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Repression of Nitrogen Fixation in *Klebsiella pneumoniae* at High Temperature

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The effect of elevated growth temperature on *nif* mRNA synthesis, *nif* protein synthesis, and nitrogenase activity was investigated in wild-type and NifL$^-$ strains of *Klebsiella pneumoniae*. Nitrogenase activity is not affected at 41°C; however, nitrogenase is not synthesized at that temperature. Transcription of three *nif* operons studied is repressed at 41°C. We show that the *nifL* protein is not required for repression by high temperature and propose that repression of *nif* at 41°C results from a reversible inactivation of the *nifA* gene product.

The *nif* (nitrogen fixation) regulon in *Klebsiella pneumoniae* consists of at least 15 genes and the site of ammonium regulation. The regulon is arranged in seven contiguous operons. Expression of the *nif* genes is complex and responds to several different environmental stimuli, including high levels of ammonium (24–26), certain amino acids (22, 29), oxygen (23), temperature (9, 30), and conditions which evoke a stringent response (18, 19). Regulation occurs at the levels of transcription, mRNA stability (J. J. Collins and W. J. Brill, submitted for publication), and protein stability (20, 21).

Regulation of *nif* expression involves the synthesis and activity of two *nif*-specific regulatory proteins. The *nifA* gene product is a positive regulator (4, 5, 21) and the *nifL* gene product is a negative regulator (10, 15) of *nif* expression. These two genes comprise a single operon whose expression is directly controlled at the operator-promoter region by elements of the *ntr* (nitrogen regulation) system (6, 7, 14, 16, 17, 27, 28). The expression of the other six *nif* operons is, in turn, regulated by the *nifA* and *nifL* gene products (16, 21).

K. pneumoniae is unable to grow on N_2 as the sole nitrogen source at 37°C, although it grows well on many sources of fixed nitrogen at this temperature (30). It has been shown that the expression of six of the *nif* operons is repressed by high temperature, whereas that of the *nifAL* operon is unaffected (30). Investigation of temperature repression has led one group to conclude that the activating activity of the *nifA* protein is temperature sensitive (3), whereas another group has suggested that the *nifL* protein acts as a repressor in response to high temperature (15).

Using a variety of *Klebsiella pneumoniae* strains and several different methods of analysis, we have shown that regulation at high temperature is a transcriptional effect which does require the activity of the *nifL* gene product and suggest that the effect is caused by a reversible inactivation of the *nifA* gene product.

MATERIALS AND METHODS

Bacterial strains. All *K. pneumoniae* strains used in this paper have been described by MacNeil and Brill (12). *Escherichia coli* K-12 strain C600 has the genotype *thr-1 leuB6 thi-1 supE44 tonA21 lacY recA* λ^r.

Media, chemicals, and isotopes. LC (1) and minimal (11) have been described previously. Antibiotics, obtained from Sigma Chemical Co., St. Louis, Mo., were added at the following concentrations (μg/ml): carbenicillin, 800; ampicillin, 60; tetracycline, 25. Acrylamide, bisacrylamide, and sodium dodecyl sulfate were obtained from Bio-Rad Laboratories, Richmond, Calif. The carrier ampholytes for isoelectric focusing were purchased from LKB Instruments, Inc., Gaithersburg, Md. [^{35}S]methionine was purchased from New England Nuclear Corp., Boston, Mass., and uniformly labeled [^{3}H]uridine, [^{32}P]dCTP, and [^{32}P]TTP were purchased from Amersham Corp., Arlington Heights, Ill. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc., Beverly, Mass. and were used according to manufacturer instructions.

Construction of pSB2001. The 3.0-kilobase *Sal*I fragment spanning part of the *nifL* gene, all of the *nifA* gene, and part of the *nifB* gene was electrophoretically purified (8). It was ligated into the *Sal*I site of pRZ4020 (the DNA for which was kindly provided by Reid Johnson). *E. coli* K-12 strain C600 was transformed with the ligation mixture by the $CaCl_2$-shock method. Ampicillin-resistant transformants were selected. Clones were initially screened for the presence of the 3.0-kilobase fragment by colony hybridization, using the labeled 3.0-kilobase fragment as the probe. Those clones demonstrating hybridization were subjected to restriction analysis, using *Sal*I and *Pvu*I maps of the *nif* region (J. J. Collins, unpublished data) to demonstrate the presence of the 3.0-kilobase fragment and determine its orientation relative to pRZ4020. *K. pneumoniae* UN was transformed with plasmid pSB2001 to produce strain UN5215.

Nitrogenase activity. *K. pneumoniae* cells were grown at 30°C to stationary phase in minimal medium with NH_4^+; carbenicillin and ampicillin were added for the growth of strain UN5215. Cells were pelleted, washed once, and diluted 10-fold into minimal medium without a nitrogen source. This 2-ml culture in a stoppered 16-mm test tube was evacuated and flushed with argon. After the cells were incubated for 1.5 h at the appropriate temperature, serine was added to 40 μg/ml, and the culture was incubated for an additional 3.5 h. The assay was initiated by the addition of 1 ml of C_2H_2, and the culture was incubated with shaking for

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TABLE 1. Acetylene-reducing activity at 30 and 41°C

Strain	Relevant phenotype	Activity (nmol of C_2H_2 min^{-1} mg of $protein^{-1}$)	
		30°C	41°C
UN	Wild type	30.6	<0.1
UN4357	$NifL^-$ (derepressed in O_2)	54.4	<0.1
UN4452	$NifL^-$ (derepressed in O_2 and NH_4^+)	46.9	0.3
UN5215	NifA protein overproduced (derepressed in O_2 and NH_4^+)	35.6	0.2

15 min. A 0.3-ml portion of the gas phase was removed for ethylene determination by flame ionization with a Packard gas chromatograph having a 1-Poropak N column. Protein concentration was determined by a modified Bradford assay (2).

Preparation of labeled extract. A 5-ml culture of *K. pneumoniae* cells was labeled and broken as described previously (20). Stationary-phase cultures of strain UN5215 were grown in the presence of carbenicillin and ampicillin.

Two-dimensional PAGE, fluorography, and autoradiography. Labeled extracts were spun in an Eppendorf microfuge for 30 s, and 50 to 100 μl of the supernatant solution was subjected to two-dimensional polyacrylamide gel electrophoresis (PAGE) as modified and described previously (20). Fluorography was performed, using liquid En^{3}Hance (New England Nuclear Corp.) according to manufacturer instructions. The enhanced gels were dried onto Whatman no. 1 filter paper with a Bio-Rad slab gel drier and placed in direct contact with Kodak X-Omat X-ray film (Eastman Kodak Co., Rochester, N.Y.) with incubation at −70°C.

***nif* mRNA measurements.** *nif* mRNA synthesis was measured by labeling samples of a *K. pneumoniae* culture derepressed for nitrogenase synthesis with [^{3}H]uridine at the times indicated in Fig. 2 and 3. Conditions for derepression, RNA labeling and extraction, and DNA-RNA hybridization, as well as for construction of *nif* DNA clones, plasmid DNA purification, and preparation of DNA filters for hybridization, have been described (Collins and Brill, submitted for publication).

RESULTS

Temperature effect on acetylene-reducing activity. Previous studies involving temperature effects on *nif* have used different high temperatures for the nonpermissive condition: 37°C (15), 39°C (9), and 41°C (30). Under our derepression conditions, incubation at 37°C produced variable results and was unsuitable for a quantitative analysis of temperature effects. We chose to use 41°C as the nonpermissive temperature as it gave very consistent results yet did not prevent growth when nitrogen fixation was not required.

Table 1 shows the results from experiments in which the entire 5-h derepression period took place at either 30 or 41°C. No acetylene-reducing activity was detected for any of the strains at the high temperature. Strains UN4357 and UN4452 are both Nif^+ revertants of Mu insertions in the *nifL* gene (12) and are therefore $NifL^-$, yet they are both subject to temperature repression. UN5215 contains a wild-type *nif* regulon on the chromosome and carries pSB2001, a multicopy plasmid from which the *nifA* gene product is synthesized constitutively from a *lacUV5* promoter. Constitutive overproduction of the *nifA*-coded gene product from the plasmid allows UN5215 to overcome repression by oxygen and ammonium as evidenced by acetylene reduction (for the ammonium effect only) and by two-dimensional PAGE (data not shown). However, UN5215 failed to reduce acetylene at 41°C, suggesting either that the activity of the nitrogenase complex is temperature sensitive or that constitutive overproduction of the *nifA*-coded protein is not sufficient for derepression of nitrogenase synthesis at high temperature.

The kinetics of temperature repression were investigated in experiments performed as described in Table 2. At various times during the derepression time course, part of the bacterial culture was shifted to 41°C and assayed for acetylene-reducing activity at times thereafter. The repression effect is best seen early in derepression when the normal increases in activity at 30°C were most dramatic. When the cultures were shifted to 41°C, the increases were eliminated. The two $NifL^-$ mutants showed the same degree of temperature repression as did the wild-type strain, indicating again that the *nifL* gene product is not required for temperature repression of *nif*.

The temperature stability of the nitrogenase complex was studied by in vitro assays of purified components. Neither the dithionite-coupled assay nor the *nifJ* protein-coupled assay is affected after a 15-min incubation at 41°C (V. K. Shah, personal communication).

Temperature effect on *nif* protein synthesis. The synthesis of the *nif* proteins was investigated by two-dimensional PAGE of radioactively labeled cell extracts. The autoradiograms of wild-type cells grown at 30°C and 41°C are shown in Fig. 1A and B, respectively. The *nifA*-coded protein is the only *nif* protein present in cultures grown at 41°C. The lack of other *nif* proteins at high temperature was also seen in autoradiograms of a UN4357 extract (data not shown). In strain UN5215, small amounts of some *nif* proteins were synthesized at 41°C but much less than the amounts synthe-

TABLE 2. Kinetics of high-temperature repression

Strain	Time of shift (h after depression)	% Final acetylene-reducing activity[a] after incubation at 41°C for (min):			
		0	30	60[b]	90
UN	2	1.0	1.2	1.4 (13.8)	0.5
	3	13.8	13.4	15.3 (63.4)	13.9
UN4357	2	5.5	2.5	2.6 (21.7)	2.0
	3	21.7	18.9	22.6 (80.6)	22.2
UN4452	2	7.4	8.3	7.5 (33.1)	7.6
	3	33.1	36.8	30.5 (76.8)	30.2

[a] Final activity is that obtained for the strain after a standard 5-h derepression at 30°C.
[b] Values within parentheses are percent final activity for parallel cultures held at 30°C for 60 min.

sized in either wild-type strain or strain UN5215 at 30°C (data not shown).

Temperature effect on *nif* mRNA synthesis. The effect of elevated temperature on *nif* mRNA synthesis was investigated. Derepressed cultures were shifted from 30 to 41°C at a time when transcription of the *nif* operons was maximal and constant, and *nif* mRNA synthesis was measured at times thereafter. The shift to 41°C rapidly repressed mRNA synthesis from the *nif* operons studied (*nifKDH*, *nifMVSU*, and *nifJ*) in the wild-type and two NifL$^-$ strains. Data for *nifKDH* are shown in Fig. 2. We conclude that transcription of the six *nif* operons regulated by the products of the *nifAL* operon is repressed at 41°C and that the *nifL* gene product is not required for this repression. *nifAL* mRNA synthesis is not repressed at 41°C (Collins and Brill, submitted for publication).

The six *nif* operons repressed at 41°C are subject to positive regulation of transcription by the *nifA* gene product and negative regulation of transcription by the *nifL* gene product in the presence of oxygen and ammonium. We have shown that the *nifL* gene product is not involved in repression at 41°C. It therefore seems likely that repression of these six operons at 41°C results from a loss of positive activation of transcription by the *nifA* gene product.

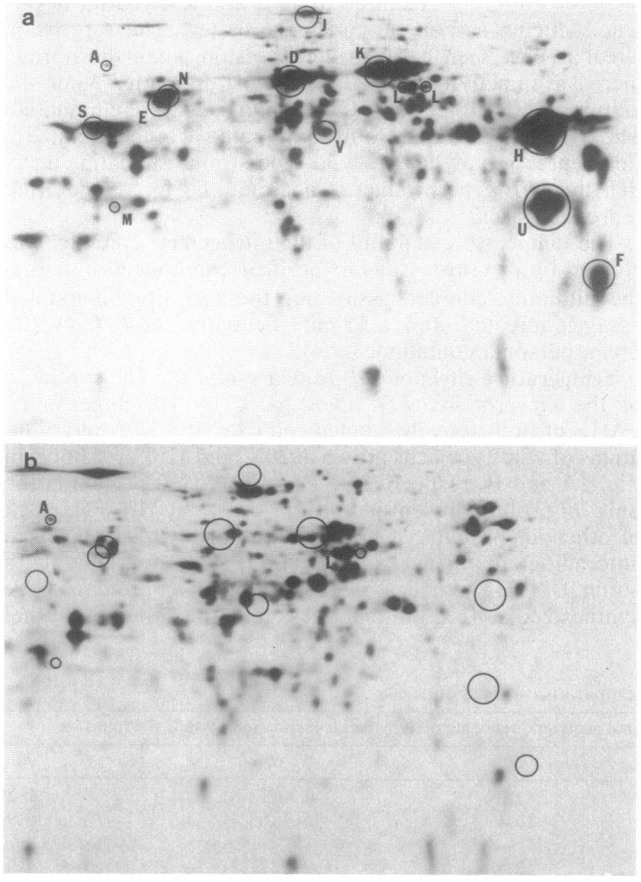

FIG. 1. Two-dimensional PAGE of [^{35}S]methionine-labeled proteins from wild-type *K. pneumoniae* labeled at 30 (a) or 41°C (b). The entire 4-h derepression period as well as the 5-min labeling and chase periods took place at the appropriate temperature. Letters indicate the *nif* gene which codes for the protein circled. *nif* proteins not detected are indicated by unlabeled circles.

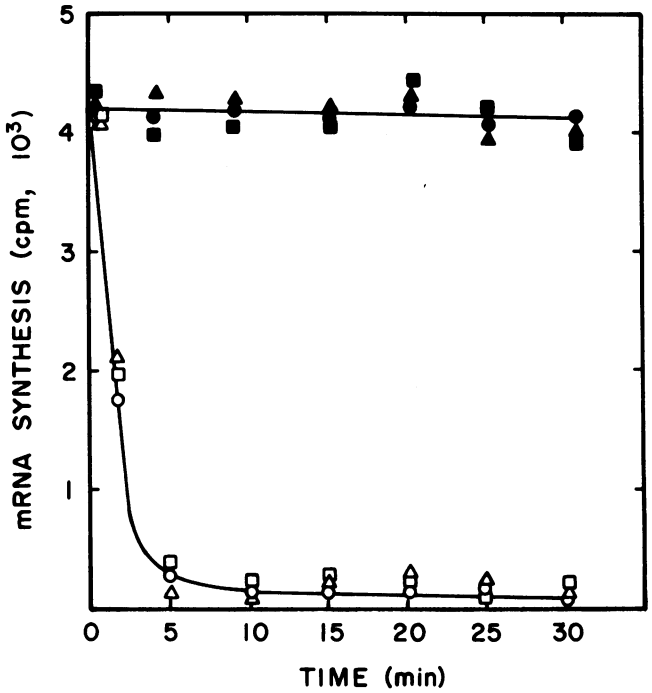

FIG. 2. Effect of increased temperature on *nifKDH* mRNA synthesis. Derepressed cultures of wild-type (circles) and NifL$^-$ strains UN4357 (triangles) and UN4452 (squares) were shifted from 30 to 41°C at zero time. Samples taken from cultures at the times shown were pulse-labeled with [^{3}H]uridine to label mRNA being synthesized. [^{3}H]RNA was extracted and hybridized with a specific DNA probe to determine *nifKDH* mRNA synthesis. Filled symbols represent cultures which remained at 30°C; open symbols represent those shifted to 41°C at zero time.

If the positive regulatory function of the *nifA* gene product is inactivated at 41°C, the following experiment shows that this inactivation is rapidly reversible. Two derepressed cultures were shifted from 30 to 41°C, after which time synthesis of *nif* mRNA was undetectable. Tetracycline was added to inhibit protein synthesis to one culture while it was at 41°C, and both cultures were returned to 30°C. mRNA synthesis from the *nifKDH*, *nifMVSU*, and *nifJ* operons was measured at times throughout the experiment. In both cultures (with and without tetracycline) *nif* mRNA synthesis resumed almost immediately upon return of the cultures to 30°C (Fig. 3). The resumption of transcription was more rapid than would be expected if new *nifA* protein synthesis were necessary and was seen in a culture where new *nifA* protein synthesis was blocked by tetracycline. Apparently, the *nifA*-coded protein which is inactivated at 41°C rapidly regains the ability to activate *nif* transcription after it is shifted back to 30°C. The *nifA* protein itself or some other feature of the interaction involved in activation of *nif* transcription by the *nifA* protein may be the actual temperature-sensitive component.

DISCUSSION

Temperature sensitivity of nitrogen fixation was studied by several approaches to deduce the nature of the temperature-sensitive component(s) of the system. No nitrogenase activity was detected when *K. pneumoniae* was derepressed at 41°C. Nitrogenase was not inactivated by high temperature incubation of in vitro assays of purified components. *nif*

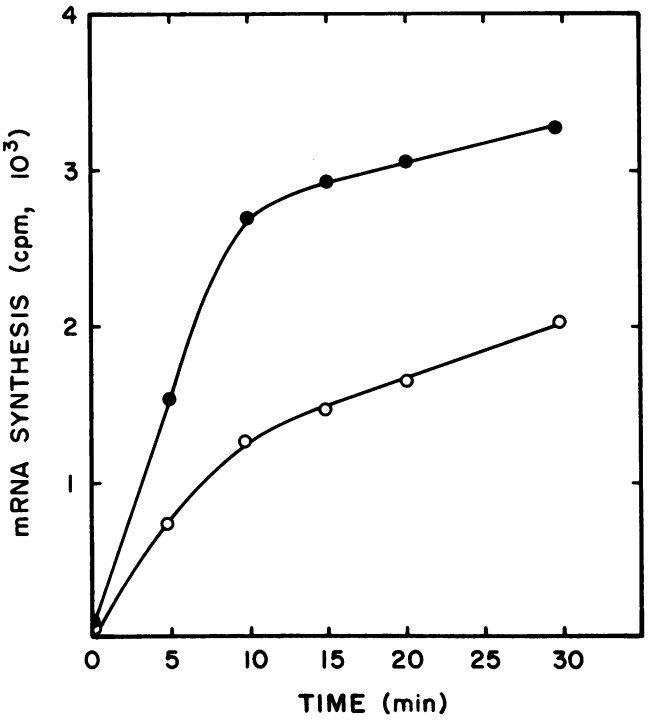

FIG. 3. *nifKDH* mRNA synthesis after temperature shift up and down. At the same time as that described in the legend to derepressed cultures of wild-type cells were shifted from 30 to 41°C to repress *nif* mRNA synthesis. While at 41°C, tetracycline (20 μg/ml) was added to one culture to prevent new protein synthesis. After 15 min, the cultures were rapidly returned to 30°C in an ice-water bath and then incubated at 30°C. Samples taken at times shown were treated as described in the legend to Fig. 2 to determine *nifKDH* mRNA synthesis in untreated (●) and tetracycline-treated (○) cultures.

protein synthesis was repressed by high temperature, as seen on two-dimensional polyacrylamide gels of labeled extracts, although the *nifA* gene product continued to be made. This result is supported by experiments in which fusions of the *lacZ* gene to the *nifAL* operon expressed β-galactosidase at 41°C, whereas expression of all other *nif-lacZ* fusions was repressed at that temperature (13). mRNA synthesis from *nif* operons regulated by *nifA* was rapidly repressed when the culture was shifted from 30 to 41°C. Temperature-repressed cultures were immediately capable of resuming transcription of *nif* upon a shift back to 30°C, even when protein synthesis was blocked by tetracycline, indicating that temperature inactivation of the sensitive component is reversible. In all experiments, the wild-type strain, NifL$^-$ mutants, and a strain constitutively overproducing the *nifA* gene product were sensitive to repression at 41°C, although the last strain was capable of a limited amount of *nif* protein synthesis at high temperature.

Merrick et al. have concluded that the *nifL* protein acts as a repressor of *nif* transcription at 37°C (15). Our data support the hypothesis that the activity of the *nifA* protein is temperature sensitive (3) and that the *nifL* protein is not required for temperature repression of the *nif* regulon. Strain UN4357, a Nif$^+$ revertant of a Mu insertion in the *nifL* gene, which lacks the *nifL* gene product, is fully repressed for *nif* mRNA and protein synthesis by incubation of cultures at 41°C. As suggested by Buchanan-Wollaston et al. (3), the *nifA* protein has some activity at 37°C. The observation that at 37°C NifL$^-$ mutants derepress to a greater extent than does the wild-type strain is probably due to a diminished requirement for *nifA* protein in the absence of functional *nifL* protein. This would explain the results of Merrick et al. (15) which led them to assign a repressing function to the *nifL* protein at 37°C.

The six *nif* operons which are repressed at high temperature require the *nifA* protein for their transcription (Collins and Brill, submitted for publication). The *nifAL* operon, which is not repressed at high temperature, does not require the *nifA* protein for its transcription (4, 11, 13, 20). These results are consistent with the proposal that loss of *nifA* protein activity is responsible for repression of *nif* at high temperature.

The data presented in this paper suggest that repression of *nif* by high temperature is exerted by preventing the interaction of the *nifA* protein with the regulatory region of the six *nif* operons whose transcription it activates. The temperature-sensitive component could be either the *nifA* protein, the conformation of the DNA with which it interacts, or the interaction itself. It has recently been suggested to us that a more global temperature regulatory protein may be responsible for the phenotype (F. Neidhart, personal communication). If there is a temperature regulatory protein that acts on a number of operons, including *nif*, it must act to prevent *nifA* protein-mediated activation of transcription, since constitutively synthesized *nifA* protein is incapable of completely overcoming 41°C repression of *nif* transcription. It seems more likely that a global regulatory molecule would act to prevent the synthesis of the *nifA* protein itself, in a manner analogous to *ntr* regulation of *nif*. No such regulation of *nifA* gene expression at 41°C was observed.

It has previously been shown that the formation of a purple pigment by *K. pneumoniae* cells grown in the presence of 6-cyanopurine requires the presence of active *nifA* protein and is also temperature sensitive (30). The *nifA* protein is the only *nif*-specific gene product required (11). The correlation between the temperature sensitivity of *nifA*-dependent activation of *nif* transcription and the *nifA*-dependent production of the purple pigment supports the hypothesis that temperature sensitivity of *nif* expression is a consequence of temperature sensitivity of *nifA* protein activity.

The large amount of energy necessary for the production of active nitrogenase favored the evolution of mechanisms for the regulation of the *nif* regulon. In an environment rich in fixed nitrogen, it is useless for the bacterium to synthesize large amounts of the unnecessary enzymes. Likewise, in the presence of oxygen, it would be wasteful to synthesize proteins destined to become irreversibly inactivated. Repression by high temperature is more difficult to rationalize. Neither nitrogenase activity nor electron transport to nitrogenase is impaired in vitro by incubation at high temperature. It is possible that a step before transport of electrons to nitrogenase, such as activation and assembly of the nitrogenase protein complex, is defective at high temperature. It is also possible that the *nifA* protein or its interaction with its target DNA is inherently temperature sensitive and that the nature of the *nifA* protein or the protein-DNA interaction prevents the evolution of temperature resistance. Since the organism is capable of growth on nitrogen sources other than N_2 at 41°C, the interaction between other proteins and the DNA coding for essential functions must not be significantly temperature sensitive, making it unlikely that the *nifA-nif* promoter DNA interaction itself is necessarily temperature sensitive. *K. pneumoniae* is found in a wide variety of

environments, including soil, plants, and animals, and therefore sees a wide range of temperature conditions. Repression by high temperature may be a signal to the bacterium that it is currently in an environment unsuitable for nitrogen fixation. The rapid reversibility of the temperature effect suggests that the environment to which the bacterium is responding during temperature repression in nature fluctuates to such an extent that the constant presence of activatable *nifA* protein is necessary for survival. Experiments are currently underway to investigate these hypotheses.

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