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Regulation of Nitrogenase Synthesis in Histidine Auxotrophs of *Klebsiella pneumoniae* with Altered Levels of Adenylate Nucleotides

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A histidine auxotrophic (*hisA*) mutant of *Klebsiella pneumoniae* is phenotypically Nif⁻ when grown with 20 μg of histidine ml^{-1} but Nif⁺ when supplied with histidine at 100 μg ml^{-1}. Reversion to Nif⁺ at 20 μg of histidine ml^{-1} occurs phenotypically by the addition of 2-thiazolyl-DL-alanine or genetically by mutation in *hisG*; 2-thiazolyl-DL-alanine inhibits and *hisG* encodes phosphoribosyl phosphotransferase, the first enzyme of the histidine biosynthetic pathway which consumes ATP. Physiological studies of the *hisA* mutant JS85 showed that after removal of NH_4^+ from a culture of the mutant grown with 20 μg of histidine ml^{-1}, synthesis of nitrogenase polypeptides occurred at a rate similar to that in the wild type for about 3 h and acetylene reduction activity reached about 10% of the fully derepressed wild-type level. Shortly thereafter the concentration of intracellular adenylates decreased; in particular, ATP fell to about 10% of normal levels. Also, nitrogenase proteins (*nifHDK* products) and the *nifJ* gene product stopped being synthesized. These effects were not due to impairment of growth or protein synthesis by histidine starvation. Inhibition of phosphoribosyl phosphotransferase with 2-thiazolyl-DL-alanine restored nitrogenase activity and synthesis, indicating that the effect of the *hisA* mutation on *nif* expression was probably a consequence of lowered energy resources that occurred during anaerobic N starvation. The loss of ATP was not associated with nitrogenase synthesis or activity, since *hisA nifA* and *hisA nifH* double mutants underwent a loss of ATP in derepressing conditions. Transcription from the *nifL*, *nifN*, and *nifH* promoters was examined in *hisA* strains with Mu d(Ap *lac*) fusions in these *nif* genes. Transcription was not significantly influenced under conditions where adenylates were decreased in concentration. Also *nif* mRNA apparently accumulated in cultures unable to synthesize nitrogenase, suggesting that translational control of *nif* gene product synthesis occurs under unfavorable energetic conditions.

Biological nitrogen fixation is an energy-demanding process. The free-living diazotroph *Klebsiella pneumoniae* uses approximately 30 molecules of ATP for each molecule of N_2 reduced to ammonia (5). It is therefore not surprising that cellular regulatory mechanisms prevent nitrogenase synthesis under conditions where its activity is unnecessary, such as when fixed nitrogen is provided in excess, or would be destroyed, such as in O_2.

Regulation of the 17 *nif* genes occurs at two transcriptional levels (see reference 2 for a review). The *ntrC* gene product, which controls several genes concerned with nitrogen metabolism, activates transcription of the *nifLA* operon under conditions of ammonium limitation. Products of the *nifLA* operon in turn regulate expression of the other *nif* transcriptional units. The *nifA* product is absolutely required for their transcription, whereas the *nifL* gene product interacts with the *nifA* product to prevent activation in the presence of fixed nitrogen (NH_4^+ or amino acids) or O_2. The *nifL* product appears also to be involved in posttranscriptional control by stabilization of *nif* mRNA; in *nifL* mutants, *nif* mRNA is more stable than in the wild type after exposure to NH_4^+ or O_2 (1).

Another factor affecting nitrogenase synthesis is the cell's energy status. A facet of this was revealed by a study of *hisA1* mutants of *K. pneumoniae* that were phenotypically Nif⁻ on solid medium with 20 μg of histidine ml^{-1} but were Nif⁺ when supplied with 100 μg of histidine ml^{-1} (7). Other histidine auxotrophs with mutations in *hisC*, *hisB*, *hisD*, *hisF*, or *hisG* were Nif⁺ even in low-histidine medium, although nitrogenase activity was slightly reduced in some of them. These results indicated that histidine starvation per se did not cause the Nif⁻ phenotype in the *hisA* mutants. In liquid cultures of one *hisA* mutant, JS85, nitrogenase activity was never more than 10% of wild-type levels when histidine was supplied at 20 μg ml^{-1} but was 100% when histidine was at 100 μg ml^{-1}. ATP levels in JS85 decreased to 10% of that of His⁺ strains during derepression and appeared to correlate with a cessation of nitrogenase synthesis.

The reversal of JS85 to Nif⁺ by the addition of adenine or 2-thiazolyl-DL-alanine (2TA) to low-histidine, N-free agar medium also indicated that histidine starvation was not the cause of the Nif⁻ phenotype (7). 2TA is an analog of histidine that is not incorporated into proteins and has no effect on protein synthesis but inhibits the activity of the first enzyme of the histidine biosynthetic pathway. This is phosphoribosyl phosphotransferase, an ATP-consuming enzyme (Fig. 1). The *hisA* mutation prevents formation of the biosynthetic intermediate 5-aminoimidazole-4-carboxamide-1-ribonucleotide, which normally restores the purine precursor pool. Thus, physiological evidence indicated that a loss of ATP or a consequent energy imbalance correlated with the Nif⁻ phenotype of JS85.

This *hisA* mutant, JS85, has now been further characterized by examining Nif⁺ revertants, by measuring adenylate levels and rates of nitrogenase synthesis, and by testing for effects of the mutation on transcription of various *nif* oper-

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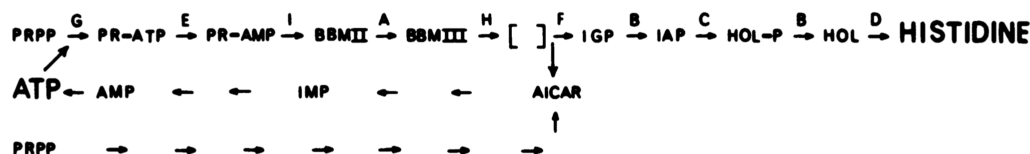

FIG. 1. Histidine and purine biosynthetic pathways. Abbreviations: PRPP, phosphoribosylpyrophosphate; PR-ATP, phosphoribosyl-ATP; PR-AMP, phosphoribosyl-AMP; BBMII, 5-(5′-phosphoribosylaminoformimino)-1-5″-phosphoribosyl)imidazole-4-carboxamide; BBMIII, 5-(5′-phosphoribulosylaminoformimino)-1-(5″-phosphoribosyl)-imidazole-4-carboxamide; [ ], unknown intermediates; IGP, imidazole glycerol phosphate; IAP, imidazole acetol phosphate; HOL-P, L-histidinol phosphate; HOL, L-histidinol; AICAR, 5-aminoimidazole-4-carboxamide-1-ribonucleotide; G, ATP phosphoribosyltransferase (EC 2.4.2.17); A, *N*-(5′-phospho-D-ribosylformimino)-5-amino-1-(5″-phosphoribosyl)-4-imidazolecarboxamide isomerase (EC 5.3.1.16); D, histidinol dehydrogenase (EC 1.1.1.23). The gene products catalyzing histidine biosynthesis are indicated above the arrows.

ons during derepression with *nif-lac* fusion strains. The results suggest that synthesis of *nif* gene products is prevented through posttranscriptional control when ATP is in short supply.

MATERIALS AND METHODS

Bacteria and plasmids. The strains of *K. pneumoniae* and plasmids carried by *Salmonella typhimurium* donor strains used in this work are shown in Table 1.

Derepression of *nif*. Exponential or stationary-phase nutrient broth cultures were diluted 1:20 into nitrogen-free medium (7) supplemented with aspartate at 100 $\mu g\ ml^{-1}$ and the appropriate amount of histidine. Incubation was at 29°C. Anaerobic conditions were obtained by sparging cultures with N_2.

Nitrogenase assay. A 4-ml volume of culture was transferred by syringe to a 7-ml bijoux bottle which had been previously flushed with N_2 gas and capped with a suba seal. Acetylene (1 ml) was injected, and the bottles were incubated for 10 to 15 min at 29°C and then stopped with 0.2 ml of 0.4% cetyltrimethylammonium bromide. Ethylene was measured on a gas chromatograph (Pye Unicam) fitted with a column of Porapak R (oven temperature, 50°C). Specific activities were calculated as nanomoles of C_2H_4 produced per minute per milligram (dry weight).

Dry weights. A measured volume of culture was collected on a Gelman metricel membrane filter (0.45-μm pore size). Filters were washed twice with 10 ml of 1% NaCl and once with 5 ml of distilled water. The filtered cells were dried to constant weight at 70°C.

Protein labeling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Proteins were labeled by incorporation of a mixture of ^{14}C-amino acids. A 1-ml sample of culture was injected into a bijoux bottle containing 2.5 μCi of ^{14}C-amino acids (50 μCi/ml) and flushed with N_2. Incorporation was allowed for 10 or 20 min at 29°C. The reaction was stopped by adding Casamino Acids (1 mg ml^{-1} in saline phosphate; Difco Laboratories). Cells were pelleted, suspended in 0.5 ml of Casamino Acids (1 mg ml^{-1}), and transferred to Eppendorf tubes.

After centrifugation for 30 s in an Eppendorf centrifuge the cells were suspended in 200 μl of sample buffer. Cells were broken by placing tubes in boiling water for 4 min. Released protein extract was transferred to a clean tube after 15 min of centrifugation. Extracts were stored at −20°C before gel electrophoresis. SDS-PAGE was done by a modified Laemmli method (11).

Estimation of total protein synthesis. Total incorporation of ^{14}C-labeled mixed amino acids was determined as trichloroacetic acid-precipitable counts. A 5-μl sample of protein extract was spotted on filter paper and soaked in 5% tri-

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype	Origin or reference
K. pneumoniae[a]		
JS85	*hisA1 rpsL4 hsdRI*	Jensen and Kennedy (7)
UNF122	*hisD2 Δlac-2002 hsdRI*	R. Dixon
JS97	*hisA1 Δlac-2002 hsdRI*	HisA1⁻ derivative from UNF122
JS104	*hisA1 Δlac-2002 hsdRI recA56 srl-300*::Tn*10*	JS97, this publication
UNF191	*nifL*::Mu d(Ap *lac*)*2792 Δlac-2002 hsdRI*	R. Dixon
UNF193	*nifA*::Mu d(Ap *lac*)*2797 Δlac-2002 hsdRI*	R. Dixon
UNF768	*hisD2 nifH*::Mu d(Ap *lac*)*183 Δlac-2002 hsdRI*	Hill et al. (6)
UNF980	*hisD2 nifN*::Mu d(Ap *lac*)*2790 Δlac-2002 hsdRI*	Hill et al. (6)
JS101	*hisA1 nifH*::Mu d(Ap *lac*)*183 Δlac-2002 hsdRI recA56 srl-300*::Tn*10*	UNF768, this work
JS102	*hisA1 nifN*::Mu d(Ap *lac*) *Δlac-2002 hsdRI recA56 srl-300*::Tn*10*	UNF980, this work
JS107	*hisA1 nifA*::Mu d(Ap *lac*)*2787 Δlac-2002 hsdRI recA srl-300*::Tn*10*	UNF193, this work
JS111	*hisA1 nifL*::Mu d(Ap *lac*)*2782 Δlac-2002 hsdRI recA56 srl-300*::Tn*10*	UNF191, this work
Plasmids[b]		
pJS1	*hisA1* Nif⁺	Derivative of pRD1 (R. Dixon), this work
F′ his⁺	F′ *hisA2406* F′ $hisD_{ab}2381$	P. Hartman
F′ hisBH2405	F′ *hisA3017* F′ $hisD_{b}2377$	P. Hartman
F′ hisB	F′ *hisF51* F′ *hisC2485*	P. Hartman
F′ hisE	F′ *ΔhisBA2633 F′* $hisD_{a}2369$	P. Hartman
F′ hisF2408	F′ *hisAF703* F′ *hisI*	P. Hartman
F′ hisG2416	F′ *hisG3025*	P. Hartman

[a] All *K. pneumoniae* strains were derived from strain M5a1.
[b] All F′ plasmids had *S. typhimurium his* mutations carried in *S. typhimurium* strains and were obtained from P. Hartman, Johns Hopkins University.

chloroacetic acid for 30 min. Filters were soaked in ethanol then acetone for 15 min each, dried, and placed in a vial containing 6 ml of scintillation cocktail [666 ml of Toluene, 332 ml of Triton X-100, 5 g of 2,5-diphenyloxazole, and 0.15 g of 1,4-bis(5-phenyloxazolyl)benzene per liter].

Nucleotide extraction and measurements. Samples were prepared by vacuum withdrawal of 1 ml of culture into a syringe containing 1 ml of ice-cold 10% trichloroacetic acid and 4 mM sodium EDTA in N_2. The mixture was immediately sprayed into a test tube through the needle. Extracts were left on ice for 15 min then diluted 50-fold into Tris-EDTA (0.1 M, pH 7.75) and stored at −20°C. All extractions were done in triplicate.

ATP was measured with the luciferin-luciferase assay (15) with an ATP monitoring kit and luminometer purchased from LKB Instruments, Inc. ADP was converted to ATP by using phosphenolpyruvate (PEP) and pyruvate kinase and was estimated as the increase over the first determined ATP level. The conversion reactions were performed in Eppendorf tubes containing equal volumes of sample and PEP buffer with 40 U of pyruvate kinase ml^{-1}. The reaction mixture was incubated at 22°C for 5 min before ATP was measured. AMP was also determined by conversion to ATP with adenylate kinase and pyruvate kinase-PEP and subtraction of the ATP and ADP levels. Equal volumes of sample and PEP buffer (with 50 U of pyruvate kinase ml^{-1}, 200 U of adenylate kinase ml^{-1}) were incubated for 5 min at 20°C before ATP was measured.

PEP buffer (0.3 mM PEP, 9 mM $MgCl_2$, 5 mM KCl, 0.1% bovine serum albumin in 0.1 M Tris–EDTA [pH 7.75]) was prepared just before measurements. Pyruvate kinase and adenylate kinase were bought as $(NH_4)_2SO_4$ solutions, the required units of activity were precipitated by a 5-min centrifugation in an Eppendorf centrifuge before being suspended in PEP buffer. Adenylate kinase preparations contained adenylates, and the background levels in the AMP conversion mixture were therefore measured and subtracted from values obtained from the experimental samples.

All estimates were done in triplicate and energy charge was calculated as ([ATP] + 0.5 [ADP])/([ATP] + [ADP] + [AMP]) from the average values.

Construction of transcriptional *nif-lacZ* fusions in *nifA*, *nifL*, *nifN*, and *nifH* strains. The defective bacteriophage Mu d(Ap *lac*) was used by Dixon et al. (3) to isolate transcriptional *lacZ* fusions in most of the *nif* genes. Hill et al. (6) then transduced certain *nif-lac* fusions to the *K. pneumoniae* chromosome to study the effect of various regulatory mutations on *nif* expression. Fusions of *nifH-lacZ183* and *nifN-lacZ2790* were thus available in a *hisD2* background, and the *hisA1* derivatives JS101 and JS102 were constructed by phage P1.Km cotransduction of *hisA1 hisD2*$^+$ into the UNF768 and UNF980 strains carrying the fusions. The *hisA1* transductants were selected for growth on histidinol and screened for inability to grow without histidine or histidinol.

The *nifL-lacZ2792* and *nifA-lacZ2797* fusions were available only in a *hisD2*$^+$ background. The Mu d(Ap *lac*) fusions were transduced directly with P1 grown on strains UNF191 and UNF193 to infect a *hisA1* Δ*lac* strain, JS97. Transductants were selected on N-deficient medium supplemented with 200 μg of histidine ml^{-1}, 50 μg of serine ml^{-1}, 100 μg of carbenicillin ml^{-1}, and 50 μg of ampicillin ml^{-1} and screened for histidine requirement. The color indicator 5-bromo-4-chloro-β-D-galactosidase was used at 40 μg ml^{-1} in the selective plates to indicate the presence of the *lacZ* gene fusion.

The sites of the *lacZ* fusions in all four of the *hisA1 nif-lacZ* fusion strains were confirmed by complementation of *nif* with pRD1 derivatives with appropriate *nif* mutations. β-Galactosidase activity was determined by the method of Miller (16).

Construction of plasmid JS1. The His$^+$ Nif$^+$ plasmid pRD1 was transferred to JS85 by conjugation. In vivo recombination between the chromosomal and plasmid *his* regions followed by homogenotization of diploids produced *hisA1* pRD1 derivatives. These were isolated after D-cycloserine enrichment for His$^-$ derivatives.

RESULTS

Characterization of Nif$^+$ revertants of JS85. Physiological evidence, summarized above, indicated that ATP consumption by phosphoribosyl phosphotransferase, the first enzyme of the histidine biosynthetic pathway encoded by *hisG*, was associated with the Nif$^-$ phenotype of JS85. Genetic evidence for this correlation was gained by examining Nif revertants of JS85, isolated as single colonies on N-free medium supplemented with 10 μg of histidine ml^{-1}. Among more than 1,200 Nif$^+$ colonies examined, some 95% were HisA$^+$ revertants. About 3% were His$^-$ Nif$^\pm$ and retained the ability to be restored to full Nif$^+$ by the addition of adenine or 100 μg of histidine ml^{-1}. Of the 22 other colonies that were His$^-$ Nif$^+$, 14 had an additional *his* mutation characterized by complementation as *hisG* by using the F′ *his* plasmids listed in Table 1. In these 14, introduction of any but the *hisG* (or *hisA*) plasmids resulted in colonies that were His$^+$. The other eight His$^-$ Nif$^+$ colonies had complex phenotypes. They required other amino acids or purines for aerobic but not anaerobic growth; they were not further characterized. In any case, the high proportion of *hisG* mutants among the His$^-$ Nif$^+$ colonies examined correlated well with the previously discussed reversal of Nif phenotype in JS85 by the addition of histidine or 2TA, which inhibit activity of phosphoribosyl phosphotransferase, or of adenine, which replenishes the purine ring lost by interruption of the histidine biosynthetic cycle.

Effect of *hisA* mutation on nitrogenase synthesis and activity and on concentration of adenylate nucleotides. The apparent correlation between the adenylate levels and nitrogenase activity and synthesis were investigated further in JS85. All three parameters were measured in batch cultures throughout *nif* derepression in medium containing high (100 μg ml^{-1}) or low (20 μg ml^{-1}) levels of histidine (Fig. 2 through 4). Rates of synthesis of *nifHDK* and *nifJ* products were determined in cultures pulse-labeled with ^{14}C-amino acids; proteins from cell extracts were separated by SDS-PAGE and examined by autoradiography. Nitrogenase synthesis was evident at 6 h, and activity was evident at 7 h, after NH_4^+ was removed from both cultures (Fig. 2 and 3). In the low-histidine culture, activity reached a maximum at 9 to 10 h and decreased thereafter. This decrease in nitrogenase activity was preceded by a drastic reduction in levels of ATP which occurred at 7 to 8 h (Fig. 3b). The level of ATP at this time was approximately 10% of the initial concentration. Amounts of ADP and AMP also decreased. About 2 h after the decline in adenylates, nitrogenase polypeptides stopped being synthesized (Fig. 4). Synthesis of the *nifH* polypeptide appeared to stop later than did synthesis of *nifK*, *nifD*, and *nifJ* products, although the rate of synthesis of this polypeptide was apparently higher throughout derepression.

In the culture of JS85 with high levels of histidine (100 μg ml^{-1}), nitrogenase activity (Fig. 2) and synthesis (data not

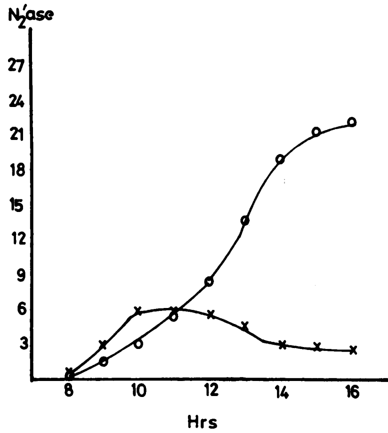

FIG. 2. Nitrogenase activities in JS85 (*hisA1*) grown and derepressed in N-free medium containing 100 μg of aspartate ml^{-1} and 100 μg of histidine ml^{-1} (O) or 20 μg of histidine ml^{-1} (×). Activity is acetylene reduction (nanomoles of C_2H_4 produced per minute per milligram) measured at times after dilution of cells into the medium. Each point represents the mean of three values obtained in two experiments.

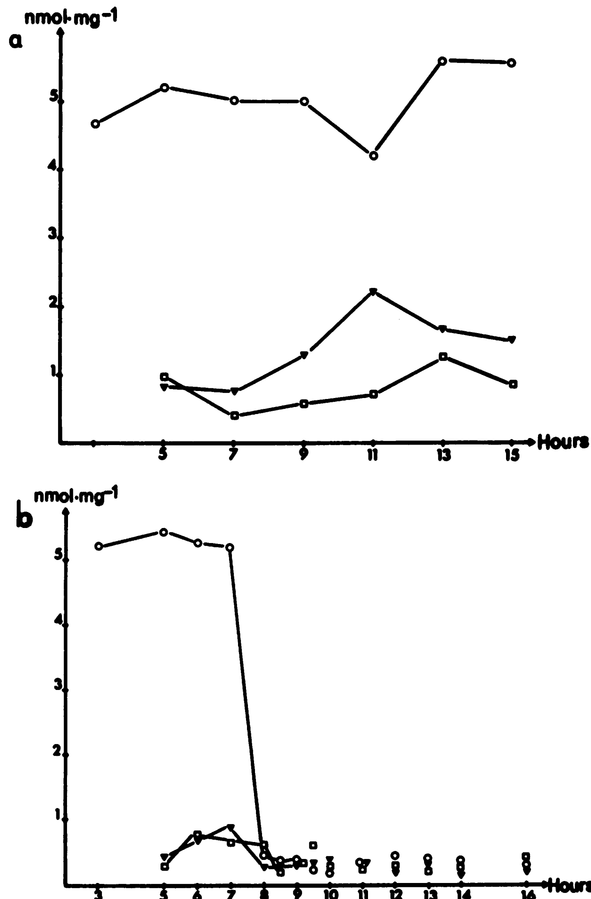

FIG. 3. Levels of intracellular adenylate nucleotides during derepression of JS85 at 100 μg of histidine ml^{-1} (a) or at 20 μg of histidine ml^{-1} (b). Symbols: concentrations of ATP (O), ADP (∇), and AMP (□).

shown) were sustained throughout the course of the 16-h experiment. Only a small and transient decrease in ATP (and concomitant increase in ADP) was observed at 11 h (Fig. 3a).

To see whether the energy demands imposed by *nif* gene expression or nitrogenase activity contributed to the loss of ATP during derepression in JS85, *hisA nifA* and *hisA nifH* double mutants were constructed. In the former, only the *nifLA* operon should be expressed, whereas in the latter nitrogenase is inactive. ATP was measured before and 15 h after the removal of NH_4^+. ATP levels decreased substantially but somewhat less than those in the *hisA* Nif^+ strain (Table 2), indicating that neither expression of *nif* genes other than *nifLA* nor nitrogenase activity is the major cause of the decline in adenylates.

Nitrogenase activity, adenylate concentrations, and protein synthesis after addition of histidine. Histidine (100 μg ml^{-1}) was added to a JS85 culture derepressed at low histidine at the time when the parallel culture supplied with high histidine from the outset was fully derepressed (15 h in Fig. 1). This addition resulted in the appearance of substantial nitrogenase activity (Fig. 5). Addition of rifampin or tetracycline 5 min before supplying histidine prevented the full restoration of nitrogenase activity. This is consistent with the gel results described above and indicate, as expected, that *nif* transcription or translation had been prevented when the ATP levels were low. The reappearance of nitrogenase synthesis and activity, as well as changes in adenylate nucleotide levels, was therefore measured after the addition of histidine to a culture of JS85 derepressed at low histidine.

At the time of addition, ATP concentration and nitrogenase activity were both less than 10% of levels found in JS85 derepressed with high histidine. After the addition of 100 μg of histidine ml^{-1} alone, nitrogenase activity increased biphasically (Fig. 5). About 60% of full activity was reached in 30 min; after a delay of another 30 min, activity increased at a slower rate than previously and reached that of the parallel culture grown with high histidine by approximately 2 h later (Fig. 5). Addition of tetracycline (100 μg ml^{-1}) (Fig. 5) or chloramphenicol (250 μg ml^{-1}) (data not shown) 5 min before histidine diminished the first phase and prevented the second phase of increase in nitrogenase activity. Addition of

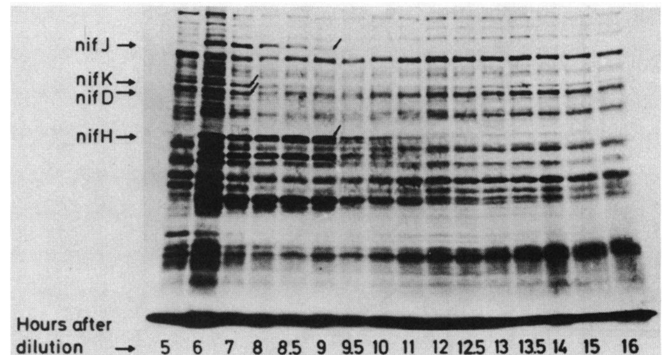

FIG. 4. Autoradiograph of radiolabeled cell extracts separated by SDS-PAGE. A 1-ml culture of JS85 was labeled with ^{14}C-labeled mixed amino acids (2.5 μCi ml^{-1}) during a derepression experiment at time intervals from 5 to 16 h after bacteria were diluted into N-deficient medium containing 20 μg of histidine ml^{-1}. Samples were prepared as described in Materials and Methods, and 15 μl was loaded in each 8-mm sample well. The positions of the *nifHDKJ* gene products are indicated by arrows.

TABLE 2. Expression of *nif-lac* transcriptional Mu d (Ap *lac*) fusions in *hisA1* strains derepressed at histidine concentrations of 100 and 20 μg ml^{-1}

Strain[b]	Histidine concn (μg ml^{-1})	β-Galactosidase activity (U)				ATP (nmol mg^{-1})		Acetylene reduction (nmol min^{-1} mg^{-1})	
		15 h		18 h					
		No plasmid	+pJS1	No plasmid	+pJS1	No plasmid	+pJS1	No plasmid	+pJS1
JS101 (*nifH-lac*)	100	1,442	2,696	888	2,822	5.1	6.2	0	43
	20	1,168	2,211	861	2,370	2.2	1.5	0	17
JS102 (*nifN-lac*)	100	1,616	391	1,880	372	5.4	4.0	0	32
	20	1,357	293	1,297	290	2.4	0.8	0	10
JS107 (*nifA-lac*)	100	371	132	392	126	3.7	4.6	0	33
	20	293	111	314	117	1.0	0.9	0	9
JS111 (*nifL-lac*)	100	467	170	474	177	5.0	4.9	0	34
	20	406	170	374	142	1.35	0.8	0	10

[a] β-Galactosidase was measured 15 and 18 h after dilution from nutrient broth; intracellular ATP concentrations and acetylene reduction were measured only at the 15-h time point, when nitrogenase activity would have been fully derepressed. The β-galactosidase activity in NH_4^+-grown cultures was <10 U.
[b] All strains were *hisA1*.

100 μg of rifampin ml^{-1} before histidine resulted in about 70% of the nitrogenase activity found in the uninhibited culture, which suggested that de novo transcription was probably required for the second stage of increase. The rifampin resistance and partial tetracycline sensitivity of the first phase of increase could indicate that *nif* mRNA translation had been prevented at low levels of ATP or that reactivation of inactive nitrogenase required protein synthesis or both.

Synthesis of nitrogenase polypeptides and the *nifJ* product was determined as previously. No *nif* products were detected in 15-h cultures of JS85 grown with low histidine. Synthesis was restored shortly after histidine was added and reached maximum rates about 1 h later (data not shown). The addition of rifampin before histidine resulted in an initially high rate of *nif* polypeptide synthesis, which diminished 40 min later. No *nif* products were detected in cultures with tetracycline (data not shown).

The addition of histidine (100 μg ml^{-1}) to JS85 derepressed at low histidine also resulted in restoration of ATP to 80% of wild-type levels within 40 min (Fig. 6). The ADP pool initially increased faster than the ATP pool (for about 15 min) before a steady, approximately wild-type level was maintained after about 40 min. A small increase in AMP was also detected after histidine addition.

Protein synthesis in JS95 was reduced to 45% of the wild-type rate after the decrease in adenylate nucleotides. After the addition of 100 μg of histidine ml^{-1}, the rate of protein synthesis increased threefold within the first 40 min and paralleled the increase in ATP concentration (data not shown). As expected, tetracycline completely prevented protein synthesis, whereas some protein synthesis occurred initially and then declined in the culture to which rifampin was added before histidine.

To confirm that the effect of the *hisA* mutation on nitrogenase synthesis was due to energy imbalance and not to amino acid starvation, the histidine analog 2TA or adenine was added to JS85 in experiments similar to those reported above. The results were similar to those obtained with histidine: high levels of nitrogenase synthesis and activity were attained soon after the addition of either compound (Fig. 7). The time scale of these experiments was too short to allow observation of the effect of histidine, 2TA, or adenine addition on the growth of JS85; however, in separate exper-

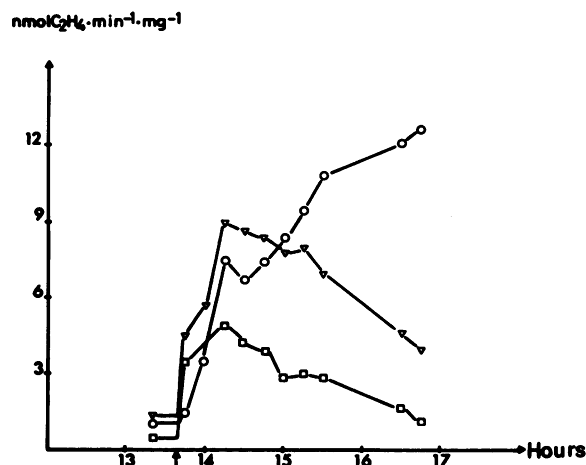

FIG. 5. Nitrogenase activity of JS85 after the addition of 100 μg of histidine ml^{-1} to cultures derepressed at 20 μg of histidine ml^{-1}. Symbols: (○) histidine added at the time indicated by the arrow, (▽) rifampin (200 μg ml^{-1}) added 5 min before histidine, (□) tetracycline (100 μg ml^{-1}) added 5 min before histidine.

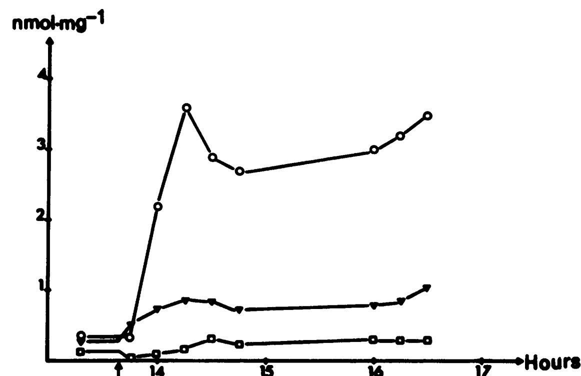

FIG. 6. Adenylate nucleotide concentrations after the addition of histidine to a culture of JS85 derepressed with 20 μg of histidine ml^{-1}. Symbols: concentrations of ATP (○), ADP (▽), and AMP (□).

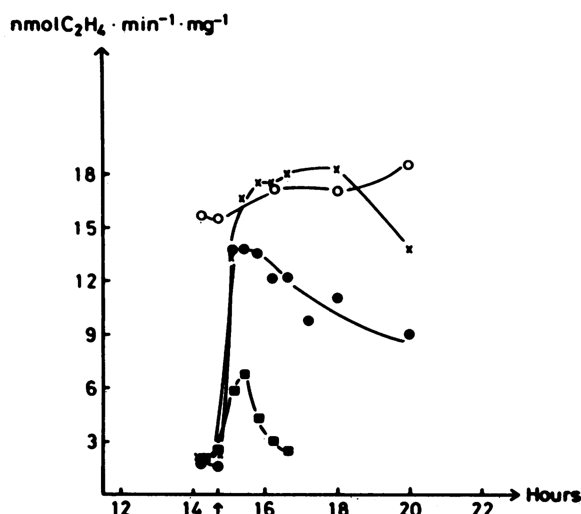

FIG. 7. Nitrogenase activity of JS85 after addition of the histidine analog 2TA, adenine, or NH_4Cl. Cultures were derepressed at 20 μg of histidine ml^{-1}, and 100 μg of 2TA ml^{-1} (×), 25 μg of adenine ml^{-1} (●), or 1 mg of NH_4Cl ml^{-1} (■) were added at the time indicated by the arrow. The control cultures (○) were derepressed with 100 μg of histidine ml^{-1} from time zero.

iments all three compounds restored the ability of JS85 to grow on N-free solid medium.

Effect of low ATP levels on expression of different *nif* genes. To determine whether transcription of *nif* genes was affected in JS85 grown with 20 μg of histidine ml^{-1}, different *nif-lac* fusions were introduced into the *hisA* background. These included Mu d(Ap *lac*) inserts in *nifL*, *nifA*, *nifH*, and *nifN*. To see whether *nif* products (or their absence) influenced expression under these conditions, merodiploid strains were made by transfer of the *hisA1* Nif^+ plasmid pJS1 into the fusion background. Expression from the *nif* promoters was determined in all fusion strains grown under the same conditions used for measuring nitrogenase synthesis and nucleotide levels described in previous experiments.

β-Galactosidase activity in the *nif-lac* fusion strains grown with 20 μg of histidine ml^{-1} ranged from 70 to 100% of that in cultures with histidine at 100 μg ml^{-1} (Table 2). No particular fusion appeared to be more affected by the *hisA1* mutation than the others, not even among the merodiploid strains. While neither ATP levels nor acetylene reduction activity was generally as low in the mutants as the levels found in JS85 in previous experiments (13 to 45% versus 10%), the failure of expression of the *nif-lac* fusions to be significantly affected suggests that the effect of the *hisA1* mutation is on a post-transcriptional step of nitrogenase synthesis.

Since rifampin prevented JS85 from reaching 100% of wild-type levels of nitrogenase activity after the addition of histidine to a culture derepressed at low histidine (Fig. 5), it is possible that *nif* transcription is needed for recovery of full activity. Addition of histidine to a *hisA nif-lacZ* fusion strain might therefore result in an increase in β-galactosidase activity coinciding with the recovery of full nitrogenase activity. This was tested in the diploid fusion strains derepressed at 20 and 100 μg of histidine ml^{-1} as above. Nitrogenase activity, β-galactosidase, and ATP were measured at 15 h, and then histidine was added to the cultures derepressed at low histidine. The three parameters were measured again at 18 h (Table 3). As expected, nearly wild-type levels of ATP were attained in the *nifH*, *nifL*, and *nifA* fusion strains within 3 h. Full nitrogenase activity was restored in the *nifH* fusion strain carrying pJS1, whereas slightly less but substantial activity appeared in the *nifL* and *nifA* fusions. However no increase in the level of β-galactosidase was observed in any of these strains or in the *nifN* fusion strain. Taken together these results indicate that *nif* transcription per se has little effect on the level of recovery of nitrogenase activity and synthesis after ATP levels are restored to normal in JS85. However, it is possible that ATP levels must be less than 10% of the wild type before a decrease in *nif* transcription is evident; in these experiments, ATP levels in cultures with 20 μg of histidine ml^{-1} were 16 to 20% of those in cultures with 100 μg of histidine ml^{-1}.

DISCUSSION

Two important questions arise from the experiments reported here with *hisA* mutant JS85 and its derivatives: why do ATP levels decrease during anaerobic NH_4^+-free growth

TABLE 3. Expression of *nif*::*lacZ* transcriptional Mu d (Ap *lac*) fusions in diploid *hisA1* strains after histidine readdition[a]

Strain[b]	Histidine	β-Galactosidase activity U		ATP (nmol mg^{-1})		Acetylene reduction (nmol min^{-1} mg^{-1})	
		15 h	18 h	15 h	18 h	15 h	18 h
JS101(pJS1) (*nifH-lac*)	100 + His	2,823	3,400	6.21	5.89	36	41
	20 + His	2,503	2,290	1.22	5.17	14	46
	20	2,437	2,517	1.11	0.88	15	22
JS102(pJS1) (*nifN-lac*)	100 + His	372	351	4.13	ND	42	ND
	20 + His	290	237	0.76	ND	14	ND
	20	ND	ND	ND	ND	ND	ND
JS107(pJS1) (*nifA-lac*)	100 + His	130	124	4.51	4.15	37	27
	20 + His	111	110	0.71	3.88	8	28
	20	120	110	0.8	0.72	9	9
JS111(pJS1) (*nifL-lac*)	100 + His	170	177	4.7	4.57	35	30
	20 + His	161	120	0.84	4.71	11	23
	20	159	145	0.76	0.65	12	13

[a] β-Galactosidase activity, intracellular ATP concentration, and acetylene reduction were measured at 15 and 18 h. Histidine (100 μg ml^{-1}) was added to the cultures derepressed at 20 and 100 μg of histidine ml^{-1} (marked 20 + His, 100 + His) just before the 15-h sampling. ND, Not determined.

[b] All strains were *hisA1*.

at 20 μg of histidine ml^{-1}, and why does nitrogenase synthesis stop in these cultures resulting in a Nif^- phenotype?

The dramatic decrease in adenylates after derepression by NH_4^+ removal in the *K. pneumoniae hisA* mutant JS85 grown with 20 μg of histidine ml^{-1} is not easily explained. The *his* operon might be more derepressed under these conditions than with 100 μg of histidine ml^{-1}, leading to higher levels of phosphoribosyl phosphotransferase and consequently more consumption of ATP by this enzyme. However, there is no obvious reason for derepression of the *his* operon; the supply of histidine is not limiting, since protein synthesis continued in these cultures, albeit at a reduced rate (45% of original), and addition of adenine or 2TA led to resumption of nitrogenase synthesis. In other cases where histidine auxotrophy has been associated with either loss of ATP or adenine auxotrophy, experimental conditions were such that derepression of the *his* operon was expected to occur, leading to increased production of the ATP-consuming *hisG* gene product, phosphoribosyl phosphotransferase (4, 8, 21, 22). One example is in *hisF cheZ* mutants of *S. typhimurium*, where loss of ATP prevented the tumbling behavior associated with chemotaxis (4). ATP only decreased in cultures where the supply of histidine was decreased from about 23 to 0 μg ml^{-1}. ATP-dependent tumbling movement was restored by addition of adenine or 2TA. In another case, *hisA* (and other *his*) mutants supplied with histidinol, a histidine source which is poorly transported at 30°C, were able to grow at this temperature only when adenine was present (8).

It seemed possible that the energy demand associated with transcription and translation of the several *nif* operons somehow triggered a loss of ATP, but this was not the case since a *hisA nifA* double mutant also showed a marked decrease in ATP levels. The energy demand of nitrogenase was also not involved, because ATP decreased in a *hisA nifH* mutant. Perhaps some other consequence of N starvation or anaerobic growth is involved. One possibility is the energy requirement associated with NH_4^+ transport in *K. pneumoniae*, which becomes more significant under NH_4^+ limitation (12). Another is the use of glutamine synthetase, an ATP-consuming enzyme, rather than glutamate dehydrogenase for NH_4^+ assimilation under conditions of N limitation (25).

Since the rapid decline in ATP levels preceded the cessation of nitrogenase synthesis by 1 to 2 h, it is unlikely that there is direct control of *nif* expression by ATP levels. Therefore nitrogenase synthesis may be affected by a particular balance of nucleotides or by regulatory nucleotides. Among the latter is ppGpp, which is known to increase in amino acid- or N-starved bacteria, including *K. pneumoniae* (13, 19). However, ppGpp is apparently not necessary for *nif* gene expression, since N-starved *K. pneumoniae* cultures supplemented with glutamine had nitrogenase activity but failed to accumulate significant ppGpp (18).

The effects of lowered ATP levels in JS85 on *nif* transcription or translation were investigated in the histidine readdition experiments. With no other additions, nitrogenase synthesis quickly resumed and adenylate pools were replenished. Nitrogenase activity reached wild-type levels in two stages of increase. Inhibition of protein synthesis with tetracycline prevented nitrogenase synthesis, significant activity was nevertheless restored, presumably from preformed nitrogenase, which was inactive due to insufficient ATP. The recovery of full activity after readdition of histidine was therefore dependent on nitrogenase synthesis. Addition of the transcriptional inhibitor rifampin, shown by Collins et al. (1) to prevent transcription of *nif* genes, did not inhibit nitrogenase synthesis (data not shown), and the nitrogenase activity peaked at a level higher than that reached during the first stage of recovery after the addition of histidine (Fig. 7). Therefore signficant *nif* mRNA accumulated during the period when nitrogenase synthesis was inhibited in JS85 and indicated that the major effect of the *hisA* mutation was to prevent translation of *nif* mRNA. Translation was not generally inhibited, since the pattern of proteins on the SDS-polyacrylamide gels shown in Fig. 4 did not observably alter except for the *nifHDK* and *nifJ* products. It is possible, however, that translation of other proteins not detected by one-dimensional SDS-PAGE was affected. Of significance here is a report by Swedes et al. (24) that in an *Escherichia coli* adenine auxotroph the rate of protein synthesis was not affected by decreased levels of ATP (10% of the wild type) as long as the energy charge was not greatly affected. Thus, low ATP levels per se do not prevent translation.

Translational control of nitrogenase synthesis in JS85 after a decline in ATP levels is supported by studies of *nif-lac* fusions. Only minor reductions in the level of transcription of the *nifLA*, *nifN*, or *nifH* genes were apparent at low levels of supplied histidine. Furthermore, readdition of histidine to cultures derepressed at low histidine did not allow the increase in β-galactosidase activity which a reinitiation of transcription would predict. The half-life of *nif* mRNA in nitrogen-starved *K. pneumoniae* cells has been estimated to be 20 to 30 min, or five- to sixfold longer than the half-life of most bacterial mRNAs (9, 10). Exposure of cultures to NH_4^+ or O_2 or high temperature (41°C) results in rapid cessation of *nif* gene transcription and also an increased rate of *nif* mRNA breakdown. The *nifL* gene product is involved in both effects, at least in response to NH_4^+ and O_2, since in *nifL* mutants *nif* transcripts continue to be made and are not degraded under these conditions (1). Since *nif* transcripts are apparently synthesized and not degraded in JS85, a $nifL^+$ strain, the *nifL* product is probably not involved in the regulating nitrogenase synthesis when cells are depleted of ATP. Translational control has been reported for a few other procaryotic systems, including ribosomal protein operons where a protein encoded in the operon blocks translation of that mRNA species (reviewed in reference 14). Blockage of mRNA translation by antisense RNA occurs with the Tn*10* transposase gene (23) and with the osmoregulatory genes *ompF* and *ompC* (17, 20). How translational control of nitrogenase synthesis occurs remains to be elucidated, but the association of low energy status with prevention of nitrogenase synthesis is a sensible way of avoiding wastage of biosynthetic capacity by futile synthesis of an enzyme that uses a great deal of ATP.

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