BIOCHIMIE — Extrait du Tome 62, n° 4, 1980, p. 267.

Printed in France.

Characterization and kinetics of the biosynthesis of some nitrogen fixation *(nif)* gene products in *Klebsiella pneumoniae.*

Jean HOUMARD ◇, Didier BOGUSZ, Régis BIGAULT and Claudine ELMERICH.

Unité de Physiologie Cellulaire, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, 28 rue du Docteur Roux, F-75724 Paris Cédex 15.

(21-12-1979).

Résumé.

Chez *Klebsiella pneumoniae*, une étude comparative des protéines synthétisées soit par une souche Nif$^+$, soit par des mutants *nif*::Mu, soit encore par des mutants portant une délétion de gènes *nif* a été réalisée par la technique des gels à deux dimensions. La comparaison des autoradiogrammes obtenus avec les extraits des cellules incubées 10 minutes en présence d'acides aminés ^{14}C a permis de repérer six produits spécifiques des gènes *nif*. En plus des produits des gènes de structure du complexe nitrogénase (*nifK*, *nifD*, *nifH*) et du produit de *nifJ* préalablement caractérisés, un polypeptide de 10.000 daltons et de pI environ 4,5 a été identifié comme produit du gène *nifF*, et un polypeptide de 22.000 daltons et de pI 5 comme produit du gène *nifU*. La biosynthèse de ces deux derniers produits est sous le contrôle du gène de régulation *nifA*, tout comme cela avait déjà été montré pour les autres produits *nif*. Les résultats de cette étude confirment les données préalablement obtenues par l'analyse génétique concernant l'organisation en unités de transcription des gènes *nif* de *Klebsiella pneumoniae*.

Une cinétique de la biosynthèse des six produits *nif* identifiés et de l'apparition de l'activité nitrogénase, lors de la dérépression des gènes *nif*, a été réalisée. Les six produits ne sont décelables qu'après 45 minutes et leur apparition est simultanée, ce qui suggère une transcription coordonnée des différentes unités. Cependant, l'activité nitrogénase n'est décelable, tant *in vivo* qu'*in vitro*, que 30 minutes plus tard. Une étude cinétique analogue a été réalisée avec la souche Nif$^+$, cultivée en conditions de fixation, à laquelle des ions NH_4^+ ont été ajoutés. Dans ces conditions, on observe un arrêt de la biosynthèse des différents produits *nif*. Cependant, cet arrêt n'est pas synchrone pour les six produits car la synthèse du produit de *nifU* n'est plus décelable 5 minutes après l'addition d'ions NH_4, celle de *nifF*, *K*, *D* et *J* ne l'est plus après 30 minutes tandis qu'on observe une synthèse résiduelle du polypeptide *nifH* après 60 minutes. Lors de la répression par les ions NH^4, il semble donc que la transcription des gènes *nif* soit bloquée et que seuls les RNA messagers préexistants continuent d'être traduits.

Mots-clés : Fixation de l'azote ; *Klebsiella pneumoniae* ; produits des gènes *nif*.

Summary.

Analysis of ^{14}C pulse-labelled proteins, synthesized by a Nif$^+$ *Klebsiella pneumoniae* strain and by a number of genetically mapped *nif*::Mu and *nif* deletion mutants, was performed by two-dimensional gel electrophoresis. By comparison of the autoradiograms, six *nif*-specific polypeptides were identified. In addition to the previously characterized *nifK*, *nifD*, *nifH* and *nifJ* products, the product of *nifF* was identified as a polypeptide of 10,000 daltons and pI about 4.5 and the product of *nifU* as a polypeptide of 22,000 daltons and pI 5. Moreover, the biosynthesis of *nifF* and *nifU* polypeptides was shown to be prevented in mutants affecting the regulatory gene *nifA*, which is known to control the biosynthesis of the other *nif* genes products so far identified. In all cases, the biochemical phenotypes of the different polar mutants were in good agreement with those expected from

◇ *To whom all correspondence should be addressed.*

O.R.S.T.O.M. Fonds Documentaire
N° : 16443
Cote : B
9 JANV. 1985

the transcriptional organization of the *nif* cluster previously established by genetic analysis.

Kinetic studies of both nitrogenase activity and of the biosynthesis of the six *nif*-specific polypeptides were performed with the Nif^+ strain, incubated either under conditions of derepression or under conditions of repression by NH_4^+ ions. Upon derepression, the biosynthesis of the six *nif* polypeptides, which belong to four different transcriptional units, seems to be coordinated since they appear simultaneously after a lag of 45 minutes. Under those conditions, both *in vivo* and *in vitro* nitrogenase activities were detectable only 30 minutes later. Upon addition of NH_4^+ ions, the biosynthesis of the six *nif* polypeptides was rapidly abolished. However, the kinetics of residual biosynthesis, probably due to the transcription of preexisting mRNAs, was not similar for the six *nif* products. The *nifU* product was no longer detectable after 5 minutes, the *nifF, K, D* and *J* products were not detectable after 30 minutes, whereas some *nifH* product was still slightly detectable after 60 minutes.

Key-words : Nitrogen fixation ; *Klebsiella pneumoniae* ; *nif* gene products.

Introduction.

Genetic studies of nitrogen fixation in *Klebsiella pneumoniae* led to the definition of 14 *nif* cistrons organized in seven transcriptional units [1-5]. Biochemical characterizations of some *nif* mutations have been reported [1, 2, 6, 7]. However, relatively few of the *nif* gene products and functions have so far been identified unambiguously. The nitrogenase complex of *Klebsiella pneumoniae* consists of two proteins Kp_1 and Kp_2 [8] which, according to Hageman and Burris [9], are termed respectively nitrogenase (Kp_1) and nitrogenase reductase (Kp_2). The nitrogenase is a tetramer made up of two non-identical subunits $\alpha_2\beta_2$ [10] ; the nitrogenase reductase is a dimer made up of identical protomers [8]. The *nifH, nifD* and *nifK* genes have been identified as the genes coding for Kp_2, $Kp_1\alpha$ and $Kp_1\beta$ subunits [1, 2, 6]. *NifA* was shown to be involved in regulation [1, 2, 6, 11], *nifB* [7] and tentatively *nifN* and *nifE* [6] in the molybdenum-iron cofactor (FeMoco) synthesis, *nifM* in the processing of Kp_2 [6]. The product of *nifJ* has been identified as a polypeptide of 120,000 daltons molecular weight [1, 2, 6] that would be required for acetylene-reducing activity *in vivo*, but not *in vitro* [6]. In addition to the products of *nifH, D, K* and *J*, the products of *nifN, nifE, nifS* and *nifF* have been identified as polypeptides of 50,000, 46,000, 18,000 and 17,000 daltons molecular weight [6]. All these products are present under anaerobic conditions, only when bacteria are grown in the absence of NH_4^+ ions.

We report here the identification of two new products, which we assigned to *nifF* and *nifU* genes, and the kinetics of derepression and repression by ammonium ions of the biosynthesis of the *nifF, nifU, nifK, nifD, nifH* and *nifJ* products.

Materials and Methods.

Bacteria and plasmids.

The *K. pneumoniae* strains are listed in table I. The genotype of the different Nif^- plasmids [2, 4] appears in table II. As a uniform nomenclature has been recently set up, plasmids previously termed pLS1, pLS17, pNC172 for example [2] are now termed pPC1, pPC17 and pPC172, with allele numbers *8001, 8017* and *8172*. All the plasmids are pCE1 derivatives. The pCE1 plasmid derives from pRD1 (amp^+ tet^+ kan^+ gnd^+

TABLE I.
Klebsiella pneumoniae strains.

Strain	Genotype or phenotype	Reference
UNF107	Δ *(gnd-his-nif) 107 rpsL*	Dixon *et al.* 1977 [11]
KP52	Δ *(gnd-his-nif) 4648 rpsL*	Streicher *et al.* 1972 [14]
UNF131	Δ *(his-nif) 2473 hsdRI rpsL*	Merrick *et al.* 1978 [1]
UNF156	Δ *nif-2479 hsdRI rpsL4*	Merrick *et al.* 1978 [1]
UNF169	Δ *nif-2482 hsdRI*	Merrick *et al.* 1978 [1]
UNF177	Δ *(his-nif) 2483 hsdRI*	Merrick *et al.* 1979 [4]

Genetic symbols are those of Bachman *et al.* [12].

his$^+$ *nif*$^+$ *shi*$^+$ *tra*$^+$ *incP*) constructed by Dixon *et al.* [13]. Unlike pRD1, pCE1 does not suppress the Mu sensitivity in *E. coli* strains [2]. In this study, all the plasmids were carried by UNF107 *K. pneumoniae nif* total deletion strain.

cell pellet was resuspended in 200 µl of the « lysis buffer » described by O'Farrell [16]. Triton X-100 was used instead of Nonidet P40. Samples were subjected to five cycles of freezing in liquid nitrogen and thawing. After centrifugation at 4,500 g for 10 minutes, supernatants were withdrawn, frozen in liquid nitrogen and kept at —20°C.

TABLE II.

Biosynthesis of nif-*specific polypeptides by* nif *mutants.*

Genotype	Gene products						Plasmids or strains (allele)
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Spot 6	
wild type	++	++	++	++	++	++	pCE1
nifB : : Mu	++	++	++	++	++	++	pPC501 (*nif-8501*) pPC80 (*nif-8080*) pPC172 (*nif-8172*)
nifA : : Mu	—	—	—	—	—	—	pPC141 (*nif-8141*) pPC1 (*nif-8001*)
nifL : : Mu	—	—	—	—	—	—	pPC112 (*nif-8112*)
nifF : : Mu	++	++	++	++	++	—	pPC503 (*nif-8503*) pPC7 (*nif-8007*)
nifM : : Mu	+	++	++	++	++	++	pPC52 (*nif-8052*) pPC100 (*nif-8100*) pPC504 (*nif-8504*)
nifS : : Mu	++	+	w	++	++	++	pPC31 (*nif-8031*) pPC41 (*nif-8041*) pPC557 (*nif-8557*)
nifU : : Mu	++	+	w	++	—	++	pPC71 (*nif-8071*) pPC560 (*nif-8560*)
nifN : : Mu	++	++	++	++	++	++	pPC92 (*nif-8092*) pPC569 (*nif-8569*) pPC570 (*nif-8570*)
nifE : : Mu	++	++	++	++	++	++	pPC40 (*nif-8040*) pPC505 (*nif-8505*)
nifK : : Mu	+	—	—	++	++	++	pPC16 (*nif-8016*) pPC21 (*nif-8021*) pPC76 (*nif-8076*)
nifD : : Mu	+	—	—	++	++	++	pPC47 (*nif-8047*) pPC117 (*nif-8117*)
nifH : : Mu	++	—	—	—	++	++	pPC11 (*nif-8011*) pPC27 (*nif-8027*)
nifJ : : Mu	—	++	++	++	++	++	pPC15 (*nif-8015*) pPC45 (*nif-8045*)
*nif*Δ (*M-J*)	—	—	—	—	—	++	pPC111.1 (*nif-8760*)
*nif*Δ (*B-V*)	—	—	—	—	—	—	KP52 (*nif-4648*)
*nif*Δ (*B-M*)	—	—	—	—	—	—	UNF131 (*nif-2473*)
*nif*Δ (*N-H*)	+	—	—	—	++	++	UNF156 (*nif-2479*)
*nif*Δ (*K-J*)	—	—	—	—	++	++	UNF169 (*nif-2482*)
*nif*Δ (*-B*)	++	++	++	++	++	++	UNF177 (*nif-2483*)
*nif*Δ (*B-J*)	—	—	—	—	—	—	UNF107 (*nif-107*)

Relative intensities were estimated by visual comparison and noted + or ++ ; — indicates that the corresponding spot was not detected ; w indicates that the spot was weakly visible.

Culture media.

Nitrogen free (NFM) and Luria Broth (LB) media have been previously described [2, 15].

Growth conditions for radioactive labelling of proteins.

The following procedure has been used for the derepression of the nitrogenase complex. Strains were grown overnight, under N_2, in 10 ml of NFM containing 19 mM NH_4Cl. After centrifugation under argon, bacteria were resuspended, at 10^9 cells per ml in anaerobic NFM supplemented with 100 µg/ml aspartate and with or without 19 mM NH_4Cl. Five milliliters of culture were then incubated with shaking, under argon, at 30°C.

Samples of 3 ml were withdrawn, usually after 3 hours of incubation, and added anaerobically to a centrifuge tube which contained 6 µCi of a ^{14}C protein hydrolysate solution (CEA, France, ref. C4B). Ten minutes later, 4 ml of NFM containing 2 mg/ml casamino-acids were added and the cells were centrifuged. Cells were washed with 5 ml of the NFM casamino-acids and centrifuged for 10 minutes at 6,500 g. Each

Assays for nitrogen fixation.

Nitrogenase activity of whole cells was estimated by the acetylene reduction test [17]. *In vitro* acetylene-reducing activities were measured as described by Eady *et al.* [8]. Ethylene produced was measured with a 204 Pye Unicam gas chromatograph, by injecting 0.25 ml gas sample into a 40 × 0.2 cm column filled with Porapak T and equilibrated at 55°C.

Two-dimensional polyacrylamide gel electrophoresis.

The isoelectric focusing was performed according to Iborra and Buhler [18] with a LKB Multiphor apparatus. The gel contained 5 per cent acrylamide, 8.3 M urea and 2 per cent ampholines pH 3.5-10 (LKB). After 1 hour of prefocusing (20 mA, 1,000 volts and 200 watts were set up as maxima on the LKB2103 power supply), about 0.1 µCi of labelled proteins were loaded onto the gel and focusing was allowed to run for 2 hours at 12°C. Gel strips of about 1 cm width

were then cut and equilibrated in SDS Ortec sample buffer for 15 minutes at 30°C. Samples were then frozen in a dry ice-acetone bath and kept at —20°C.

The SDS gel electrophoresis, in the second dimension, was performed as described by Laemmli [19]. Either 10 or 13 per cent acrylamide running gels (about 10 cm height) were used. The height of the stacking gel (4.5 per cent acrylamide) was 1 cm. The focusing strip was set up on the top of the stacking gel and layered by a 2 per cent agarose solution (made up in 25 mM Tris-glycine buffer pH 8.6, containing 0.1 per cent SDS).

Molecular weights were determined using bovine serum albumin (67,000), egg albumin (45,000), α-chymotrypsinogen A (25,700), soybean trypsin inhibitor (21,500), ribonuclease (14,700) and horse heart cytochrome c (13,700) as standards in the second dimension. For pI determinations, focusing strips were sliced and the profile of the gradient was established by measuring the pH of each slice. In addition, the *nifH, D, K* and *J* products, of which molecular weights and pIs have been previously reported [1, 2, 6], were used as internal controls.

Scintillating autoradiography was performed according to Bonner and Laskey [20]. After water precipitation of 2,5-diphenyloxazole (PPO) and washings, the gel was soaked in a glycerol : acetic acid : water (1:10:89 v/v) solution for 1 hour at room temperature and dried between two cellophane sheets, under a hood. Whenever necessary, protein staining was achieved using Coomassie brilliant blue R250.

Kinetics of the derepression of nif *genes.*

A fermentor containing 1.5 liter of NFM supplemented with 19 mM NH_4Cl was inoculated with 7.5 ml of a LB grown UNF107 (pCE1) exponential culture. Initial absorbance was 0.02 at 570 nm. Cells were grown overnight at 23°C under N_2. After centrifugation under argon, bacteria were resuspended at 10^9 cells per ml in a fermentor containing 1.5 liter of anaerobic NFM supplemented with 100 μg/ml aspartate. Cells were incubated under argon at 30°C. Aliquots were withdrawn at given times : 3 ml samples for radioactive labelling (as described above), 10 ml samples for *in vivo* assays of nitrogenase activity and samples of about 150 ml for *in vitro* assays of nitrogenase activity. The latter samples were centrifuged under argon for 10 minutes at 6,500 g. The cell pellets were frozen in liquid nitrogen and stored at —20°C.

Crude extracts were obtained by disrupting the cells with glass beads in a Mickle apparatus (Gomshall, Surrey). Cell pellets were resuspended in 4 ml of 100 mM Tris-HCl pH 8.7 buffer, containing 1 mM sodium dithionite and 0.1 g/l dithiothreitol. After addition of 4 ml glass beads, the suspension was shaken three times for 1 minute under argon. After centrifugation at 1,000 g for 5 minutes, crude extracts were withdrawn and assayed for nitrogenase activity. Protein concentration was determined with Coomassie blue G250, using bovine serum albumin as a standard [21].

Repression by ammonium ions.

K. pneumoniae UNF107(pCE1) was grown in a 1.5 liter fermentor, under conditions of nitrogen fixation. When cell concentration of about 10^9 cells per ml was reached, ammonium ions (19 mM final concentration) were added. At different times, 10 ml samples were withdrawn for *in vivo* assay of nitrogenase activity and 3 ml samples for radioactive pulse labelling.

Results.

Identification of nif *gene products.*

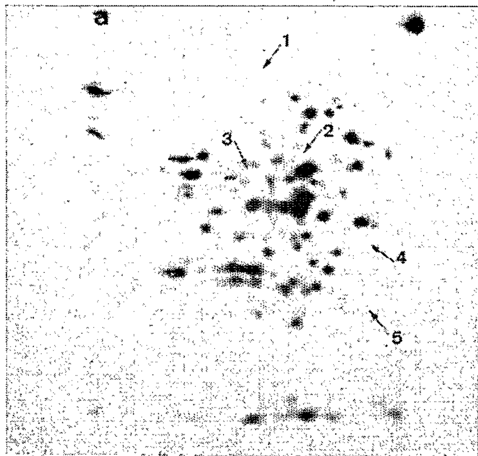
The methodology was based on the comparison of autoradiograms of two-dimensional gels of pulse-labelled proteins synthesized by the wild type strain and by *nif* mutants incubated under conditions of derepression or repression by ammonium ions of nitrogen fixation.

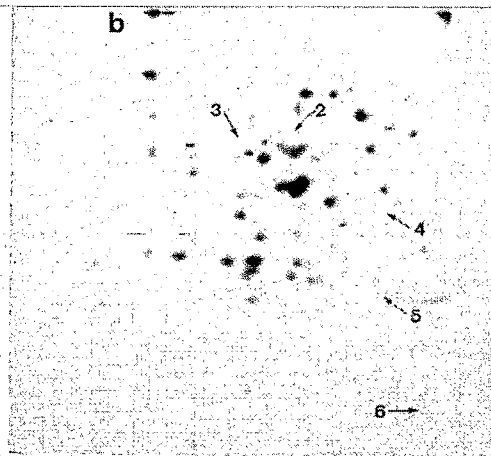
As shown in figure 1, with the wild type UNF107(pCE1), six spots, present when bacteria were incubated under conditions of nitrogen fixation, were missing when bacteria were grown in the presence of NH_4^+ ions. Five spots can be seen on both 10 per cent and 13 per cent acrylamide gel autoradiograms, spots 1 to 5 in figure 1c and spots 2 to 6 in figure 1d. None of the six polypeptides, can be detected with UNF107, a *nif* total deletion, incubated either with (data not shown) or without ammonia (fig. 1a and b). It can thus be assumed that the six spots correspond to shown or without ammonia (fig. 1a and b). It thus can be assumed that the six spots correspond to *nif*-specific polypeptides. Coelectrophoresis of pulse-labelled UNF107(pCE1) extracts with pure Kp_1 and Kp_2 proteins showed that spots 2 and 3 correspond to Kp_1 α and β subunits and that spot 4 corresponds to Kp_2 subunit.

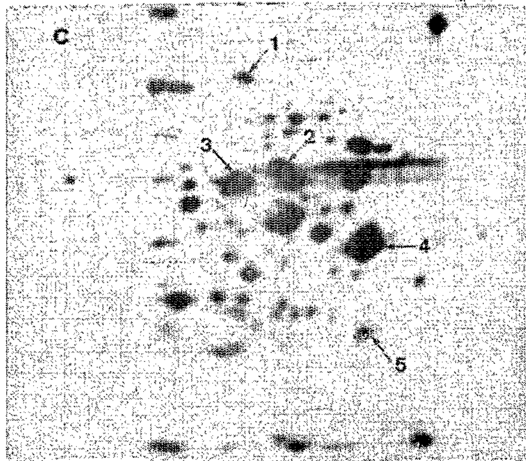
Autoradiograms of gels obtained with extracts from *nif*::Mu and *nif* deletion mutants were examined for the presence of each of the six *nif*-specific spots defined above. According to the results reported in table II, it was possible to assign spot 1 to *nifJ*, spots 2 and 3 to *nifK* and *D*, spot 4 to *nifH*, spot 5 to *nifU* and spot 6 to *nifF*.

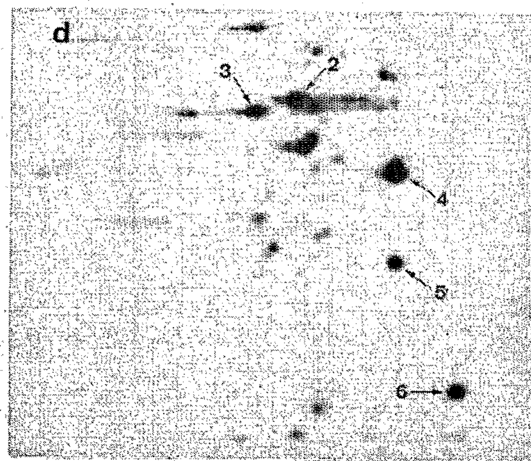
Fig. 1. — *Two-dimensional gel autoradiograms of* ^{14}C *proteins synthesized by the Nif⁻ strain UNF107 and the Nif⁺ strain UNF107 (pCE1).*

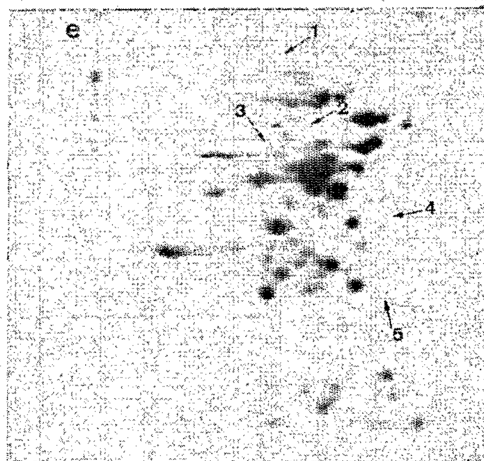
a and b : UNF107 ; c, d, e and f : UNF107 (pCE1).
a, b, c and d : cells incubated under argon in NFM + 100 μg/ml aspartate.
e and f : cells incubated under argon in NFM + 100 μg/ml aspartate + 19 mM NH_4Cl.
a, c and e : 10 per cent polyacrylamide SDS gels.
b, d and f : 13 per cent polyacrylamide SDS gels.
Arrows indicate the location of presumed *nif*-specific polypeptides.

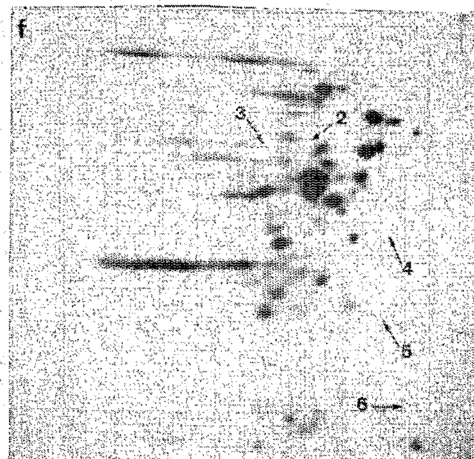

a
1
2
3
4
5


b
2
3
4
5
6


c
1
2
3
4
5


d
2
3
4
5
6


e
1
2
3
4
5


f
2
3
4
5
6

— The phenotypes of *nifB*::Mu, *nifM*::Mu, *nifN*::Mu and *nifE*::Mu mutants could not be distinguished from the phenotype of the wild type ;

— results concerning Mu insertions in *nifK*, *nifD*, *nifH* and *nifJ* were in agreement with those previously reported using one-dimensional gels

TABLE III.

Kinetics of the biosynthesis of nif-*specific polypeptides under conditions of derepression.*

Time after NH_4^+ exhaustion (minutes)	Gene products					
	nifF	*nifU*	*nifK*	*nifD*	*nifH*	*nifJ*
0	—	—	—	—	—	—
5	—	—	—	—	—	—
10	—	—	—	—	—	—
15	—	—	—	—	—	—
30	—	—	—	—	—	—
45	W	+	+	+	+	+
60	++	++	++	++	++	++
90	++	++	++	++	++	++
each hour from 120 to 360	++	++	++	++	++	++

Relative intensities were estimated by visual comparison and noted + or ++ ; — indicates that the corresponding spot was not detected. NH_4^+ repressed cells were resuspended in NFM at zero time.

TABLE IV.

Kinetics of repression by NH_4^+ ions of the biosynthesis of nif-*specific polypeptides.*

Time after NH_4^+ addition (minutes)	Gene products					
	nifF	*nifU*	*nifK*	*nifD*	*nifH*	*nifJ*
0	++	++	++	++	++	++
2	++	++	++	++	++	++
5	++	—	++	++	++	++
10	++	—	+	+	++	+
15	+	—	+	+	+	+
30	—	—	—	—	+	—
60	—	—	—	—	+	—

Relative intensities were estimated by visual comparison and noted + or ++ ; — indicates that the corresponding spot was not detected.

— an entirely negative phenotype was observed in *nifA*::Mu,*nifL*::Mu mutants and in mutants carrying deletions that covered the *nif*A/L region ;

— spot 6 is the only missing spot in *nifF*::Mu mutants ;

— in *nifS* and *nifU* mutants, low amounts of the polypeptides corresponding to spots 2 and 3 were present and, in *nifU* mutants, spot 5 was totally missing ;

[1, 2] on which bands corresponding to spots 1, 2, 3 and 4 had been characterized. In those mutants, the polypeptides corresponding to spots 5 and 6 were present ;

— as expected, phenotypes of the deletion mutants were in agreement with those of Mu insertion mutants.

Molecular weight and pI determinations showed that the *nifU* product is a polypeptide of about 22,000 daltons, pI 5 and that the *nifF* product is

a polypeptide of about 10,000 daltons and pI 4.5. Molecular weight and pI determinations of the *nifK, D, H* and *J* products were similar to those previously reported [1, 2, 6].

Kinetics of the derepression of nif *genes.*

It can be seen in table III that the spots corresponding to the six identified polypeptides appeared simultaneously on the autoradiograms, when bacteria had been incubated for 45 minutes under conditions of derepression in NFM. The intensity of the spots reached a maximum after one hour and did not change up to 6 hours. This suggests that the rate of biosynthesis of the six polypeptides was maximum after 1 hour and remained constant afterwards.

In the same experiment, the appearance of the acetylene-reducing activity was measured both *in vivo* and *in vitro* (data not shown). Activity was first detected after 75 minutes of derepression, increased linearly from 2 hours up to 4 hours and then remained constant. A lag between the appearance of Kp_1 and Kp_2 subunits and the detection of acetylene-reducing activity was previously reported with intact cells [22]. In addition, we found that both *in vivo* and *in vitro* activities appeared simultaneously.

Repression by ammonium ions.

Ammonium chloride (19 mM) was added to the culture vessel of a N_2-fixing culture of *Klebsiella pneumoniae* UNF107(pCE1) at zero time. Kinetic studies of both the acetylene-reducing activity and of the biosynthesis of *nif*-specific polypeptides were performed.

Addition of NH_4^+ ions resulted in a slight increase of the total acetylene-reducing activity of the culture during the first 10 minutes, followed by a plateau up to at least 3 hours. Decrease of the specific activity was directly related to dilution due to cell division (generation time of about 2 hours). These results were in agreement with data previously reported by Tubb and Postgate [23].

The effect of NH_4^+ addition on the biosynthesis of the six *nif*-specific polypeptides is shown in table IV. The *nifU* product was no longer detectable on the autoradiograms 5 minutes after NH_4^+ addition. The *nifF, K, D* and *J* products were no more detectable after 30 minutes, while some *nifH* product was still synthesized after 60 minutes.

Discussion.

By two-dimensional electrophoresis of extracts of *nif*::Mu and *nif* deletion mutants of *Klebsiella pneumoniae* pulse-labelled with ^{14}C aminoacids, the products of *nifF, U, K, D, H* and *J* were characterized. Products of *nifK, D, H* and *J* are now well identified [1, 2, 6]. In addition Roberts *et al.* [6] previously reported the characterization, by two-dimensional gel electrophoresis, of the products of *nifF, S, N* and *E*. According to these authors, the *nifF* product would be a polypeptide of 17,000 daltons and of pI 5, whereas results reported here lead to the conclusion that the *nifF* product would have a molecular weight of 10,000 daltons and a pI about 4.5. A new *nif* product, that of *nifU*, was identified as a polypeptide of 22,000 daltons and of pI 5.

Our characterization of *nifF* product was based on the phenotypes of pPC7 and pPC503 mutants which were identified as *nifF* by complementation analysis and deletion mapping [4]. None of the mutants complements the *nifF4066* point mutation described by St. John *et al.* [7]. Moreover crude extracts of strains carrying pPC7 (*nifF8007*::Mu) exhibit a high acetylene-reducing activity *in vitro* (unpublished results). This is in agreement with the usual phenotype of *nifF* mutants [6, 7, 11]. No obvious explanation can therefore account for the discrepancies between the results of Roberts *et al.* and those reported here.

As we did not show that point mutations in *nifF* and *nifU* altered the electrophoretic mobility of the corresponding polypeptides, we cannot rule out the possibility that *nifF* and *nifU* are regulatory genes involved in the biosynthesis of the identified polypeptides. Among all the *nif*::Mu insertions, only *nifA*::Mu in addition to *nifF*::Mu and *nifU*::Mu lead to the disappearance of the polypeptides assigned to *nifF* and *nifU*. Consequently if the polypeptides were not the products of *nifF* and *nifU*, they could only be the products of unidentified *nif* genes.

Genetic studies have shown that the *nif* genes are organized in seven transcriptional units : *nifQB, nifAL, nifF, nifMVSU, nifNE, nifKDH* and *nifJ* [3, 4]. The first six units were shown to be transcribed in the same direction from *nifH* towards *nifQ* [3, 4, 24]. The existence of *nifKDH* and *nifJ* as independent units, as well as the direction of transcription within the *nifKDH* operon, has been already confirmed by biochemical studies [1, 2, 6]. Results reported here are in agreement with *nifF* belonging to a transcriptional

unit independent of *nifMVSU*, as Mu inserts in *nifF* do not prevent the expression of *nifU*, nor Mu inserts in any of the *nifM*, *nifS* or *nifU* genes the expression of *nifF*. Moreover, our results agree with *nifNE* and *nifMVSU* being separate units and with *nifMVSU* transcribed from *nifU* to *nifM* as in both *nifN*::Mu and *nifS*::Mu mutants the *nifU* product is present.

Identification of *nifA* as a regulatory gene was first based on results reported by Dixon *et al.* who observed that point mutations in *nifA* prevented the biosynthesis of both Kp_1 and Kp_2 subunits [11]. This observation was confirmed with insertion mutants and it was shown that insertions in *nifA* prevented not only the expression of *nifK, D* and *H*, but also that of *nifJ* [1, 2, 6] and of *nifF, S, N* and *E* [6]. Results reported here showed that none of the *nif*-specific polypeptides that we identified, including the *nifF* and *nifU* products, were synthesized by *nifA*::Mu mutants nor by mutants carrying a deletion of the *nifA/L* region. It thus appears that the *nifA* product is required for the biosynthesis of all the *nif* polypeptides identified so far.

Concerning the organization of the transcriptional unit *nifKDH*, a difficulty previously mentioned [1, 2, 6, 11] was met once more. Indeed, insertion mutants in *nifK* synthesized the *nifH* product but not the product of *nifD*. Two hypotheses were proposed to account for this observation : either the absence of the *nifD* product is due to an antipolarity effect [1], or the α subunit of the nitrogenase is unstable in the absence of the β subunit [1, 2, 6]. Furthermore, Mu insertions in *nifU* and *nifS* were shown here to interfere with the presence of Kp_1 subunits. Such an effect has been reported by Roberts *et al.* [6] with *nifS*::Mu mutants, but was not observed with insertions in *nifM* or *nifV* [6, this report]. It is not yet possible to decide whether only the *nifS* product or both the *nifU* and *nifS* products are required for the presence of « normal » amounts of Kp_1 subunits since insertions in *nifU* have a polar effect on *nifS*. Among all the *nif*::Mu mutants studied, only insertions in *nifA/L, nifS* and *nifU* displayed a pleiotropic effect on the presence of Kp_1 subunits. This raises the question of whether *nifS* and *nifU* play a regulatory role similar to that of *nifA* in the expression of *nifK* and *nifD*. Since « normal » amounts of Kp_2 subunits are synthesized in *nifS* and *nifU* mutants, it is difficult to imagine such a regulatory role at the transcriptional level. A more likely explanation might be that either the *nifS* product or both the *nifU* and *nifS* products are involved in maturation of the *nifK* and *nifD* products.

The kinetic study of the appearance of the *nif* products and of nitrogenase activity during derepression showed that the products of *nifF, U, K, D, H* and *J* were detectable simultaneously after 45 minutes of derepression whereas nitrogenase activity was detectable only 30 minutes later. The six *nif* polypeptides belong to four independent transcriptional units but nevertheless their biosynthesis appear to be coordinated. One may thus imagine that the first event of the derepression is the synthesis of a specific positive activator (the *nifA* product for example) and that once the effector is present, the transcription of all the other *nif* transcriptional units proceeds concomitantly. Flemming and Haselkorn working with blue green algae [25] and Eady *et al.* with *Klebsiella pneumoniae* [22] have shown that, during the derepression of nitrogen fixation, the components of the nitrogenase complex are detectable long before the appearance of enzymic activity. Data reported here confirm this observation and show that the *nifF, U* and *J* products appear concomitantly with the *nifK, D* and *H* polypeptides. It was suggested that the lag between the appearance of Kp_1 and Kp_2 subunits and that of nitrogenase activity measured *in vivo* might be due to the lack of the specific electron donor [22]. Our results do not support this hypothesis as the biosynthesis of the *nifF* product is not particularly delayed and as the appearance of nitrogenase activity measured in crude extracts, using dithionite as the electron donor, is concomitant with the appearance of the activity in whole cells. As mentioned by Eady *et al.* [22], the possibility that the lag is due to the slow assembly of Kp_1 or Kp_2 from their respective subunits cannot be excluded.

As previously reported [22, 23], addition of NH_4^+ ions to a N_2-fixing culture of *Klebsiella pneumoniae* was found to have no effect on the preexisting nitrogenase activity which remains at a constant level. However, as shown in table IV, the biosynthesis of *nif*-specific polypeptides is rapidly abolished. It thus seems that transcription of the *nif* genes is turned off when NH_4^+ ions are added and that the observed biosyntheses correspond to the translation of preexisting mRNAs. Nevertheless, the kinetics of the residual biosyntheses is not similar for all the *nif* products studied. The *nifU* product is no longer synthesized 5 minutes after NH_4^+ ions addition whereas some *nifH* product is still synthesized at a low rate

after 60 minutes. All our results are in agreement with the hypothesis of Tubb and Postgate [23] who postulate that the repression by ammonium is effective at the level of mRNA synthesis. However the mechanism by which repression is mediated is not clearly understood.

Acknowledgements.

The authors wish to thank Drs R. A. Dixon and M. Merrick for gift of bacterial strains, Dr. J.-P. Aubert for helpful discussions and Dr. J. Shapiro for critical reading of the manuscript. D. Bogusz was the recipient of an O.R.S.T.O.M. studentship. R. Bigault was on leave from the Entreprise Minière et Chimique (E.M.C.). This investigation was supported by research funds from the University Paris VII and by a grant from the D.G.R.S.T. (No. 78-7-0440).

REFERENCES.

1. Merrick, M., Filser, M., Kennedy, C. & Dixon, R. (1978) *Molec. gen. Genet.*, **165**, 103-111.
2. Elmerich, C., Houmard, J., Sibold, L., Manheimer, I. & Charpin, N. (1978) *Molec. gen. Genet.*, **165**, 181-189.
3. MacNeil, T., MacNeil, D., Roberts, G. P., Supiano, M. & Brill, W. J. (1978) *J. Bacteriol.*, **136**, 253-266.
4. Merrick, M., Filser, M., Dixon, R., Elmerich, C., Sibold, L. & Houmard, J. (1980) *J. Gen. Microbiol.*, **117**, 509-520.
5. Elmerich, C. (1979) *Physiol. Vég.*, **17**, 883-906.
6. Roberts, G. P., MacNeil, T., MacNeil, D. & Brill, W. J. (1978) *J. Bacteriol.*, **136**, 267-279.
7. St. John, R. T., Johnston, M. H., Seidman, C., Garfinkel, D., Gordon, J. K., Shah, V. K. & Brill, W. J. (1975) *J. Bacteriol.*, **121**, 759-765.
8. Eady, R. R., Smith, B. E., Cook, K. A. & Postgate, J. R. (1972) *Biochem. J.*, **128**, 655-675.
9. Hageman, R. V. & Burris, R. H. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 2699-2702.
10. Kennedy, C., Eady, R. R., Kondorosi, E. & Klavans-Rekosh, D. (1976) *Biochem. J.*, **155**, 383-389.
11. Dixon, R. A., Kennedy, C., Kondorosi, A., Krishnapillai, V. & Merrick, M. (1977) *Molec. gen. Genet.*, **157**, 189-198.
12. Bachman, B. J., Low, K. B. & Taylor, A. L. (1976) *Bacteriol. Rev.*, **40**, 116-167.
13. Dixon, R., Cannon, F. C. & Kondorosi, A. (1976) *Nature*, **260**, 268-271.
14. Streicher, S. L., Gurney, E. G. & Valentine, R. C. (1972) *Nature*, **239**, 495-499.
15. Cannon, F. C., Dixon, R. A., Postgate, J. R. & Primrose, S. B. (1974) *J. Gen. Microbiol.*, **80**, 227-239.
16. O'Farrell, P. H. (1975) *J. Biol. Chem.*, **250**, 4007-4021.
17. Postgate, J. R. (1972) *in « Methods in Microbiology »* (Norris, J. R. and Ribbons, D. W., eds), vol. 6B, pp. 343-356, Academic Press, New York and London.
18. Iborra, F. & Buhler, J. M. (1976) *Anal. Biochem.*, **74**, 503-511.
19. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
20. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.*, **46**, 83-88.
21. Sedmak, J. J. & Grossberg, S. E. (1977) *Anal. Biochem.*, **79**, 544-552.
22. Eady, R. R., Issack, R., Kennedy, C., Postgate, J. R. & Ratcliffe, H. D. (1978) *J. Gen. Microbiol.*, **104**, 277-285.
23. Tubb, R. S. & Postgate, J. R. (1973) *J. Gen. Microbiol.*, **79**, 103-117.
24. Janssen, K. A., Riedel, G. E., Ausubel, F. M. & Cannon, F. C. (1980) *in « Nitrogen Fixation »* (Newton, W. E. & Orme-Johnson, W. H., eds), vol. 1, pp. 85-93, University Park Press, Baltimore.
25. Flemming, H. & Haselkorn, R. (1974) *Cell*, **3**, 159-170.