LUND, P. (1974). L-Glutamine: determination with glutaminase and glutamate dehydrogenase. In *Methods of Enzymatic Analysis*, vol. 4, pp. 1719–1722. Edited by H. U. Bergmeyer. New York: Academic Press.
- LUNDIN, A., RICKARDSSON, A. & THORE, A. (1976). Continuous monitoring of ATP converting reactions by purified firefly luciferase. *Analytical Biochemistry* **75**, 611–620.
- MERRICK, M. J. (1982). A new model for nitrogen control. *Nature, London* **297**, 362–363.
- MERRICK, M., HILL, S., HENNECKE, H., HAHN, M., DIXON, R. & KENNEDY, C. (1982). Repressor properties of *nifL* gene product in *Klebsiella pneumoniae*. *Molecular and General Genetics* **185**, 75–81.
- NAIR, M. B. & EADY, R. R. (1984). Enhanced level of *nif* expression in *K. pneumoniae* without accumulation of ppGpp. In *Advances in Nitrogen Fixation Research*, p. 743. Edited by C. Veeger, & W. E. Newton. The Hague: Nijhoff/Junk; Wageningen: Pudoc.
- OHMORI, M. & HATTORI, A. (1974). Effect of ammonia on nitrogen fixation by the blue-green alga *Anabaena cylindrica*. *Plant and Cell Physiology* **15**, 131–142.
- OW, D. W., SUNDARESAN, V., ROTHSTEIN, D. M., BROWN, S. E. & AUSUBEL, F. M. (1983). Promoters regulated by the *glnG* (*ntnC*) and *nifA* gene products share a heptameric consensus sequence in the –15 region. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 2524–2528.
- PAO, C. & DYESS, B. (1981). Stringent control of RNA synthesis in the absence of guanosine 5′di 3′diphosphate. *Journal of Biological Chemistry* **256**, 2252–2257.
- PAO, C. & GALLANT, J. (1979). A new nucleotide involved in the stringent response in *Escherichia coli*. *Journal of Biological Chemistry* **254**, 688–692.
- REISENBERG, D., ERDEI, S., KONDOROSI, E. & KARI, C. (1982). Positive involvement of ppGpp in derepression of *nif* operon in *Klebsiella pneumoniae*. *Molecular and General Genetics* **185**, 198–204.
- RYALS, J., LITTLE, R. & BREMER, H. (1982a). Control of RNA synthesis in *Escherichia coli* after a shift to higher temperature. *Journal of Bacteriology* **151**, 1425–1432.
- RYALS, J., LITTLE, R. & BREMER, H. (1982b). Control of rRNA and tRNA synthesis in *Escherichia coli* by guanosine tetraphosphate. *Journal of Bacteriology* **151**, 1261–1268.
- SHANMUGAM, K. T. & MORANDI, C. (1976). Amino acids as repressors of nitrogenase biosynthesis in *Klebsiella pneumoniae*. *Biochimica et biophysica acta* **437**, 322–332.
- SPADARO, A., SPENA, A., SANTONASTASO, V. & DONINI, P. (1981). Stringency without ppGpp accumulation. *Nature, London* **291**, 256–258.
- TRAVERS, A. A., KARI, C. & MACE, H. A. F. (1981). Transcriptional regulation by bacterial RNA polymerase. *Symposia of the Society for General Microbiology* **31**, 113–130.
- TRAVERS, A. A., LAMOND, A. I. & MACE, H. A. F. (1982). ppGpp regulates the binding of two RNA polymerase molecules of the *tyrT* promoter. *Nucleic Acids Research* **10**, 5043–5057.
- ZUMFT, W. G. & NEUMANN, S. (1983). Changes in guanosine tetraphosphate level during nitrogenase expression in the phototrophic bacterium *Rhodospseudomonas palustris*. *FEBS Letters* **154**, 121–126.