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6-Cyanopurine, a Color Indicator Useful for Isolating Mutations in the *nif* (Nitrogen Fixation) Genes of *Klebsiella pneumoniae*

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6-Cyanopurine (6-CP) can be used as a color indicator for certain classes of *nif* (N_2 fixation) mutations in *Klebsiella pneumoniae*. Under N_2-fixing conditions, Nif^+ colonies and most Nif^- colonies are purple on media containing 6-CP. Twenty-two Nif^- mutants with altered color on medium containing 6-CP were isolated. All white mutants contained mutations in the regulatory genes, *nifA-nifL*. Mutants which were more darkly colored than the wild type had mutations distributed among six *nif* genes. Medium with 6-CP was used to isolate Nif^- mutants with deletions internal to the *nif* genes, and 6-CP was used to identify strains derepressed for nitrogenase synthesis in the presence of NH_4^+.

Most studies investigating the genetics of N_2 fixation have utilized *Klebsiella pneumoniae*. Since this organism is closely related to *Escherichia coli*, genetic techniques developed for *E. coli* have been applied to *K. pneumoniae*. Phage P1 transduction (12, 20, 22), conjugation (8, 15), phage Mu mutagenesis (2, 17), fine-structure mapping (16), and complementation analysis (7, 16) have been performed with the *nif* (N_2 fixation) genes. Such studies have shown that N_2 fixation is a complex process involving 14 genes (16) clustered near the *his* locus (22).

The genetic and biochemical analyses of many microbial systems have been facilitated by the use of media incorporating analogs to select or screen for various mutants. Substrate analogs would be useful for isolating and characterizing Nif mutants, but no such analogs have yet been described. Nitrogenase, besides reducing N_2 to NH_4^+, also is capable of reducing other triple-bonded compounds such as acetylene, azide, and cyanide (reviewed in reference 5). We screened many compounds with side groups having these structures. Although none appeared to be substrates for the enzyme, one, 6-cyanopurine (6-CP), has been useful for isolating several classes of Nif mutants.

MATERIALS AND METHODS

Media and chemicals. LC, a rich medium (2), and K, an N-free minimal medium (15), have been described. KN medium is K medium which includes 0.2% ammonium acetate. This concentration is sufficient to completely repress nitrogenase synthesis. When required, the medium was supplemented with 20 μg of an amino acid or 1 μg of a vitamin per ml. Glucose was substituted for sucrose when the medium was used for growing *E. coli*. CP medium was made from agar-containing K medium lacking $FeCl_3$, but containing 50 μg of L-serine and 125 μg of 6-CP per ml. The 6-CP is made fresh as a 10-mg/ml stock solution in distilled water and must be added after the autoclaved medium has cooled to 45°C. H medium is agar-containing K medium supplemented with 200 μg of L-histidine per ml as the N source. Agar media contained 15 g of agar (Difco Laboratories, Detroit, Mich.) per liter except K medium, which contained 13 g of purified agar (Difco) per liter. 6-Bromopurine, 6-thiopurine, and 6-carboxypurine were obtained from Sigma Chemical Co., St. Louis, Mo. All other analogs were obtained from Aldrich Chemical Co., Milwaukee, Wis. Additional supplies of 6-CP also were obtained from Burroughs Wellcome, Triangle Park, N.C. Diethyl sulfate (DES) was obtained from Eastman Kodak Co., Rochester, N.Y.

Bacterial strains. The wild-type *K. pneumoniae* strain was M5a1 (strain UN) obtained from P. W. Wilson. Mutant strains UN106 (*nifB4106*), UN116 (*nifH4116*), UN179 (*nif-4179*), and UN209 (*hisD4226*) have been described previously (15, 20). UN365 is derepressed for the synthesis of nitrogenase in the presence of NH_4^+ at 1% of the level observed when UN365 is grown on N_2 (J. K. Gordon, Ph.D. thesis, University of Wisconsin, Madison, 1975). UN562 is deletion 16 Δ(*rfb gnd his nif*) obtained from R. C. Valentine (22). *E. coli* strains used were UQ27 (His^-) and UQ29, which is UQ27 containing the plasmid pTM4010 (15). This plasmid, a derivative of pRD1 (6, 7), contains the genes *gnd*, *rfb*, *his*, *nif*, and *shi* of *K. pneumoniae* and codes for resistance to the antibiotics carbenicillin, tetracycline, and kanomycin (6). *K. pneumoniae* and *E. coli* strains containing pTM4010, unlike strains containing pRD1, remain sensitive to phages Mu and P1.

Mutagenesis. Overnight LC cultures were treated

with DES (19), and survivors were grown to stationary phase in KN medium and plated on CP plates.

Isolation of strains with *nif* deletions. Heat induction of strains with Mu insertions in *his* or *nif* were performed as described (2) with the following modifications. One-tenth milliliter of heat-killed *E. coli*, prepared by heating 30 ml of an overnight culture of a Mu-sensitive *E. coli* strain at 70°C for 1 h and resuspending the killed cells in 10 ml of buffer, was substituted for Mu-specific antiserum. Survivors from heat induction of Mu lysogens were spread on KN or CP plates.

Transductions. Transductions were performed as previously described (25), using phage P1*kc h1* (16), a spontaneous mutant of P1*kc* with an extended *K. pneumoniae* host range.

Acetylene reduction assay of nitrogenase. Cultures were grown overnight in KN medium, suspended in 0.05 M phosphate, pH 7.8, diluted 1:20 into K medium (or, for putative Nif constitutive strains, into KN medium), incubated anaerobically at 30°C for 5 h, and then assayed for the rate of acetylene reduction (3).

Testing compounds for an interaction with the Nif system. To compare the effect of the chemicals, a Nif^+ and several Nif^- strains were each spread (10^8 cells) onto KN and H plates, and 20 mg of the compound being tested was added to the center of the plate. These plates were incubated anaerobically for 5 days at 30°C. Zones of inhibition were measured. In addition, any color development on the test plates was noted.

Pigmentation of strains on CP medium. The 6-CP-associated color of various strains was determined by two methods. When testing media or isolating mutant strains, overnight LC cultures were diluted and plated to yield 200 to 400 single colonies on CP plates. When retesting strains isolated as single colonies on CP plates or when testing mutant strains previously isolated, overnight LC cultures without dilution were spotted on CP plates. After 5 days of anaerobic incubation at 30°C, the color of each strain was noted. This was determined immediately after opening the anaerobic jars because the color disappears after several hours in air. Strains which were as purple as the wild type are designated as Cpu^+, those which were a darker purple than the wild type are Cpu^d, and those which remained white (or occasionally formed a very faint purple color) are Cpu^-. If CP plates had more than 400 colonies, no color developed within colonies at the center of the plate. A Cpu^+ and a Cpu^- strain were always spotted as controls.

RESULTS

Screening of chemicals for interaction with the Nif system. The ability of 43 chemicals, containing triple bonds, to alter growth of Nif^+ and Nif^- strains of *K. pneumoniae* was investigated. 3-Aminobenzonitrile, 4-(aminomethyl)-cyclohexanecarbonitrile, anisonitrile, sodium azide, 2-benzimidazolylacetonitrile, 2-benzoylpropionitrile, sodium cyanide, 1-cyanoacetylpiperidine, 1-cyanoacetylpyrrolidine, 2-cyanoamino-4-6-dimethylpyrimidine, *N*-(2-cyanoethyl)pyrrole, 6-cyanopurine, 3-cyanopyridine, 3-cyclohexene-1-carbonitrile, fumaronitrile, β-iminohydrocinnamonitrile, 1-methoxy-1-buten-3-yne, and succinonitrile showed varying amounts of toxicity, but none caused a differential toxicity among the wild type and mutant strains UN106, UN116, and UN562. Acrylonitrile, 5-amino-4-cyano-1-phenyl-3-pyrazoleacetonitrile, amygdalin, 3-anilinopropionitrile, benzimidazolecarbonitrile, benzoyl-2-phenylpropionitrile, benzyl cyanide, *N*-benzylpropargylamine, 4-bromobenzonitrile, butylisocyanide, 2-chloroacrylonitrile, cyanoacetylurea, 3-cyano-4,6-dimethyl-2-hydroxypyrimidine, 5-cyano-1,3-dimethyl-4-nitropyrazole, *N*-(2-cyanoethyl)-glycine, 1-cyanonaphthalene, 1-cyclohexylisocyanide, hexylisocyanide, *n*-methylpropargylamine, monopropargylamine, phenylacetylene, *n*-(propargyloxy)-phthalimide, piperonylonitrile, steronitrile, and tolylmethylisocyanide were not toxic. When 6-CP was added to H plates, strains UN106 and UN116 and the wild type developed a nondiffusible purple color, but strain UN562 with a total deletion of *nif* remained white. No color developed in any of the four strains on KN plates containing 6-CP. This suggested that 6-CP might be useful as a color indicator of *nif* gene expression.

Optimum conditions for color development. To study the physiological and genetic basis of the color change in the presence of 6-CP, the medium composition was altered to determine optimal conditions for pigmentation. H medium used in the screening tests contained L-histidine as an N source. Nif^- strains grew poorly on K medium with 100 μg of 6-CP per ml, but Nif^+ and Nif^- strains grew well on KN plates with or without 100 μg of 6-CP per ml. This indicates that the compound is a poor N source and, therefore, unlikely to repress nitrogenase synthesis. Furthermore, the addition of 100 μg of 6-CP per ml to derepressed cultures of the wild type did not inhibit the acetylene-reducing ability of nitrogenase. The purple color was observed in the Nif^+ strain on K medium with 125 μg of 6-CP per ml and on medium further supplemented with 50 μg of amino acids per ml instead of L-histidine. The color development on plates containing amino acids which are good N sources for the Nif^- strains varied. Glutamine produced the most intense color with the wild type, but poor color with the Nif^- strains. When L-serine was added to the medium, the wild type was almost as deeply pigmented as with the glutamine plates, but strains UN106 and UN116 were also deeply colored. On all media, strain UN562 was white.

Fe and Mo form complexes with cyanide (4) and might react with 6-CP. K medium is supplemented with both of these metals because they are contained in nitrogenase. In plates containing K medium with 125 μg of 6-CP and 50 μg of serine per ml, the Mo and Fe concentration was altered. When the Mo concentration was varied from no addition to 20-fold the concentration in K medium, the color of the test strains was unchanged. Although increasing the Fe 2-fold had no effect, eliminating the Fe increased the pigmentation of UN, UN106, and UN116 significantly. UN562 remained white. Growth was not altered on K medium lacking Fe. Presumably Fe sufficient for growth contaminated the other medium constituents.

On K plates containing 50 μg of L-serine per ml and lacking added Fe, addition of less than 100 μg of 6-CP per ml gave little or no pigmentation with the four test strains and showed no inhibition of growth, whereas on medium with concentrations of 6-CP between 100 μg/ml and 1 mg/ml, UN, UN106, and UN116 were darkly colored. Concentrations greater than 1 mg of 6-CP per ml inhibited growth. Subsequent studies, therefore, used CP plates which consistently stained Nif^+ and most Nif^- strains purple, but did not stain UN562.

Strains grown in liquid CP medium did not become pigmented. This hindered efforts to obtain sufficient quantities of the pigment for biochemical studies. However, pigment was obtained fom wild-type cells scraped off CP plates, and the absorbance spectrum on a sonically prepared lysate was determined. Over a range from 320 to 800 nm there was a single broad peak with a maximum absorbance at approximately 545 nm.

Effect of analogs of 6-CP. CP medium containing 6-aminopurine (adenine), 6-bromopurine, 6-carboxypurine, 6-thiopurine, guanine, hypoxanthine, xanthine, or sodium cyanide instead of 6-CP failed to color the colonies of Nif^+ or Nif^- strains. Pigmentation on CP plates is inhibited by the addition of adenine, 6-bromopurine, or hypoxanthine. This inhibition is probably not due to repression of *nif* by the purine because strain UN365, which is constitutive for nitrogenase synthesis (Gordon, Ph.D thesis), shows a similar inhibition. However, unlike the wild type, coloration in strain UN365 is not inhibited when excess NH_4^+ is included in CP plates.

Effect of 6-CP on Nif^+ *E. coli*. 6-CP pigmentation is not limited to *K. pneumoniae*. When the *K. pneumoniae nif* genes were transferred into *E. coli* strain UQ27, the resulting Nif^+ strain (UQ29) stained purple on CP plates. The Nif^- *E. coli* strain was not stained. Furthermore, as observed in wild-type *K. pneumoniae*, NH_4^+ inhibited pigmentation in UQ29.

Isolation of mutants with altered pigmentation on CP media. The wild type and Nif^- strains UN106 and UN116 were pigmented (Cpu^+), but the strain with a total deletion of *nif* (UN562) was white (Cpu^-). This suggests that one or more products coded by the *nif* genes may be required for formation of the purple pigment. This was investigated by isolating and characterizing mutants with altered color on CP plates. Cultures of the wild type were mutagenized with DES, and survivors were spread on CP media. Two classes of colonies were isolated. One class was a darker purple (Cpu^d) than the majority of colonies, and the other class was white (Cpu^-). In 7 experiments, 11 of 89 Cpu^d colonies were Nif^- and 13 of 30 Cpu^- mutants were Nif^-. These *nif* mutations were ordered by deletion mapping and were assigned to genes by complementation analyses as described in the accompanying paper (16). The *nif* mutations in the Cpu^d strains were located in six genes (Fig. 1). Four of the Cpu^- Nif^- mutants may be siblings because they came from the same selection and their *nif* mutations mapped in the same deletion interval of *nifA*. Consequently, only nine independent *nif* mutations from the Cpu^- Nif^- strains are shown in Fig. 1. The mutations in eight of the Cpu^- Nif^- strains were located within *nifA*, and the mutation *nifL4373* in one Cpu^- Nif^- strain was in *nifL*. However, this *nifL* mutation is polar onto the *nifA* gene (16). In addition, all 23 strains isolated in the accompanying paper (16) with mutations in *nifA* were Cpu^-. These results indicate that the *nifA* product is required for development of the 6-CP-related pigment.

The product of the *nifA* gene is probably a regulatory element because strains with *nifA* mutations produce no detectable *nif*-specific proteins (18). To determine whether any other *nif* genes are required for the development of color, 120 Nif^- strains with Mu insertions in the 14 *nif* genes (16) were spot tested on CP plates. Mu lysogens were chosen because insertion of phage Mu into a gene will inactivate that gene (24) and Mu insertions are strongly polar (11). All six strains with Mu inserted into *nifA* and all 18 strains with Mu inserted into *nifL* (which are polar onto *nifA*) were Cpu^-, but all other Mu lysogens were Cpu^+.

The mutations in three strains that were Cpu^- Nif^+ were tested for linkage to *hisD4226*. If a mutation conferring the Cpu^- phenotype to a Nif^+ strain is within the *nif* cluster, it should be detected as Cpu^- among the His^+ transductants

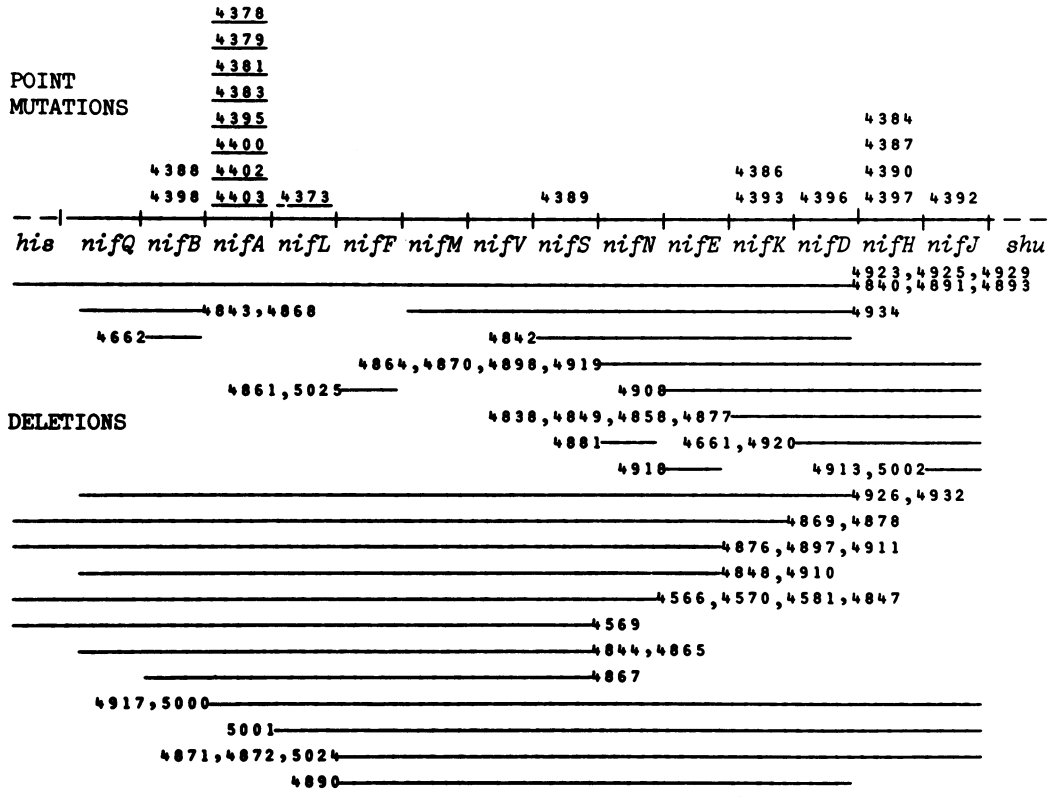

FIG. 1. *Mapping of mutations in strains with altered color on CP medium. Point mutations were assigned to nif genes by mapping and complementation analyses (16). Cpu⁻ strains contained the underlined alleles. Cpu^d strains contained the other nif point mutations. Deleted DNA in Cpu⁻ strains is indicated by a black line. The allele of each deletion is at an end of that deletion.*

using UN209 (*hisD4226*) as the recipient, because mutations in the 14 *nif* genes are 32 to 84% cotransducible with this *his* allele (16). However, no Cpu⁻ His⁺ transductants were observed among 200 transductants tested from three Nif⁺ Cpu⁻ strains.

6-CP is an analog of adenine and, like several other base analogs (2-aminopurine and 5-bromouracil), might induce mutations. Consequently, the screening of cultures on CP plates could have induced mutations leading to a Cpu^d or Cpu⁻ phenotype as well as other uncharacterized mutations. However, the reversion frequency of five *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced amino acid auxotrophs of *K. pneumoniae* was the same on KN media with or without 6-CP. In addition, 6-CP did not increase the reversion frequency of two histidine auxotrophs of *Salmonella typhimurium* used as indicators to test the potency of mutagens (1). One of these auxotrophs is revertible by base substitution mutagens, and the other is revertible by frameshift-inducing mutagens.

Isolation of strains with deletions in *nif*. The above experiments identified a single *nif* locus (*nifA-nifL*) involved in the Cpu⁺ phenotype and presented an opportunity to identify deletions within *nif*. Any strain containing a deletion which removes all or part of *nifA-nifL* would be expected to be Cpu⁻. Some heat-resistant mutants derived from heat-induced cultures of Mu *cts* lysogens contain deletions of the prophage and neighboring bacterial genes (10). To obtain strains containing *nif* deletions, Cpu⁻ colonies were isolated from survivors of heat inductions of 33 Cpu⁺ Mu *cts* lysogens with Mu insertions in *nif* or *his*. Cpu⁻ colonies appeared at frequencies between 10^{-1} and 10^{-3}. In 20 heat induction experiments, 65% (99/152) of the Cpu⁻ clones contained *nif* deletions. In comparison, when a random sample of survivors from five heat selections were tested, only 0.8% (8/877) contained *nif* deletions.

Using the above procedure, 36 independent strains with deletions in *nif*, but not eliminating *his*, were isolated. In addition, 16 independent strains with *his-nif* deletions were isolated by adding L-histidine to the CP plates and then

scoring Cpu⁻ strains for His⁻. The deletions in the His⁺ and His⁻ strains were mapped by crossing them with plasmids containing point mutations in each *nif* gene. A detailed map of these deletions is given in the accompanying paper (16), and a summary is presented in Fig. 1. All eight strains containing *Δnif-4566*, *Δnif-4569*, *Δnif-4570*, *Δnif-4581*, *Δnif-4662*, *Δnif-4843*, *Δnif-4844*, and *Δnif-4868* isolated as Cpu⁻, among heat-resistant survivors from Mu lysogens with insertions in *his* or *nifB*, contained deletions into or through *nifA-nifL*. However, only 25/44 deletions originating from Mu insertions in *nifF* to *nifJ* extended into *nifA-nifL*. When strains containing deletions which did not extend into *nifA-nifL* were spot tested on CP plates, all but one, UN1914 (*Δnif-4871*), developed a faint but observable purple color. On the other hand, all 33 strains containing deletions into *nifA-nifL* remained white upon retesting.

Isolation of strains with altered regulation of *nif*. As discussed above, Nif⁻ Cpu⁻ strains have *nifA* mutations. These strains have no acetylene-reducing activity and are pleiotropic negative, having no Nif proteins detectable on two-dimensional polyacrylamide gels (18). Consequently, the *nifA* product has been proposed to be involved in regulation of the *nif* operons (7, 18). In addition to these regulatory mutants, 6-CP can be used to identify strains which are derepressed for the synthesis of nitrogenase. Although wild-type and Nif⁻ strains are Cpu⁻ when the medium contains excess NH_4^+ (CPN medium), UN365, which is a derepressed strain, was Cpu⁺ on that medium.

Additional derepressed strains were isolated using CPN plates. Some revertants of UN179 (Nif⁻, Hut⁻, and Put⁻) reduce acetylene in the presence of NH_4^+ (Gordon, Ph.D. thesis). Thirty-four revertants selected as Nif⁺, Hut⁺, or Put⁺ were purified and tested for acetylene reduction in the presence of NH_4^+ as well as their color reaction on CPN plates. Ten of these revertants were Cpu⁺ on CPN plates, and only these revertants reduced acetylene in the presence of excess NH_4^+, at rates between 0.1 and 5% of the wild type derepressed in N-free medium. These 10 strains were simultaneously Hut⁺, Put⁺, and Nif⁺, suggesting that the mutation causing derepression was not *nif* specific. These strains grew as well as the wild type on KN plates, but growth was less on CPN plates.

DISCUSSION

The rationale for screening compounds having potential Nif-specific effects was based on the fact that nitrogenase readily reduces many triple-bonded substrates other than N_2. Of the compounds tested, only 6-CP gave a useful effect. When incorporated into solid medium, 6-CP is a color indicator for expression of some *nif* genes. The unexpected finding, however, was that the Cpu⁺ phenotype (under N-limiting conditions) did not require that an active nitrogenase be made. Even when the *nifD*, *nifK*, and *nifH* genes, coding for the nitrogenase proteins (18), were deleted or inactivated by a Mu insertion, the cells still were pigmented. Therefore, pigment formation is not catalyzed by a nitrogenase-specific reduction of the C–N triple bond in 6-CP. The mechanism of pigment formation and the structure of the pigment are not known.

Previously, 6-CP has been described as an inhibitor of adenine phosphoribosyl transferase and a substrate for xanthine oxidase and aldehyde oxidase (9, 13). In *K. pneumoniae*, 6-CP may also interact with some aspect of purine metabolism since adenine and hypoxanthine inhibit formation of the pigment. The loss of pigmentation in Cpu⁺ strains upon exposure to air may be due to a change in the cells' metabolism or to oxygen lability of the pigment.

6-CP is useful for isolating Nif strains with mutations in many *nif* genes since a high proportion of strains isolated with altered color on CP plates are Nif⁻. Mutations in the Nif⁻ Cpu^d strains mapped in several *nif* genes, whereas Nif⁻ strains with *nifA* mutations (and with mutations which are polar onto *nifA*) were white on CP plates. Since the *nifA* protein is a regulator of *nif* (7, 18), it may be required for transcription of a gene whose product is required for formation of the purple pigment. Alternatively, the *nifA* protein may have an enzymatic function involved in pigment formation. Since nonpolar *nifL* mutants have not been isolated and characterized (16), the role of *nifL* in pigment formation is unknown. The other 12 *nif* genes are not essential for pigment formation since strains with Mu insertions in these genes are Cpu⁺. Mutations in Nif⁺ strains which failed to develop color on CP plates were unlinked to the *nif* cluster. Thus at least two gene products are involved in pigment formation.

The correlation of the Cpu⁻ phenotype with mutations at a single *nif* locus was exploited to obtain *nif* deletions. In contrast to methods in which *his-nif* deletions (2) and *rfb-gnd-his-nif* deletions (21) were isolated, most Cpu⁻ strains obtained by the method described here contained deletions only within the *nif* genes. Moreover, this procedure enables one to rapidly screen many more isolates; Cpu⁻ colonies as infrequent as 10^{-3} were easily identified. In addition to the expected class of Cpu⁻ strains with deletions into *nifA-nifL*, strains with deletions which did not extend into *nifA-nifL* were iso-

lated as Cpu⁻. Further testing showed that these latter mutants were slightly pigmented. The reduced pigmentation in these strains may reflect an alteration in *nif* regulation, since other *nif* proteins beside the product of *nifA* are involved in *nif* regulation, as discussed in the accompanying paper (18). Alternatively, there may be another gene involved in pigment formation which was not identified among the *nif* complementation groups (16). The deletions isolated on CP plates have been useful for mapping over 300 *nif* mutations (16).

6-CP was used to isolate Nif regulatory mutants, including strains that were pleiotropic negative and those which were derepressed for nitrogenase synthesis on NH_4^+. 6-CP may be considered a color indicator of *nifA* expression. However, the isolation of Nif⁻ Cpu^d strains and Cpu⁻ strains with deletions that do not extend into *nifA* (Fig. 1) demonstrates a broad usefulness of 6-CP for investigating Nif. Because glutamine synthetase is required for expression of *nif* (23), 6-CP may also be useful for isolating mutants with defects in the *gln* genes. In fact, several GlnC mutants (Gordon, Ph.D. thesis) were Cpu⁺ on CPN plates. Since *E. coli* strains with *K. pneumoniae nif* genes also developed color on CP plates, 6-CP may be useful for isolating and characterizing Nif mutants in other N_2-fixing bacteria. This could be especially useful for *Rhizobium*, where an asymbiotic assay for *nif* expression has been developed (14).

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LITERATURE CITED

1. **Ames, B. N., F. D. Lee, and W. E. Durston.** 1973. An improved bacteria test system for the detection and classification of mutagens and carcinogens. Proc. Natl. Acad. Sci. U.S.A. **70**:782–786.
2. **Bachhuber, M., W. J. Brill, and M. M. Howe.** 1976. Use of bacteriophage Mu to isolate deletions in the *his-nif* region of *Klebsiella pneumoniae*. J. Bacteriol. **128**:749–753.
3. **Brill, W. J., A. L. Steiner, and V. K. Shah.** 1974. Effect of molybdenum starvation and tungsten on the synthesis of nitrogenase components in *Klebsiella pneumoniae*. J. Bacteriol. **118**:986–989.
4. **Britten, D.** 1967. Structural chemistry of the C ≡ N group, p. 109–150. *In* J. E. Dunitz and J. Ibers (ed.), Perspectives in structural chemistry, vol. 1. John Wiley and Sons, Inc., New York.
5. **Burns, R. C., and R. W. F. Hardy.** 1975. Nitrogen fixation in bacteria and higher plants. Springer-Verlag, New York.
6. **Dixon, R. A., F. C. Cannon, and A. Kondorosi.** 1976. Construction of a P plasmid carrying nitrogen fixation genes from *Klebsiella pneumoniae*. Nature (London) **260**:268–271.
7. **Dixon, R., C. Kennedy, A. Kondorosi, V. Krishnapillai, and M. Merrick.** 1977. Complementation analysis of *Klebsiella pneumoniae* mutants defective in nitrogen fixation. 1977. Mol. Gen. Genet. **57**:189–198.
8. **Dixon, R. A., and J. R. Postgate.** 1971. Transfer of nitrogen fixation genes by conjugation in *Klebsiella pneumoniae*. Nature (London) **234**:47–48.
9. **Gadd, R. E. A., and J. F. Henderson.** 1970. Studies of the binding of adenine to adenine phosphoribosyltransferase. Can. J. Biochem. **48**:295–301.
10. **Howe, M. M.** 1973. Prophage deletion mapping of bacteriophage Mu-1. Virology **54**:93–101.
11. **Jordan, E., H. Saedler, and P. Starlinger.** 1968. 0° and strong polar mutations in the *gal* operon are insertions. Mol. Gen. Genet. **102**:353–363.
12. **Kennedy, C.** 1977. Linkage map of the nitrogen fixation (*nif*) genes in *Klebsiella pneumoniae*. Mol. Gen. Genet. **157**:199–204.
13. **Krenitsky, T. A., S. M. Neil, G. B. Elion, and G. H. Hutchings.** 1972. A comparison of the specificity of xanthine oxidase and aldehyde oxidase. Arch. Biochem. Biophys. **150**:585–599.
14. **Kurz, W. G. W., and T. A. G. LaRue.** 1975. Nitrogenase activity in rhizobia in the absence of plant host. Nature (London) New Biol. **246**:153–154.
15. **MacNeil, T., W. J. Brill, and M. M. Howe.** 1978. Bacteriophage Mu-induced deletions in a plasmid containing the *nif* (N_2 fixation) genes of *Klebsiella pneumoniae*. J. Bacteriol. **134**:821–829.
16. **MacNeil, T., D. MacNeil, G. P. Roberts, M. A. Supiano, and W. J. Brill.** 1978. Fine-structure mapping and complementation analysis of *nif* (nitrogen fixation) genes in *Klebsiella pneumoniae*. J. Bacteriol. **136**:253–266.
17. **Rao, R. N.** 1976. Mutational alteration of a nitrogen fixing bacterium to sensitivity to infection by bacteriophage Mu: isolation of *nif* mutations in *Klebsiella pneumoniae* M5a1 induced by Mu. J. Bacteriol. **128**:356–362.
18. **Roberts, G. P., T. MacNeil, D. MacNeil, and W. J. Brill.** 1978. Regulation and characterization of protein products coded by the *nif* (nitrogen fixation) genes of *Klebsiella pneumoniae*. J. Bacteriol. **136**:266–278.
19. **Roth, J. R.** 1970. Genetic techniques in studies of bacterial metabolism. Methods Enzymol. **17A**:3–35.
20. **St. John, R. T., H. M. Johnston, C. Seidman, D. Garfinkel, J. K. Gordon, V. K. Shah, and W. J. Brill.** 1975. Biochemistry and genetics of *Klebsiella pneumoniae* mutant strains unable to fix N_2. J. Bacteriol. **121**:759–765.
21. **Shanmugam, K. T., A. S. Loo, and R. C. Valentine.** 1974. Deletion mutants of nitrogen fixation in *Klebsiella pneumoniae*: mapping of a cluster of *nif* genes essential for nitrogenase activity. Biochim. Biophys. Acta **338**:545–553.
22. **Streicher, S., E. Gurney, and R. C. Valentine.** 1971. Transduction of the nitrogen fixation genes in *Klebsiella pneumoniae*. Proc. Natl. Acad. Sci. U.S.A. **68**:1174–1177.
23. **Streicher, S. L., K. T. Shanmugam, F. Ausubel, C. Morandi, and R. B. Goldberg.** 1974. Regulation of nitrogen fixation in *Klebsiella pneumoniae*: evidence for a role of glutamine synthetase as a regulator of nitrogenase synthesis. J. Bacteriol. **120**:815–821.
24. **Taylor, A. L.** 1963. Bacteriophage-induced mutation in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **50**:1043–1051.
25. **Wolf, B., A. Newman, and D. A. Glaser.** 1968. On the origin and direction of replication of the *Escherichia coli* K12 chromosome. J. Mol. Biol. **33**:611–629.