Biochimica et Biophysica Acta, 408 (1975) 101–111

BBA 46995

REGULATION OF NITROGEN FIXATION. NITROGENASE-DEREPRESSED MUTANTS OF *KLEBSIELLA PNEUMONIAE*

K. T. SHANMUGAM*, IRENE CHAN and CARLO MORANDI*

Department of Chemistry, University of California, San Diego, La Jolla, Calif. 92037 (U.S.A.)

(Received April 22nd, 1975)

SUMMARY

1. A new procedure is described for selecting nitrogenase-derepressed mutants based on the method of Brenchley et al. (Brenchley, J. E., Prival, M. J. and Magasanik, B. (1973) J. Biol. Chem. 248, 6122–6128) for isolating histidase-constitutive mutants of a non-N_2-fixing bacterium.

2. Nitrogenase levels of the new mutants in the presence of NH_4^+ were as high as 100 % of the nitrogenase activity detected in the absence of NH_4^+.

3. Biochemical characterization of these nitrogen fixation (*nif*) derepressed mutants reveals that they fall into three classes. Three mutants (strains SK-24, 28 and 29), requiring glutamate for growth, synthesize nitrogenase and glutamine synthetase constitutively (in the presence of NH_4^+). A second class of mutants (strains SK-27 and 37) requiring glutamine for growth produces derepressed levels of nitrogenase activity and synthesized catalytically inactive glutamine synthetase protein, as determined immunologically. A third class of glutamine-requiring, nitrogenase-derepressed mutants (strain SK-25 and 26) synthesizes neither a catalytically active glutamine synthetase enzyme nor an immunologically cross-reactive glutamine synthetase protein.

4. F-prime complementation analysis reveals that the mutant strains SK-25, 26, 27, 37 map in a segment of the *Klebsiella* chromosome corresponding to the region coding for glutamine synthetase. Since the mutant strains SK-27 and SK-37 produce inactive glutamine synthetase protein, it is concluded that these mutations map within the glutamine synthetase structural gene.

INTRODUCTION

During the last 15 years, nitrogenase has been studied extensively because of its essential role in biological nitrogen fixation. In 1971 an *nif* gene cluster which codes for the synthesis of this crucial enzyme was discovered and studied in *Klebsiella pneumoniae* [1, 2]. Mutants derepressed for nitrogenase biosynthesis in the presence

* Present address: Plant Growth Laboratory, University of California, Davis, Calif. 95616 (U.S.A.).

of NH_4^+, a repressor, have been described in *Azotobacter vinelandii* [3]. Recently, regulatory genes which govern the levels of nitrogenase in the cell have been identified in *K. pneumoniae* and glutamine synthetase has been implicated as a genetic modulator of *nif* gene expression [4, 5]. Streicher et al. [4] constructed three glutamine synthetase constitutive mutants (Gln C^-) of *K. pneumoniae* by transferring the Gln C^- property from *Klebsiella aerogenes* using the phage P_1. Two of these mutants produced nitrogenase in the presence of NH_4^+ to a maximum level of 3 and 30 % respectively, compared to the levels in the absence of NH_4^+, while the third mutant produced no nitrogenase activity in the presence of NH_4^+. In this communication we describe a new procedure for isolation of nitrogenase-derepressed mutants of *K. pneumoniae*, a method which may be applicable for producing nitrogenase-derepressed mutants of a variety of types of N_2-fixing organisms. The procedure is based on a method used by Brenchley et al. [6] for selecting histidase-derepressed mutants of the non-N_2-fixing organism, *K. aerogenes*. The properties of seven nitrogen fixation (*nif*) derepressed mutants of *K. pneumoniae* which continue to synthesize nitrogenase, in the presence of NH_4^+, at levels as high as 100 % of those synthesized under derepressed conditions (in the absence of NH_4^+) are described.

MATERIALS AND METHODS

Bacterial strains. Klebsiella pneumoniae strains M5Al, Asm^- mutants derived from strain M5Al [7] and glutamine- or glutamate-requiring auxotrophs derived from Asm^- mutants were used in this study. A summary of the properties of pertinent strains is given in Table I.

Media. The bacterial culture media used (L-broth, sucrose minimal medium) were as described before [8]. Supplements such as L-glutamate, L-aspartate or L-glutamine (freshly prepared by filter sterilization; Calbiochem, A grade) were added to the minimal medium to a final concentration of 100 μg/ml. For ammonia-supported growth, $(NH_4)_2SO_4$ at a final concentration of 1 mg/ml was used.

Cultivation of bacteria. All bacteria used for enzyme assays were cultured anaerobically at room temperature for 18 h (until the mid-exponential phase of growth). Cells were grown in 1-liter Erlenmeyer flasks containing 500 ml of sucrose minimal medium and were constantly sparged with a stream of N_2.

Mutagenesis and selection. To obtain nitrogenase-derepressed mutants, cultures of either strain Asm-1, Asm-3 or Asm-24 [7] were treated with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG, Aldrich Chemical Co.), according to the procedure of Adelberg et al. [9], with modifications as follows: cells grown overnight at 37 °C in 10 ml of sucrose minimal medium in screw-cap tubes (16 × 150 mm) were harvested by centrifugation at 12 000 × *g* for 5 min and washed three times with 10 ml saline to remove any traces of the medium. For mutagenesis, washed cells were incubated in 10 ml of saline containing 300 μg/ml of NTG for 30 min at room temperature. To remove NTG the cells were washed by centrifugation (three times with 10-ml aliquots of saline) and finally resuspended in 1 ml of saline solution. To allow segregation of mutagenized genomes to occur cells were grown in L-broth for 2 h. This cell suspension was appropriately diluted and plated on L-broth plates to obtain about 100 clones per plate. Auxotrophic mutants in the population were isolated using replica-plating techniques. Glutamine or glutamate auxotrophs were selected and tested for dere-

pressed synthesis of nitrogenase. Those glutamine or glutamate auxotrophs which are simultaneously derepressed for nitrogenase were used in this study.

Enzyme assays. Nitrogenase activity in whole cells was determined using the acetylene reduction procedure as described earlier [8]. Cell-free crude enzyme extracts were obtained following disruption using a French pressure cell [4]. Glutamine synthetase activity in the crude extracts was determined according to the procedure of Shapiro and Stadtman [10], by measuring the amount of γ-glutamyl hydroxamate produced at 37 °C. Both glutamate synthase and glutamate dehydrogenase activities were determined in freshly prepared extracts by following the rate of NADPH oxidation in appropriate assay mixtures at room temperature according to the procedures of Prusiner et al. [11]. Protein was determined by the method of Lowry et al. [12] using bovine serum albumin as a standard.

Genetic mapping. F-prime (F′) complementation analysis was carried out as described before [8], in order to map the mutations leading to nitrogenase derepression. F′105 and F′133 derived from *Escherichia coli* (kindly supplied by Dr B. Bachmann, Coli Genetic Stock Center) carrying *E. coli* chromosomal markers between 75.5–77.5 min and 72–77.5 min respectively, of the genetic map [13, 14] were used in these experiments. Prototrophic colonies which appeared on sucrose minimal medium after 48–72 h at 37 °C were scored as evidence for transfer of the glutamine synthetase genes on the episome. Several of these *Klebsiella/E. coli* hybrids were isolated for biochemical and genetic analysis.

The presence of glutamine synthetase genes carried by the episome was detected by using acridine orange curing [15] or alternatively by scoring for the loss of the glutamine synthetase activity following growth for several generations in L-broth. For acridine orange curing, an overnight culture, suspected to carry an episome, was diluted 10^{-4} into L-broth containing 100 μg/ml acridine orange, 0.3 % sucrose, and 200 μg/ml L-glutamine and allowed to multiply in this medium for 24 h at 37 °C. The second procedure for curing was similar except that the acridine orange and glutamine were omitted.

Immunodiffusion experiments. Glutamine synthetase antibody was prepared according to the procedure of Tronick et al. [16]. Rabbits were immunized with 10 mg of pure *E. coli* glutamine synthetase ($E_{\bar{n}} = 1.4$) (kindly supplied by E. R. Stadtman and R. E. Miller) mixed with an equal volume of Freund's adjuvant (Difco) by injection of the antigen into the foot pads and back. Antibody levels in the serum were boosted twice, at three-week intervals, by injecting 5 mg of glutamine synthetase, mixed with an equal volume of Freund's incomplete adjuvant. Ten days after the second booster, rabbits were sacrificed and bled by heart puncture and the serum containing antibody against glutamine synthetase was obtained. The presence of glutamine synthetase antigen in crude bacterial extracts was determined by immunodiffusion in Ouchterlony-type plates [16]. These plates (Hyland Laboratories, Costa Mesa, Calif.) were equilibrated with 0.01 M imidazole buffer, pH 7.0, containing 0.15 M NaCl for 1 h. Ten μl of serum was placed in the center well. Ten μl of crude extracts (about 350–400 μg protein) obtained from various nitrogenase-derepressed mutants grown under nitrogen-fixing conditions, were placed in the respective outer wells. The presence or absence of precipitin band(s) corresponding to glutamine synthetase antigen-antibody complex was scored after 16-18 h incubation at room temperature. Purified *E. coli* and *K. pneumoniae* glutamine synthetases were used as control.

RESULTS

Isolation of nitrogenase-derepressed mutants

The rationale for isolation of nitrogenase-derepressed mutants of *K. pneumoniae* is as follows: *K. pneumoniae* is capable of utilizing one of two different enzyme systems involving a total of three enzymes for assimilation of NH_4^+ into glutamate (depending on the source of nitrogen in the medium [7, 17]).

TABLE I

VARIOUS STRAINS USED IN THIS STUDY

Abbreviations (phenotypic expressions) used: Asm^-, lacks glutamate synthase activity; Gln^+, glutamine synthetase activity; Gln^-, lacks glutamine synthetase activity and protein; $GlnC^-$, synthesizes derepressed levels of glutamine synthetase in the presence of NH_4^+; Gln $(AC)^-$, produces catalytically inactive glutamine synthetase protein; Nif, nitrogen fixation; Nif C^-, constitutive synthesis of nitrogenase in the presence of NH_4^+.

Strain	Parent	Phenotype	Growth requirements	Source
Klebsiella pneumoniae				
M5A1	Wild type	Nif^+	None	
Asm-1	M5A1	Nif^+, Asm^-	Glutamate*	
Asm-3	M5A1	Nif^+, Asm^-	Glutamate*	
Asm-24	M5A1	Nif^+, Asm^-	Glutamate*	glutamine synthetase protein mutagenesis
SK-24	Asm-1	Nif C^-, Gln C^-, Asm^-	Glutamate**	
SK-25	Asm-1	Nif C^-, Gln^-, Asm^-	Glutamine	
SK-26	Asm-1	Nif C^-, Gln^-, Asm^-	Glutamine	
SK-27	Asm-1	Nif C^-, Gln $(AC)^-$, Asm^-	Glutamine	
SK-28	Asm-3	Nif C^-, Gln C^-, Asm^-	Glutamate**	
SK-29	Asm-24	Nif C^-, Gln C^-, Asm^-	Glutamate**	
SK-31	SK-37	Nif^+, Gln^+, Asm^-	Glutamate*	Sexual transfer of F′ from KLF33/JC1553 → SK-37
SK-32	SK-25	Nif^+, Gln^+, Asm^-	Glutamate*	Sexual transfer of F′133 → SK-25
SK-33	SK-25	Nif^+, Gln^+, Asm^-	Glutamate*	Sexual transfer of F′133 → SK-25
SK-34	SK-26	Nif^+, Gln^+, Asm^-	Glutamate*	Sexual transfer of F′133 → SK-26
SK-35	SK-26	Nif^+, Gln^+, Asm^-	Glutamate*	Sexual transfer of F′133 → SK 26
SK 36	SK 27	Nif^+, Gln^+, Asm^-	Glutamate*	Sexual transfer of F′133 → SK-27
SK-37	SK-24	Nif C^-, Gln $(AC)^-$, Asm^-	Glutamine	Spontaneous
Escherichia coli				
KLF5/AB2463		F′105 (CGSC No. 4252)		Dr B. Bachmann
KLF33/JC1553		F′133 (CGSC No. 4265)		Dr B. Bachmann

* Requires glutamate (as a nitrogen source) only during growth in sucrose/minimal medium containing less than 1 mM NH_4^+.

** Either aspartate or glutamine will replace glutamate.

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADPH \rightarrow \text{L-glutamate} + NADP^+ \quad (1)$$

$$\text{L-glutamate} + NH_4^+ + ATP \rightarrow \text{L-glutamine} + ADP + P_i \quad (2)$$

$$\text{L-glutamine} + \alpha\text{-ketoglutarate} + NADPH \rightarrow 2\ \text{L-glutamate} + NADP^+ \quad (3)$$

Reaction 1, catalyzed by glutamate dehydrogenase, is the major source of glutamate for the cell when the concentration of NH_4^+ in the medium is higher than 1 mM [7]. Under nitrogen-fixing conditions (in the absence of added NH_4^+, under N_2), NH_4^+ produced by nitrogenase is assimilated into glutamate through Reactions 2 and 3 catalyzed by glutamine synthetase and glutamate synthase respectively [4, 18, 19].

It has been shown previously that Gln C^- mutants producing constitutive levels of glutamine synthetase (Reaction 2) are simultaneously derepressed for nitrogenase activity [4]. These mutants have no detectable glutamate dehydrogenase activity (Reaction 1). Brenchley et al. [6] have isolated Gln C^- mutants as revertants from Asm^- mutants (which lack glutamate synthase activity, Reaction 3) of *K. aerogenes*, a non-nitrogen-fixing organism. These mutants are found to be glutamate auxotrophs. Using this rationale we have isolated glutamate auxotrophs from Asm^- mutants of *K. pneumoniae*. About 50 % of these mutants were found to be derepressed for nitrogenase and glutamine synthetase biosynthesis (Nif C^-, Gln C^-). In addition to the Nif C^-, Gln C^- phenotype, two new classes of nitrogenase derepressed mutants were also obtained as glutamine auxotrophs, lacking glutamine synthetase activity, from Asm^- mutants. A summary of the properties of these strains is presented in Table I. In the case of Asm-1, about 5–10 % of the auxotrophic colonies were found to be glutamate or glutamine auxotrophs with about half of these (3/106 auxotrophs) being derepressed for nitrogenase biosynthesis. Nitrogenase-derepressed mutants were also isolated starting with strain Asm-3 and strain Asm-24 (strains SK-28 and 29, respectively of Table I).

Biochemical properties

Specific activities of nitrogenase levels in the mutants and their parental strains are presented in Table II. Nitrogenase activities, in the presence of NH_4^+, in the mutants (strains SK-24, 25, 26, 27, 28, 29 and 37) were 65–100 % of the value compared to those observed in the absence of NH_4^+. Under the same conditions, the parental strains produced no nitrogenase activity in the presence of NH_4^+. Nitrogenase activity was also determined in these mutants as a function of NH_4^+ concentration (data not presented). The derepressed mutants induced about 6–8 units of nitrogenase activity in the concentration range of 0–15 mM of NH_4^+. The parental strains (M5A1 and Asm^- strains) were completely repressed by concentrations of NH_4^+ as low as 1.5 mM. Because of the previously observed correlation between high glutamine synthetase activity and nitrogenase derepression, the specific activities of glutamine synthetase and other ammonia-assimilatory enzymes, such as glutamate synthase and glutamate dehydrogenase of nitrogenase derepressed mutants and their parental strains were determined (see Table II). Note that glutamine synthetase activity is fully derepressed (in the presence of NH_4^+) in all three of the glutamate-requiring strains (strains SK-24, 28 and 29). Glutamate dehydrogenase activity is not detected in these three strains, a property shared with other glutamine synthetase-constitutive mutants (see Magasanik et al. [17] for a discussion of the possible role of

TABLE II

SPECIFIC ACTIVITIES OF NITROGENASE, GLUTAMINE SYNTHETASE, GLUTAMATE SYNTHASE AND GLUTAMATE DEHYDROGENASE IN VARIOUS STRAINS OF *K. PNEUMONIAE*

Cultures were grown starting with a 1 % inoculum of a culture grown in L-broth, in sucrose/minimal medium with additions as indicated. L-Glutamate or L-glutamine was added at a concentration of 100 μg/ml. The concentration of $(NH_4)_2SO_4$ was 1 mg/ml. n.d., not determined

Strain	Additions to the medium	Nitrogenase (μmol ethylene formed/per mg cell protein)	(nmol/min per mg protein)		
			Glutamine synthetase	Glutamate synthase	Glutamate dehydrogenase
M5A1	Glu	6.75	878	37	< 5
	Glu+NH_4^+	0.00	229	53	126
Asm-1	Glu	5.11	653	< 5	18
	Glu+NH_4^+	0.00	147	< 5	116
	Gln	4.87	941	< 5	27
	Gln+NH_4^+	0.00	129	< 5	117
Asm-3	Glu	7.71	963	< 5	20
	Glu+NH_4^+	0.00	171	< 5	156
Asm-24	Glu	6.90	643	< 5	20
	Glu+NH_4^+	0.00	165	< 5	150
SK-24	Glu	4.13	684	< 5	< 5
	Glu+NH_4^+	2.70	866	< 5	< 5
SK-28	Glu	6.58	1006	< 5	< 5
	Glu+NH_4^+	4.36	1055	< 5	< 5
SK-29	Glu	6.25	739	< 5	< 5
	Glu+NH_4^+	6.61	627	< 5	< 5
SK-25	Gln	7.40	0	< 5	12
	Gln+NH_4^+	7.26	0	< 5	16
SK-26	Gln	8.86	0	< 5	17
	Gln+NH_4^+	5.79	0	< 5	18
SK-27	Gln	6.11	0	< 5	17
	Gln+NH_4^+	4.64	0	< 5	15
SK-37	Gln	4.61	0	n.d.	n.d.
	Gln+NH_4^+	3.55	0	n.d.	n.d.

glutamine synthetase in the repression of glutamate dehydrogenase). Glutamate synthase activity is not observed with any of the nitrogenase-derepressed mutants, a property of the parental strains. In contrast to the nitrogenase derepressed mutants such as strain SK-24, glutamine-requiring mutants (SK-25, 26, 27 and 37) produce no detectable glutamine synthetase activity. Low but significant levels of glutamate dehydrogenase activities were detected in the three strains. The specific activities of the four enzymes in the parental strains are given for comparison. To test the possibility that glutamine added to the medium as a growth supplement might have acted as a repressor of glutamine synthetase in the nitrogenase-derepressed mutants requiring glutamine, glutamine synthetase activity was determined in the parent, Asm-1, in the presence and absence of glutamine. As presented in Table II (strain Asm-1), addition of glutamine to the growth medium did not repress glutamine synthetase activity.

In order to rule out the possibility that catalytically active glutamine synthetase

was produced during a specific stage of growth, in the glutamine-requiring strains (strains SK-25, 26, 27 and 37), the levels of glutamine synthetase were monitored throughout the growth cycle in SK-25. No glutamine synthetase activity was detected at any time during the growth period. The nitrogenase induction pattern was similar to the parent strain. Low levels of glutamate dehydrogenase were observed late in the growth period.

Immunological experiments

During their studies of the regulation of the histidase utilization (*hut*) operon, Magasanik et al. [17] described glutamine-requiring mutants of *K. aerogenes*, a non-nitrogen-fixing organism, which produced catalytically inactive glutamine synthetase protein which was detected immunologically. This raised the possibility that the nitrogenase derepressed, glutamine-requiring mutants of *K. pneumoniae* (strains SK-25, 26, 27 and 37) may also produce glutamine synthetase protein lacking catalytic activity. Extracts prepared from the nitrogenase derepressed mutants, strains SK-25, 26 and 27, were tested for the presence of immunologically active glutamine synthetase protein (antigenic cross-reacting material). A precipitin band corresponding to glutamine synthetase protein was observed with extracts of strain SK-27 (Fig. 1). A similar precipitin band was also observed with the extracts of strain SK-37 (data not presented). No detectable precipitin band was observed with the extracts of strain

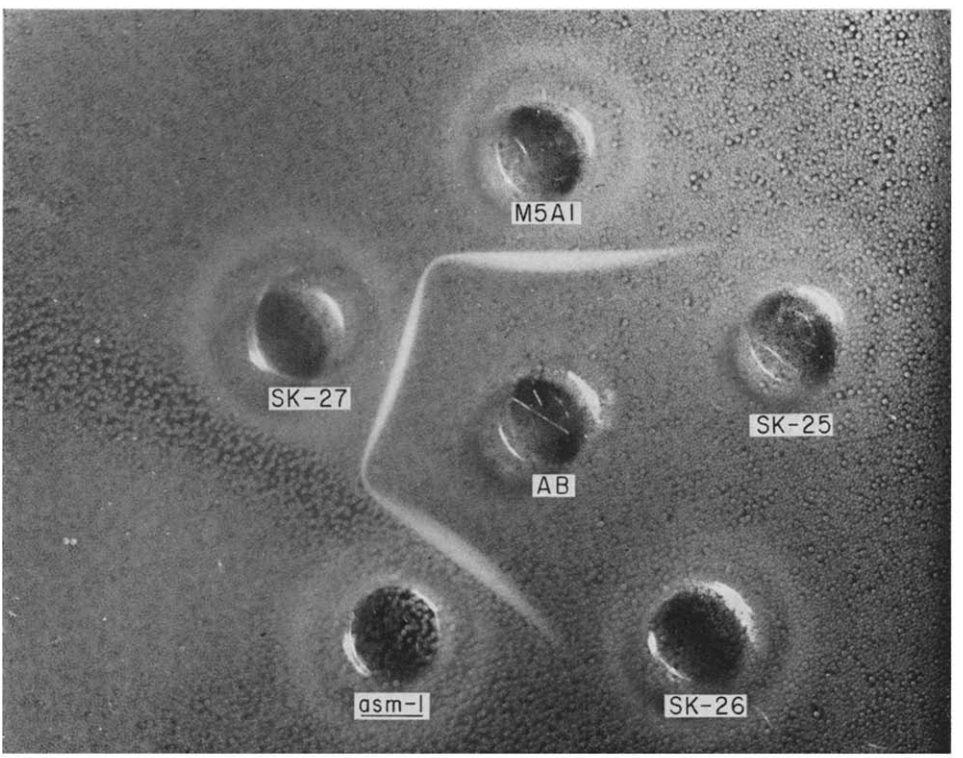

Fig. 1. Immunodiffusion analysis for the presence of glutamine synthetase protein in extracts of nitrogenase-derepressed mutants. See text for experimental details.

SK-25 or strain SK-26. Extracts of strains M5Al, Asm-l and SK-27 gave one band each corresponding to the presence of a homologous antigen in all three extracts.

A second procedure involving the use of antibody neutralization by glutamine synthetase protein [17, 20] was also used to confirm the absence of the antigen in the extracts of the strains SK-25 and 26. In this experiment, extracts of strain SK-25 or strain SK-26 added to diluted samples of the glutamine synthetase immune serum were found not to decrease the effectiveness of the antibody for inactivation of glutamine synthetase catalytic activity. Under the same conditions, extracts of strain SK-27 completely neutralized the antibody.

Mapping of nitrogenase regulatory genes

Because of the relatedness between *E. coli* and *K. pneumoniae*, it is possible to localize Gln^- or Glu^- mutations on the chromosome of *K. pneumoniae*, through complementation by *E. coli*, F-prime genetic elements. Using this technique, we have observed that glutamine-independent colonies can be obtained from Gln^- strains of *K. pneumoniae*, by constructing hybrid clones carrying *E. coli* F'133 [4], which harbors the chromosomal markers between 72 and 77.5 min of the genetic map [13]. F' complementation analysis was carried out with the glutamate- or glutamine-dependent mutants described above, in order to map the nitrogenase regulatory mutations on the *K. pneumoniae* genome. As seen from Table III, strains lacking glutamine synthetase activity (strains SK-25, 26, 27 and 37) were complemented by the episome, F'133, suggesting a location of nitrogenase regulatory genes in or near the glutamine synthetase genes on the chromosome. Hybrids derived from strains such as strain SK-27 are readily cured of the Gln^+ property (Table III) if they are tested immediately after their initial selection. Prolonged storage in a selective medium (in order to maintain the episome) leads to an eventual loss of curability even in the presence of acridine orange.

It was not possible using these techniques to map the nitrogenase regulatory loci of strains SK-24, 28 and 29, because of the inability to obtain any Glu^+ proto-

TABLE III

F-PRIME COMPLEMENTATION ANALYSIS OF NITROGENASE-DEREPRESSED MUTANTS

Conjugation experiments and curing experiments were conducted as described in Methods. +, restoration; n.d. = not determined; –, Not applicable.

Recipient	Relevant Gln phenotype	Number of prototrophic colonies with F'133*	Spontaneous reversion frequency ($X10^9$)	Percent curing
SK-24	$Gln\ C^-$	0	1.57	–
SK-25	Gln^-	540	< 0.1	0
SK-26	Gln^-	500	< 0.1	42**
SK-27	$Gln\ (AC)^-$	680	0.47	40
SK-28	$Gln\ C^-$	0	< 0.1	–
SK-29	$Gln\ C^-$	0	< 0.1	–
SK-37	$Gln\ (AC)^-$	+	n.d.	94

* Glutamate or glutamine prototrophs per ml.

** Some of the hybrid clones are curable (42 %) while others are not.

trophic colonies with *E. coli* episome F'133. However, after several days of incubation, a small number of colonies did appear in some of the crosses between strain SK-24 and F'133. Segregant colonies obtained from these hybrids (strain SK-37) were found to require glutamine instead of glutamate (see Table I) and were found to be derepressed for nitrogenase. Strain SK-37 was found to produce glutamine synthetase protein.

The inability to obtain any glutamate-independent prototrophic colonies in the crosses between GlnC$^-$ strains and F'133 could be due to the inability of these recipient strains to inherit F'133. To rule out this possibility, strains carrying Ilv$^-$ or Gln$^-$ mutations were constructed from strain SK-24. Upon sexual transfer of F'133, prototrophic colonies for isoleucine and valine or glutamine independence were obtained. None of these were found to be glutamate independent.

Biochemical properties of *K. pneumoniae*/F'133 hybrids are presented in Table IV. In contrast to the parental strains (see Table II), all hybrid strains produced glutamine synthetase (presumably the *E. coli* enzyme) as well as glutamate dehydrogenase. It should be emphasized that addition of NH_4^+ to the sucrose minimal medium, completely repressed nitrogenase induction (Nif C$^+$ phenotype) in the hybrid strains SK-32 and 35. Strains SK-31, 33, 34 and 36 were found to induce traces of nitrogenase activity in the presence of NH_4^+ (about 1–2 % of the derepressed value). In *K. pneumoniae*/*E. coli* F'133 hybrids, nitrogenase activity is detected, even when the glutamine synthetase activity (produced under the influence of *E. coli*, *gln* genes) is considerably lower than 350 units, in contrast to previous observations where no nitrogenase activity was detected when the glutamine synthetase levels decreased below 350 units [18].

K. pneumoniae/F'133 hybrids, in contrast to the parental strains, produced glutamate dehydrogenase activity even in the absence of NH_4^+ (Table IV). The

TABLE IV

NITROGENASE, GLUTAMINE SYNTHETASE AND GLUTAMATE DEHYDROGENASE ACTIVITIES OF NIF C$^-$-F'133 HYBRIDS OF K. *PNEUMONIAE*

The conditions were the same as for Table II.

Strain	NH_4^+ in the medium	Nitrogenase (μmol/h per mg cell protein)	(nmol/min per mg protein)	
			Glutamine synthetase	Glutamate dehydrogenase
SK-31	−	4.60	130	181
	+	0.06	101	128
SK-32	−	5.40	128	77
	+	0.00	109	72
SK-33	−	4.53	121	191
	+	0.12	94	72
SK-34	−	5.90	163	233
	+	0.15	172	89
SK-35	−	6.80	111	269
	+	0.00	120	100
SK-36	−	6.40	349	235
	+	0.20	72	118

presence of unusually high levels of glutamate dehydrogenase in the hybrids grown in sucrose/minimal medium without NH_4^+ (Table IV) might be related to the low levels of glutamine synthetase, since an inverse relationship between the levels of these two enzymes has been observed [4, 17]. Derepressed synthesis of nitrogenase was again observed in the segregant Gln^- clones which have been cured of the F'133. Similar genetic and biochemical results were obtained when the *E. coli* episome F'105 was used instead of F'133 for F' complementation analysis.

DISCUSSION

Evidence supporting the role of glutamine synthetase in the regulation of nitrogenase can be summarized as follows: (a) mutations affecting the structure of glutamine synthetase leading to the production of catalytically inactive glutamine synthetase protein (strain SK-27) results in the derepression of nitrogenase biosynthesis (Fig. 1 and Table II); (b) strains which produce derepressed levels of glutamine synthetase in the presence of NH_4^+ produce derepressed levels of nitrogenase (Table II) [5]; (c) some mutations abolishing glutamine synthetase activity abolish nitrogenase activity [4].

However, the finding that certain nitrogenase-derepressed mutants, isolated as Gln^- strains synthesize no detectable levels of glutamine synthetase protein (strains SK-25 and 26) may have at least three explanations: (i) that catalytically inactive, glutamine synthetase protein-negative, glutamine synthetase is present in these strains (see ref. 20 for further discussion); (ii) there is a substitute for glutamine synthetase which regulates nitrogenase biosynthesis in its absence, and (iii) glutamine synthetase does not directly regulate nitrogenase expression. Obviously, additional experiments are necessary to distinguish among the three possibilities. These glutamine synthetase, glutamine synthetase protein-negative strains share certain properties with the other classes of nitrogenase-derepressed mutants, i.e., lack of glutamate dehydrogenase activity even in the presence of NH_4^+ a property of Gln C^- and Gln (AC^-) strains. As determined by F' complementation analysis, these mutants also map in the same chromosomal region in which the Gln (AC^-) strains map (Table III). A similar mutant of *K. aerogenes* (MK 9021) which produces no detectable glutamine synthetase antigen has been mapped in the *gln* A region by Magasanik and his co-workers [20, 21].

Upon introduction of the *E. coli* episome, F'133, which carries the glutamine synthetase genes, glutamine-independent colonies can be obtained in both types of glutamine-requiring strains (strains SK-25, 26, 27, and 37). Following the introduction of F'Gln of *E. coli*, nitrogenase regulation returns to that of Nif C^+ phenotype (repression by NH_4^+), indicating that the Nif C^+ phenotype from *E. coli* is dominant over the Nif C^- property of *K. pneumoniae* in these Gln^- strains (Table IV). Catalytically active glutamine synthetase is produced by these hybrids, presumably the *E. coli* enzyme. It has been observed before that no nitrogenase activity is detected in *K. pneumoniae* when the glutamine synthetase levels decreased below 350 enzyme units [18]. The presence of normal levels of nitrogenase activity in the hybrids which synthetize less than 350 units of glutamine synthetase activity (Table IV) might be due to structural differences in the regulatory functions of the *E. coli* versus *K. pneumoniae* glutamine synthetases.

In the future, it may be possible to isolate nitrogenase-regulatory mutants of other agronomically important species of nitrogen-fixing bacteria, such as *Rhizobium* sp., *Spirillum lipoferum* (forming associative, N_2-fixing symbiosis on the roots of tropical grasses) [22], and blue-green algae by alteration of their glutamine synthetase systems.

ACKNOWLEDGEMENTS

We are indebted to Dr R. C. Valentine for encouragement, advice and support. We thank Dr E. R. Stadtman for providing antibody against glutamine synthetase, Dr R. E. Miller for help in the purification of glutamine synthetase and Dr M.D. Kamen for continued encouragement and generous use of equipment. This work was supported by Grant BMS72-02243 from the National Science Foundation and Contract AG No. 12-14-1001-397 from the U.S. Department of Agriculture to R. C. Valentine, who is also a recipient of a USPHS Career Development Award (AI 16595). C.M. is a recipient of a European Molecular Biology Organization (EMBO) fellowship.

REFERENCES

1 Streicher, S., Gurney, E. and Valentine, R. C. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1174–1177
2 Dixon, R. A. and Postgate, J. R. (1972) Nature 237, 102–103
3 Gordon, J. K. and Brill, W. J. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3501–3503
4 Streicher, S. L., Shanmugam, K. T., Ausubel, F., Morandi, C. and Goldberg, R. B. (1974) J. Bacteriol. 120, 815–821
5 Tubb, R. (1974) Nature 251, 481–485
6 Brenchley, J. E., Prival, M. J. and Magasanik, B. (1973) J. Biol. Chem. 248, 6122–6128
7 Nagatani, H., Shimizu, M. and Valentine, R. C. (1971) Arch. Mikrobiol. 79, 164–175
8 Shanmugam, K. T., Loo, A. and Valentine, R. C. (1974) Biochem. Biophys. Acta 338, 545–553
9 Adelberg, E. A., Mandel, M. and Chen, G. C. C. (1965) Biochem. Biophys. Res. Commun. 18, 788–795
10 Shapiro, B. M. and Stadtman, E. R. (1972) in Methods in Enzymology (Tabor, H. and Tabor, C. W., eds), Vol. 27A, pp. 910–922, Academic Press, New York
11 Prusiner, S., Miller, R. E. and Valentine, R. C. (1972) Proc. Natl. Acad. Sci. U.S. 69, 2922–2926
12 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
13 Low, K. B. (1972) Bacteriol. Rev. 36, 587–607
14 Taylor, A. L. and Trotter, C. D. (1972) Bacteriol. Rev. 36, 504–524
15 Miller, J. H. (1972) Experiments in Molecular Genetics, pp. 104–106, Cold Spring Harbor Laboratory, New York
16 Tronick, S. R., Ciardi, J. E. and Stadtman, E. R. (1973) J. Bacteriol. 115, 858–868
17 Magasanik, B., Prival, M. J., Brenchley, J. E., Tyler, B. M., De Leo, A. B., Streicher, S. L., Bender, R. A. and Paris, C. G. (1974) in Current Topics in Cellular Regulation (Horecker, B. L. and Stadtman, E. R., eds), Vol. 8, pp. 119–138, Academic Press, New York
18 Shanmugam, K. T., Streicher, S. L., Morandi, C., Ausubel, F., Goldberg, R. B. and Valentine, R. C. (1975) in Symposium on Dinitrogen Fixation, Kettering Foundation, in press
19 Tempest, D. W., Meers, J. L. and Brown, C. M. (1970) Biochem. J. 117, 405–407
20 De Leo, A. B. and Magasanik, B. (1975) J. Bacteriol. 121, 313–319
21 Streicher, S. L., Bender, R. A. and Magasanik, B. (1975) J. Bacteriol. 121, 320–331
22 Day, J. M. and Dobereiner, J. (1975) in Symposium on Dinitrogen Fixation, Kettering Foundation, in press