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Constitutive expression of nitrogen fixation (*nif*) genes of *Klebsiella pneumoniae* due to a DNA duplication

Lionel Sibold* and Claudine Elmerich

Unité de Physiologie Cellulaire, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, 75724 Paris Cedex 15, France

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A spontaneous mutant of *Klebsiella pneumoniae* exhibiting nitrogen fixing activity in the presence of ammonia was isolated from a *nifL*::Mu mutant. The main features of the *nif* constitutive mutation, designated *nif-8388*, were as follows: (i) neither ammonia nor bases repressed, but amino acids partially repressed, nitrogen fixation; (ii) the mutation caused an escape from the regulatory effect of *glnA* and *glnG* mutations of *K. pneumoniae* but not that of a *glnF* mutation; (iii) it enabled the activation of the *nifH-lac* fusion in the presence of oxygen with or without ammonia and a *nifL-lac* fusion in the presence of ammonia without oxygen; (iv) the mutation allowed nitrogen fixation at 37°C when plasmid-borne. Restriction analysis and Southern hybridization using Mu DNA and the 8.1-kb *nifQBALF Eco*RI fragment as probes demonstrated that the *nif-8388* mutation was a tandem duplication of 10 kb in the *nifL* region in which no Mu DNA was present. This duplication led to an operon fusion between *nifLA* and *his* since Nif^c expression was shown to be increased with a specific inducer of the *his* operon. These results provide further evidence that the *nifA* product is a *nif*-specific activator, and that the *nifL* product is involved in oxygen repression and temperature control. In addition, they suggest that there is an autoactivation of *nifLA* transcription by the *nifA* product and that *glnF* could act in *nif* regulation by a mechanism other than the *glnG*-mediated control of *nifLA* transcription.

Key words: gln regulatory genes/*Klebsiella pneumoniae*/*nif* constitutive expression/phage Mu

Introduction

In *Klebsiella pneumoniae* a cluster of 17 *nif* genes organized in seven transcriptional units, and located on the chromosome near the *his* operon, is involved in the reduction of molecular nitrogen to ammonia (MacNeil *et al.*, 1978; Merrick *et al.*, 1980; Pühler and Klipp, 1981; Sibold, 1982). In the wild-type strain, nitrogen fixation ability is repressed by a variety of nitrogen sources, including ammonia, nitrate, and amino acids and also by oxygen and by temperature >37°C (Tubb and Postgate, 1973; Eady *et al.*, 1978; Hennecke and Shanmugam, 1979). Studies on the regulation of *nif* gene expression have identified two regulatory mechanisms: a *nif*-specific mechanism functioning through the products of the *nifLA* operon (Dixon *et al.*, 1977, 1980; MacNeil and Brill, 1980; MacNeil *et al.*, 1981; Sibold *et al.*, 1981; Hill *et al.*, 1981; Merrick *et al.*, 1982; Buchanan-Wollaston *et al.*, 1981a, 1981b) and a non-*nif*-specific mechanism due to regulation of an overall system of utilization of the nitrogen source involving *gln* genes (Streicher *et al.*, 1974; Shanmugam *et al.*, 1975; Ausubel *et al.*, 1979; Leonardo and Goldberg, 1980; de Bruijn and Ausubel, 1981; Espin *et al.*, 1981, 1982).

To study the mechanism of *nifLA* regulation, we previously tested a collection of Nif$^-$ mutants (Elmerich *et al.*, 1978; Houmard *et al.*, 1980; Merrick *et al.*, 1980; Sibold, 1982) to see if any could revert to a Nif constitutive (Nif^c) phenotype, i.e., strains fixing nitrogen in the presence of NH_4^+ ions. Nif$^+$ revertants were obtained for mutations in most of the *nif* genes. Only in the case of *nifL*::Mu mutations were revertants found which were Nif^c. Preliminary studies of one of these mutants showed that the mutation (*nif-8388*) was *nif* specific and allowed nitrogen fixation to escape ammonia-induced repression up to 200 mM and to escape the regulatory effect of the *glnA* GlnR(Nif$^-$) mutation of the *K. pneumoniae* KP5060 strain. This strongly suggested that the *nifA* product was the only necessary positive effector of the transcription of the other *nif* operons and that the *nifLA* operon was the only target of the *gln*-mediated control (Sibold *et al.*, 1981; Elmerich *et al.*, 1981). Similar conclusions obtained with the same type of mutants were also reached by MacNeil and Brill (1980).

In previous reports, the *nif*^{c} mutations were not characterized at the molecular level. In this paper, we show that the *nif-8388* mutation is a consequence of a DNA duplication which fused the *nifA* gene to the adjacent *his* promoter. Physiological properties of the Nif^c mutant are in agreement with the previously reported functions of *nifA* and *nifL* products being respectively an activator and a repressor of *nif* transcription (Buchanan-Wollaston *et al.*, 1981a, 1981b; Hill *et al.*, 1981; Merrick *et al.*, 1982). In addition, the results suggest that the *glnF* gene product may be required for *nif* gene expression.

Results

Instability of the Nif^c mutation

The genealogy of plasmids and strains carrying the *nif-8388* mutation or its derivatives is given in Figure 1. Plasmid pPC852 is the initial Nif^c mutant, carrying the *nif-8388* mutation, which was derived from the *nifL8552*::Mu*cts62* insertion. This plasmid contained a Mu prophage. Strain PC88 and plasmid pPC868 (steps 1 and 2) were obtained by cotransduction of the Nif^c phenotype with the His$^+$ marker in strains UNF5023 and UNF107(pCE2), respectively. Analysis of the transductants showed that the *nif-8388* mutation was *nif* specific, dissociable from the presence of the Mu prophage, and likely located in the *nifLA* region (Sibold *et al.*, 1981). Results reported in Table I show that the Nif^c phenotype conferred by the *nif-8388* mutation was stable only in a *recA* background. Strains PC88 and UNF107(pPC868) segregated Nif$^+$ clones such as plasmid pPC870 which is indistinguishable from pCE1 (step 3), while strain UNF107(pPC852) segregated Nif$^-$ clones such as plasmid pPC853 (step 4). In no case was the Nif$^-$ phenotype due to a loss of the plasmid, since segregants were still His$^+$, Km^R, and Tc^R. Genetic analysis of plasmid pPC853 showed that

*To whom reprint requests should be sent.

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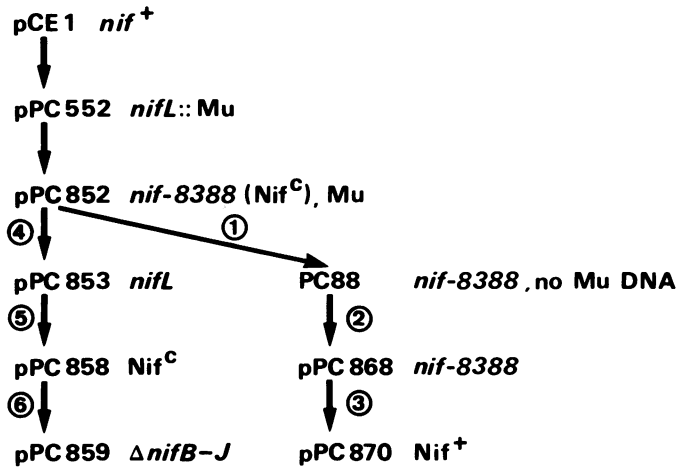

Fig. 1. Transfer of mutation *nif-8388* and derivatives of pPC852. **Steps 1** and **2**: P1 transduction. **Steps 3, 4,** and **6**: segregation. **Step 5**: reversion (see text for details). All strains derived from **steps 1–6** were Mu sensitive.

Table I. Stability of the *nif-8388* mutation

Strain	Phenotype	Frequency of Nif⁻ segregants after 30 generations in complete medium[a]	Frequency of Nif⁺ among Nifᶜ after 30 generations in complete medium[b]
UNF5023	Nif⁺	$<10^{-3}$	–
PC88	Nifᶜ	$<10^{-3}$	14/100
PC88*recA*	Nifᶜ	$<10^{-3}$	0/50
UNF107(pCE1)	Nif⁺	$<10^{-3}$	–
UNF107(pPC852)	Nifᶜ	$25-50 \times 10^{-2}$	ND
UNF107*recA*(pPC852)	Nifᶜ	$<10^{-3}$	ND
UNF107(pPC868)	Nifᶜ	$<10^{-3}$	33/50
UNF107*recA*(pPC868)	Nifᶜ	$<10^{-3}$	0/50

[a]Frequency of Nif⁻ segregants was determined by replicating ~10^3 colonies onto nitrogen-free medium.
[b]Nifᶜ phenotype of individual colonies was determined by the acetylene reduction test.
ND: not determined.

the plasmid carried a *nifL* mutation located in the same deletion interval as the original *nifL8552* insertion. Strains carrying pPC853 were Mu sensitive. Spontaneous Nif⁺ revertants were obtained from UNF107(pPC853) at a frequency of 10^{-8} (step 5). All of them were Mu sensitive and Nifᶜ to a low extent. For example, plasmid pPC858 conferred a nitrogenase activity in the presence of NH_4^+ ions, which was no more than 2% of the wild-type in the absence of ammonia. One spontaneous Nif⁻ mutant plasmid, pPC859, derived from pPC858 at a frequency of 0.2% (step 6), was found to be deleted from *nifB* through *nifJ*.

Physical analysis of plasmids carrying the nif-8388 mutation

Plasmids pCE1, pPC552, pPC852, pPC853, pPC858, pPC859, pPC868, and pPC870 (see Figure 1) were purified from the *recA* strain JC5466 and the restriction patterns generated by *Eco*RI, *Hind*III (and in some cases by *Sma*I) were compared to those of pCE1. Additional fragments were found for all plasmids except for pPC870, which had the same restriction patterns as pCE1, and for pPC859, which had fewer restriction fragments. Restriction fragments containing Mu DNA were identified by hybridization with a Mu

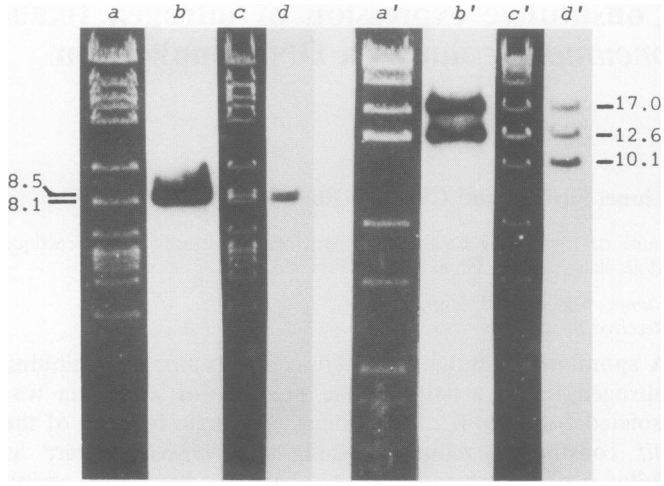

Fig. 2. Restriction patterns of pCE1 and pPC868 by *Eco*RI and *Hind*III and hybridization with the 8.1-kb *nifQBALF Eco*RI fragment. **a**: pCE1 digested by *Eco*RI. **c**: pPC868 digested by *Eco*RI. **a'**: pCE1 digested by *Hind*III. **c'**: pPC868 digested by *Hind*III. **b,d,b',d'**: corresponding autoradiograms of **a,c,a',c'**. The position of hybridizing fragments, with lengths given in kilobases, is indicated.

DNA probe. Plasmids pPC552, pPC852, pPC853, pPC858, and pPC859 contained Mu DNA, whereas plasmid pCE1, pPC868, and pPC870 as well as DNA from strain PC88 were devoid of Mu sequences. The *nifQBALF* 8.1-kb *Eco*RI fragment purified from recombinant plasmid pCRA37 was used as a DNA probe to identify the restriction fragments containing the corresponding genes. This fragment does not carry any *his* gene (see Figure 3). The wild-type pCE1 hybridized with a single 8.1-kb *Eco*RI fragment, with two *Hind*III fragments of 12.6 and 17 kb (see Figures 2 and 3) and three *Sma*I fragments of 3.3, 3.8, and 7.3 kb (data not shown).

Comparison of the restriction patterns and hybridization data established the structure of the *nif* region for all plasmids, except that of pPC858 which was not understood. The structures of the most interesting plasmids are presented schematically in Figure 3. Plasmid pPC870 which did not differ from pCE1 and plasmid pPC859 which carried a *nifB* to *J* deletion are not reported.

Plasmids pPC552 and pPC853, which are both *nifL* mutants, had almost the same structure. In pPC552, the Mu prophage was physically localized in *nifL* and its orientation was determined. The *c*-terminal end was mapped at 1.3 kb from the next *Eco*RI site and the *S*-terminal end at 4.7 kb from the next *Hind*III site. In pPC853, a Mu prophage is also present at the same location, but it appears to contain a 0.6-kb deletion in the *c*-terminal end, which is compatible with the Mu-sensitive phenotype.

The structures of plasmids pPC852 and pPC868, which both carry the *nif-8388* mutation, were more difficult to establish. Plasmid pPC868 had the same *Eco*RI, *Hind*III, and *Sma*I restriction patterns as pCE1, but contained one additional fragment of 8.5, 10, and 10 kb, respectively. Hybridization using the *nif* probe showed that plasmid pPC868 had a wild-type set of *nif* genes and that the additional fragments contained *nif* DNA (see Figure 2). Plasmid pPC852 had the same *Eco*RI and *Hind*III restriction patterns as pPC853 but in each case the additional *nif* fragments detected in pPC868 were also present. The supplementary 10-kb *Hind*III fragment from pPC868 was electroeluted from

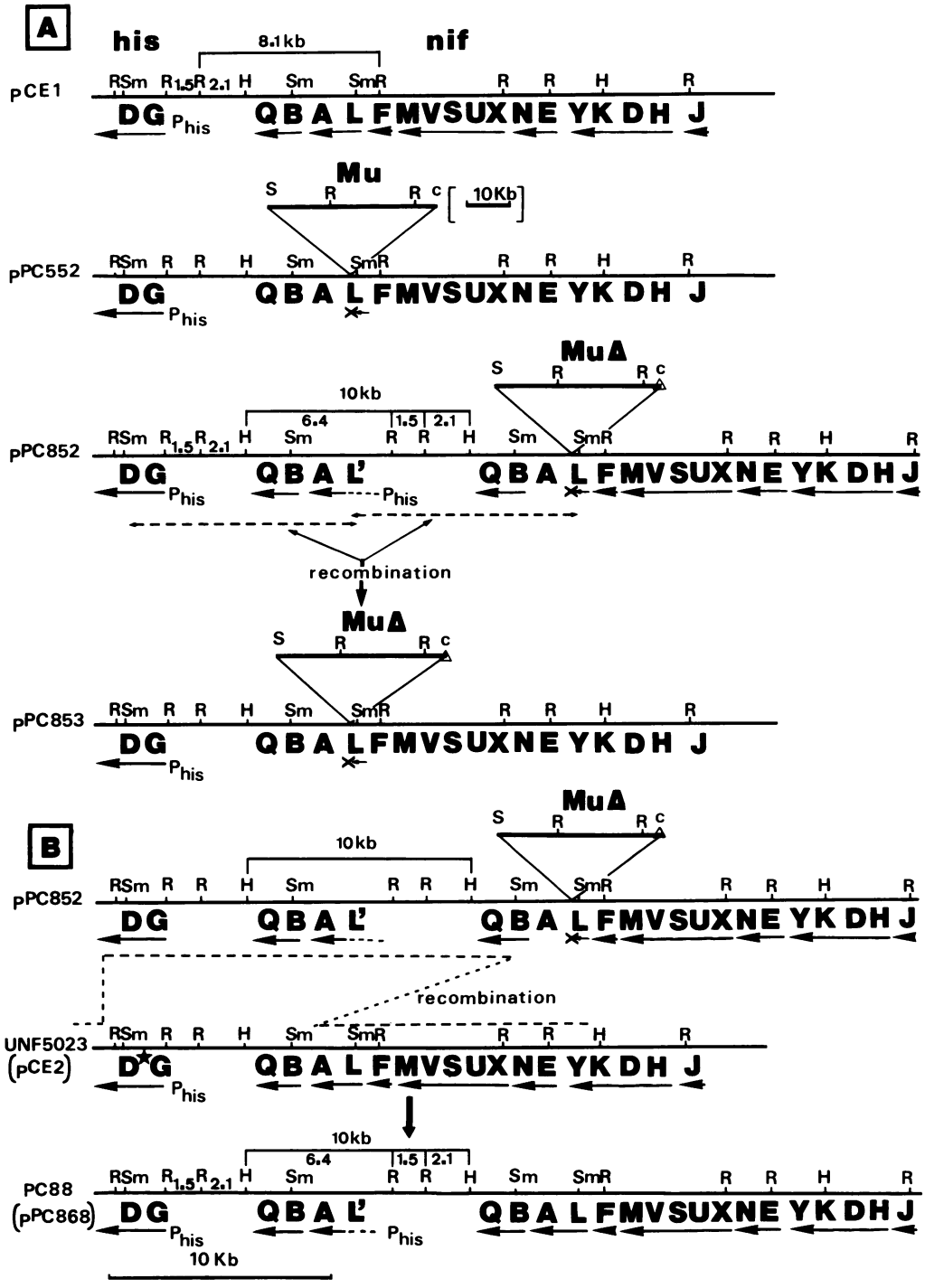

Fig. 3. Physical structures of the *his-nif* region of plasmids pPC552, pPC852, pPC853, and pPC868 as compared to pCE1. Restriction sites: H: *Hind*III, R: *Eco*RI, Sm: *Sma*I. Arrows indicate the direction of transcription of the *nif* and *hisGD* genes. S and c are respectively the *S* end and the immunity end of the Mu prophage. Mu△ stands for a defective prophage which presumably carries a deletion (△) in the *c* region (see text). X←: indicates that transcription is blocked by the insertion of Mu DNA. <— — —>: extent of the tandem duplication. kb: kilobase-pairs. D*: *hisD2* mutation. A: structures of pCE1, pPC552, pPC852, and pPC853. B: formation of PC88 and pPC868.

an agarose gel and digested by *Eco*RI. Three fragments of 6.4, 2.1, and 1.5 kb were generated. The 6.4 and 2.1 kb fragments hybridized with the *nif* probe. The 1.5 kb *Eco*RI fragment, which corresponded to the fragment located between *hisG* and *nifQ* (see Figure 3), has been duplicated in the mutant but had no homology with the *nif* probe used.

Knowing the restriction sites in the *his* operon (Rodriguez *et al.*, 1981) and in the *nif* genes (Riedel *et al.*, 1979; Pühler and Klipp, 1981) the appearance of the additional *Eco*RI, *Hind*III, and *Sma*I fragments could be explained by a tandem DNA duplication of ~10 kb in the plasmids conferring the Nif^c phenotype as schematized in Figure 3.

Physiological properties of strains carrying the nif-8388 mutation

Influence of nitrogen sources. Under conditions which totally repressed nitrogenase biosynthesis in the wild-type strain, the activity of strains carrying the *nif-8388* mutation

Table II. Influence of various nitrogen sources on nitrogenase derepression of *K. pneumoniae* strains with or without the *nif-8388* mutation

Strain	*nif* genotype	Addition to nitrogen-free medium									Luria broth
		Aspartate 0.75 mM	NH_4Cl 2 mM	NH_4Cl 20 mM	Glutamate 20 mM	Histidine 10 mM	Aspartate 20 mM	Glutamine 10 mM	Casamino acids 5 mg/ml	Bases ACGU 2 mM each	
PC8	*nif*$^+$	100 (75)	5	0	60	36	2	0	0	0	0
PC88*recA*	*nif-8388*	100 (50)	41	7	85	96	12	11	0.4	40	0
UNF107*recA*(pCE1)	△*nif*/*nif*$^+$	100 (85)	43	0	47	38	8	0	0	0	0
UNF107*recA*(pPC868)	△*nif*/*nif-8388*	100 (63)	100	56	98	24	5	84	10	86	0

Nitrogenase-specific activity was measured after overnight derepression. Activities are expressed as % of the activity (nmol C_2H_4/min/mg protein), shown in brackets, in NFM aspartate.
0 is the limit of sensitivity of the assay, i.e., 0.01 nmol/min/mg protein.

Table III. Nitrogenase activity of *K. pneumoniae gln* strains with or without the *nif-8388* mutation

Strain	Relevant genotype	Nitrogenase activity: nmol C_2H_4/min/mg protein					
		Plasmid					
		None		pCE1		pPC868	
		NFM		NFM		NFM	
		Aspartate 0.75 mM	NH_4Cl 20 mM	Aspartate 0.75 mM	NH_4Cl 20 mM	Aspartate 0.75 mM	NH_4Cl 20 mM
UNF5023	*nif*$^+$	60	0	44	0	53	40
UNF107	△*nif*	0	0	85	0	63	40
KP5060	*glnA100*	0	0	0	0	90	62
KG6099	*glnA302*	0	0	0	0	34	19
KG7040	*glnA402*	0	0	0	0	70	66
KG7039	*glnB402*	72	0	39	0	45	39
KG7069	*glnB502*	66	0	14	0	63	51
KG6238	*glnF251*	0	0	0	0	0	0
KG7209	*glnG351*	0	0	0	0	60	55

All strains were rendered *recA* as described by MacNeil *et al.* (1978).
0 is the limit of sensitivity of the assay i.e., 0.01 nmol/min/mg protein.

varied largely depending on the nitrogen source and on the chromosomal or plasmid location of the mutation. As shown in Table II, complete medium was the only condition where nitrogenase of the mutant was totally repressed. In the presence of NH_4^+ ions or bases, nitrogenase activity was not repressed when mutation *nif-8388* was plasmid-borne and was partially repressed when located on the chromosome. Amino acids such as glutamic acid or histidine, which are poorly assimilated, had little effect on the wild-type, whereas aspartic acid or glutamine were strongly repressive. Strains carrying the *nif-8388* mutation behaved as the wild-type in the presence of glutamic acid, histidine, or aspartic acid. Repression by glutamine was partially overcome.

Expression of the nif-8388 mutation in gln(Nif$^-$) regulatory mutants. Plasmid pPC868 was introduced by conjugation into *K. pneumoniae* Gln$^-$(Nif$^-$) mutants carrying mutations in the *glnA, glnB, glnF,* or *glnG* (also called *glnR* in some reports). As shown in Table III and in agreement with Streicher *et al.* (1974) and Leonardo and Goldberg (1980), the *glnA, glnG,* and *glnF* mutants did not fix nitrogen in the absence of ammonia. In our hands, the *glnB* mutants were not Nif$^-$ as previously reported (Leonardo and Goldberg, 1980). Except for strain UNF107, the Nif phenotype of the strains was not modified after introduction of pCE1. The presence of pPC868 modified the phenotype of the *glnA, glnB,* and *glnG* strains, which all became Nif^c whereas the *glnF* mutant remained Nif$^-$.

Effect of oxygen on transcription of nif operons. To study the effect of oxygen on *nif* transcription, plasmids pCE1 or pPC868 were introduced into strains carrying *nifL-lac*(UNF743) or *nifH-lac*(UNF766) fusions and β-galactosidase activity was assayed. In addition, the *nif-8388* mutation was transduced with P1 into strain UNF766 and Lac$^+$ phenotype was selected on minimal 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) plates, to yield strain PC100. The results are reported in Table IV. For strain UNF766 and UNF766(pCE1) the β-galactosidase activity was maximal only under conditions permissive for nitrogenase activity; otherwise it was repressed. In the presence of the *nif-8388* mutation, in either *cis* (PC100) or *trans* [UNF766(pPC868)] positions the β-galactosidase biosynthesis escaped ammonia and oxygen repression. In the two strains, the level of β-galactosidase activity in Luria broth was significantly higher than the basal level observed with either

Table IV. β-Galactosidase expression from the *nifL* and *nifH* promoters in *K. pneumoniae* strains with or without the *nif-8388* mutation

Strain[a]	Relevant *nif* genotype Chromosome/plasmid	β-Galactosidase activity					
		N_2 bubbling			Air bubbling		
		NFM aspartate 0.75 mM	NFM NH_4Cl 20 mM	Luria broth	NFM aspartate 0.75 mM	NFM NH_4Cl 20 mM	Luria broth
UNF766	*nifH-lac*	1285	4	9	37	24	7
PC100	*nif-8388 nifH-lac*	1243	982	17	694	939	65
UNF766(pCE1)	*nifH-lac/nif*$^+$	605	32	6	29	4	3
UNF766(pPC868)	*nifH-lac/nif-8388*	994	1474	33	624	642	67
UNF743	*nifL-lac*	108	8	8	42	25	7
UNF743(pCE1)	*nifL-lac/nif*$^+$	140	20	15	27	4	4
UNF743(pPC868)	*nifL-lac/nif-8388*	105	50	ND	40	47	ND

β-Galactosidase-specific activity, expressed in units defined by Miller (1972), was measured after 5–6 h vigorous bubbling with N_2 or air.
ND: not determined.
[a]Strains UNF766 and PC100 were rendered *recA*.

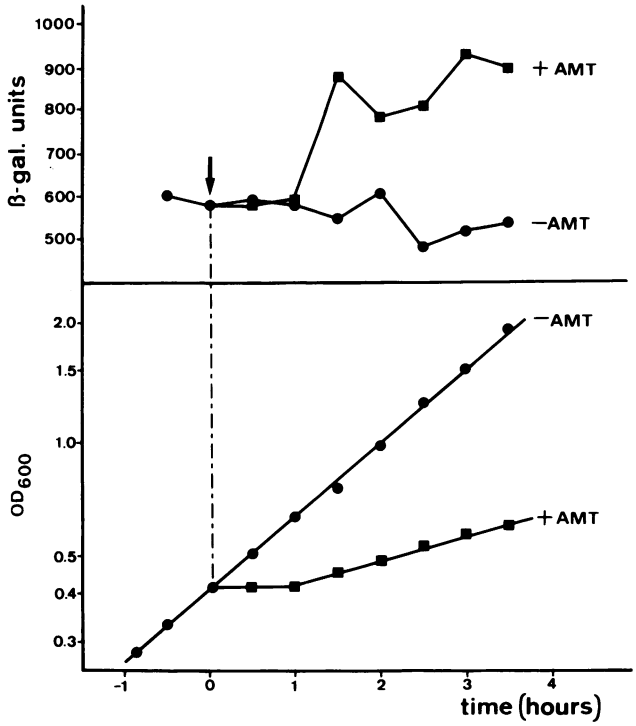

Fig. 4. Derepression with AMT of β-galactosidase in a *nifH-lac* fusion strain carrying the *nif-8388* mutation. Strain PC100 (*nifH-lac nif-8388 recA*) was grown aerobically at 30°C in nitrogen-free medium supplemented with 40 mM NH_4Cl (●——●). When OD_{600} reached 0.41 (↓) AMT (20 mM) and adenine (0.4 mM) were added to half of the culture (■——■).

strain UNF766 or UNF766(pCE1).

When strains carrying a *nifL-lac* fusion were examined, the maximum β-galactosidase activity observed in NFM aspartate (see Materials and methods) was ~10–20% of the maximum level obtained with the *nifH-lac* fusion. As previously reported, transcription from *nifLA* promoter was partially independent of oxygen repression (Dixon *et al.*, 1980; Hill *et al.*, 1981; Merrick *et al.*, 1982). This was also observed for strains carrying the *nif-8388* mutation. Thus, the level of β-galactosidase activity in NFM aspartate in the presence of oxygen was 20–40% of that observed in anaerobiosis. Results obtained in the presence of 20 mM NH_4^+ suggested that the *nifL-lac* strain containing the *nif-8388* mutation was less sensitive to repression particularly under N_2 bubbling conditions.

Influence of temperature. The *K. pneumoniae* wild-type strain did not exhibit nitrogenase activity after overnight growth at 37°C in NFM aspartate. With strain PC88*recA* which carries *nif-8388* on the chromosome, no nitrogenase activity was observed at 37°C. However, with strains UNF107*recA*(pPC868), carrying *nif-8388* on a plasmid, derepression of nitrogenase activity was observed at 37°C up to 60% of the level obtained at 30°C. In the presence of 20 mM ammonia the level reached at 37°C was no more than 8% of the level at 30°C.

Activation of nifH-lac expression with a his-specific transcription inducer. Physical analysis of the *nif-8388* mutation suggested that expression of *nifA* was under the control of a promoter located in the duplicated sequence or at the junction of the fusion. From the physical structure presented in Figure 3 it appeared that the hypothetical promoter could be the *his* promoter. In order to test this assumption we investigated the effect of 3-amino-1,2,4-triazole (AMT), a specific inducer of *his* transcription (Fernandez *et al.*, 1975), on the β-galactosidase activity of strain PC100 (*nifH-lac, nif-8388*) in the presence of 40 mM NH_4^+ ions and of oxygen. As shown in Figure 4, addition of AMT drastically affected the growth rate; however, after 1 h lag β-galactosidase-specific activity was about doubled as compared to the control without AMT. This effect was not observed when the strain did not carry the *nif-8388* mutation.

Discussion

In this paper, the main features and the physical structure of a mutation (*nif-8388*) which leads to a Nif^c phenotype are described.

RecA-dependent instability of the strains carrying the *nif-8388* mutation can be explained since it was found that the mutation corresponded to a tandem DNA duplication which could be excised in Rec^+ hosts by homologous recombination. The molecular events leading to each of the plasmid derivatives described in Figure 1 could be tentatively interpreted as schematized in Figure 3. Plasmid pPC552 contained a single Mu prophage inserted in *nifL* which is responsible for the Nif^- phenotype. Strains containing pPC852 are still Mu producers and the physical analysis revealed the presence of two Mu prophages in the plasmid, a defective one localized in *nifL*, and another one localized outside the *nif* region. Thus, spontaneous *nifL* mutant derivatives of pPC852, such as

pPC853 occurred in Rec$^+$ host by precise excision of the duplicated sequence (see Figure 3A). Molecular events which yield pPC858 or pPC859 are not understood and will not be discussed here. By P1 transduction of the duplicated region into strain UNF5023 and plasmid pCE2, the duplication was reconstructed without introduction of any Mu sequence. Thus, plasmid pPC868 and strain PC88 did not contain the original *nifL* mutation. This is why only Nif$^+$ segregants such as pPC870 were obtained by loss of the duplicated sequence.

It is tempting to speculate that the original Mu prophage in plasmid pPC552 provoked the DNA duplication event. Usually in Mu-induced DNA rearrangements, two Mu prophages are found at the extremities of the illegitimate recombination sites (Toussaint *et al.*, 1977). However, if such a structure had occurred it would have caused a Nif$^-$ and not a Nif^c phenotype. Therefore, we have no explanation for the molecular events which are responsible for the actual structure of plasmid pPC852. The structure of the supplementary 10-kb *Hind*III fragment of pPC868 (Figures 2 and 3) strongly suggests that DNA duplication occurred from the point of insertion of the originl Mu in *nifL (nif8552*::Mu) and resulted in a fusion within the *Eco*RI fragment carrying the *hisGD* genes (Figure 3) (Rodriguez *et al.*, 1981) which also contains a part of the *his* promoter. As previously suggested from physiological studies (Sibold *et al.*, 1981), the constitutive mutation dissociated *nifA* from its own promoter. The results reported here are in agreement with *nifA* being transcribed under the control of the *his* promoter since AMT increased *nif* transcription in strains that carry the *nif-8388* mutation (Figure 4).

Thus, by its structure and resulting physiological properties, the *nif-8388* mutation, which corresponded to the fusion of *nifA* to an exogenous promoter, is similar to the *in vitro* cloning of *nifA* into multicopy plasmids recently reported by Buchanan-Wollaston *et al.* (1981b). Interestingly, some phenotypes conferred by mutation *nif-8388* allow further comments on *nif* gene regulation. In the current model, *nifA* and *nifL* products are respectively an activator (Dixon *et al.*, 1980; MacNeil and Brill, 1980; Sibold *et al.*, 1981; Buchanan-Wollaston *et al.*, 1981a, 1981b) and a repressor (Hill *et al.*, 1981; Buchanan-Wollaston *et al.*, 1981a; Merrick *et al.*, 1982) of *nif* transcription. *Nif* transcription is in turn controlled in response to nitrogen sources by the *glnG* product (Leonardo and Goldberg, 1980; Espin *et al.*, 1981; de Bruijn and Ausubel, 1981) at the level of the *nifLA* promoter (MacNeil and Brill, 1980; Sibold *et al.*, 1981; Buchanan-Wollaston *et al.*, 1981b).

The effect of nitrogen sources on nitrogenase biosynthesis in the wild-type strain (see Table II) is in agreement with previous reports and with the fact that under strong repressive conditions (e.g., 20 mM NH_4^+ ions, 10 mM glutamine, Luria broth) *nifLA* is not transcribed (Merrick *et al.*, 1982). The nitrogenase level of strains carrying the *nif-8388* mutation, can be accounted for by the fact that the *his* operon, in *Salmonella typhimurium* and *Escherichia coli* is known to be partially repressed in rich medium and never totally derepressed in minimal medium (Brenner and Ames, 1971; Bruni *et al.*, 1980). However, the total repression observed in Luria broth was unexpected (as well as the repression observed in 20 mM aspartate). We could ask whether amino acids have an effect on the activity of the *nifA* product, independent of transcription of the *his-nifA* fusion. Results obtained with the *nifH-lac* fusion also show that the *nif-8388* mutation overcomes repression by NH_4^+ ions and oxygen but not that by Luria broth (Table IV).

Another important feature is that the *nif-8388* mutation is *trans* dominant over the wild-type allele regardless of the conditions used (see Tables II and IV). This could be questioned since the *nifL* product has been proposed by Hill *et al.* (1981) to have some repressor properties. In studying *nifL* mutants which partially overcome oxygen repression, these authors proposed that the *nifL* product might act as a repressor in the presence of oxygen, since the wild-type allele was *trans* dominant over the mutant allele. Similarly, some *nifL* mutants displayed nitrogenase activity in the presence of 3 mM NH_4^+ ions (Merrick *et al.*, 1982). One can tentatively explain the difference between these results on *trans* dominance by suggesting that in strains containing the *nif-8388* mutation, the *nifA* product, which is not produced from its own promoter, is made in excess with respect to the *nifL* product. Indeed the level of transcription from the *nifL* promoter in the presence of oxygen (or even in its absence) is relatively low as compared to that of the other transcriptional units (Dixon *et al.*, 1980; Hill *et al.*, 1981; Merrick *et al.*, 1982; cf. Table IV). Moreover, the introduction of the *nif-8388* mutation into the *nifL-lac* fusion (Table IV) does not modify the basal level of *nifLA* transcription in NFM aspartate with or without oxygen. It is interesting to note that, in the presence of ammonia and without oxygen, the relative level of *nifL-lac* expression is higher with the *nif-8388* mutation. This was confirmed by using a multicopy plasmid that carries the *nif-8388* mutation (Sibold *et al.*, 1983). These observations suggest that the *nifA* product can stimulate, directly or indirectly, its own transcription even though it was found not to be necessary for its transcription (Dixon *et al.*, 1980). Thus, stimulation of *nifA* transcription by its product could be an advantage in the early stages of derepression.

The role of *nifLA* products in temperature regulation is not clear (Zhu and Brill, 1981; Buchanan-Wollaston *et al.*, 1981b; Merrick *et al.*, 1982) and the reason why the *nif-8388* mutation confers thermoresistance only when plasmid-borne requires further investigation. The different *nif* expression depending on the chromosomal or plasmidic location is not a specific feature of the *nif-8388* mutation since this was found also with pCE1. A similar observation was reported in the case of *nifUSVM* expression (Sibold, 1982).

Total repression of *nif* genes in strains carrying the *nif-8388* mutation was observed not only in Luria broth (see Tables II and IV) but also in a strain with *glnF251* background even under derepressing conditions. In *K. pneumoniae, E. coli,* and *S. typhimurium*, the glutamine synthetase biosynthesis is under the control of the *glnF* (or *ntrA*) gene product through conversion of the *glnR/G* (*ntrBC*) product into an activator form (Leonardo and Goldberg, 1980; MacFarland *et al.*, 1981). In *K. pneumoniae*, recent results suggested that the *ntrC* gene product alone was sufficient for *nif* expression (Espin *et al.*, 1982). Since the *nif-8388* mutation is independent of the *glnG* control (see Table III), the finding that the mutant is totally Nif$^-$ in a *glnF* background is an important new feature in the regulation of *nif* expression. Since it is unlikely that *glnF* controls the *his* transcription, we propose that the *glnF* product is necessary for the activity of the *nifLA* products. No mechanism of action can yet be proposed. One possibility could be that the *glnF* product is involved in the production of a specific low mol. wt. effector which would act by modulating the activity of the *nifA* product.

Table V. Bacterial strains and plasmids

Strain	Genotype or phenotype	Source or reference
E. coli		
JC5466	*his trp recA56 rpsE*	Cannon *et al.* (1976)
K. pneumoniae		
UNF5023	*hisD2 hsdR1 rpsL4*	Dixon *et al.* (1977)
PC8	*hsdR1 rpsL4 recA56...srl-300::*Tn*10*	This work
UNF107	△*-107 (gnd his nif* total deletion) *rpsL1*	Dixon *et al.* (1977)
UNF743	*hsdR1* △*lac-2002 nifL2782*::MudAp*lac recA56...srl-300*::Tn*10*	Dixon *et al.* (1980)
UNF766	*hisD2* △*lac-2002 nifH2783*::MudAp*lac*	Dixon *et al.* (1980)
PC100	△*lac-2002 nif-8388 nifH2783*::MudAp*lac recA56...srl-300*::Tn*10*	This work
PC88	*hsdR1 nif-8388 rpsL4*	Sibold *et al.* (1981)
KP5060	*glnA100 hisD2 hsdR1*	Streicher *et al.* (1974)
KG6099	*glnA302 hisD2 metB102*	Leonardo and Goldberg (1980)
KG6238	*glnF251*::Mu*cts62*	Leonardo and Goldberg (1980)
KG7039	*glnB402 hutC200 metB101 rha-101*	Leonardo and Goldberg (1980)
KG7040	*glnA402 hutC200 metB101 rha-101*	Leonardo and Goldberg (1980)
KG7069	*glnB502 hutC200 metB101 rha-101*	Leonardo and Goldberg (1980)
KG7209	*glnG351*::Mu*cts62 hutC200 metB101 rha-101*	Leonardo and Goldberg (1980)
Plasmid		
pCE1	*amp*$^+$ *kan*$^+$ *tet*$^+$ *gnd*$^+$ *his*$^+$ *nif*$^+$ *tra*$^+$ *incP*	Elmerich *et al.* (1978)
pCE2	as pCE1 but *hisD2*	This laboratory
pPC552	as pCE1 but *nifL8552*::Mu*cts*	Merrick *et al.* (1980)
pPC852	as pCE1 but *nif-8388*	Sibold *et al.* (1981)
pCRA37	contains the 8.1-kb *nifQBALF Eco*RI fragment	Cannon *et al.* (1977)

Materials and methods

Bacterial strains, phages, and plasmids

The bacterial strains and plasmids used are listed in Table V. The phages used are P1Kmc*1r100* (Kennedy, 1977), Muc$^+$, and Mu*cts62* (Elmerich *et al.*, 1978).

Media and chemicals

Luria broth, minimal and nitrogen-free (NFM) media have been described earlier (Elmerich *et al.*, 1978). Nitrogen-free liquid medium was supplemented with 100 μg/ml aspartate or with other nitrogen sources as indicated in the text. Tryptophan was added at 50 μg/ml, glutamine at 200 μg/ml, and other amino acids at 25 μg/ml. Antibiotics were added at the following concentrations (μg/ml): kanamycin (Km): 20, spectinomycin (Spc): 100, streptomycin (Sm): 250, tetracycline (Tc): 10. AMT and X-gal were from Sigma Chemical Co.

Genetic techniques

Conjugation, P1-mediated transduction, bacteriophage Mu production, and sensitivity tests were performed as previously described (Elmerich *et al.*, 1978; Merrick *et al.*, 1980). The reversion test for Nif$^+$ phenotype was performed as described by Sibold *et al.* (1981). *RecA* strains were constructed according to MacNeil *et al.* (1978). Lac$^+$ phenotype was checked on solid medium containing 30 μg/ml X-gal.

Enzyme assays

Nitrogenase activity of whole cells was assayed by the acetylene reduction test as described by Elmerich *et al.* (1978). Specific activities were determined as follows: overnight cultures in Luria broth medium at 30°C were inoculated into argon-filled side-arm flasks containing nitrogen-free medium supplemented as indicated, after 16–20 h incubation at 30°C, acetylene reduction was measured when OD_{600} was between 1 and 2. The Nif^c phenotype was qualitatively assayed as follows: 0.25 ml of Luria broth grown cultures were inoculated into Bijou bottles containing 7 ml nitrogen-free medium supplemented with 20 mM NH_4Cl, and acetylene reduction was measured after 5–6 h growth at 30°C. β-Galactosidase was assayed using the procedure and units of activity described by Miller (1972). The effect of oxygen was tested either on overnight cultures grown with agitation in air or in short-term derepression after 5–6 h vigorous bubbling of 5 ml cultures with air in a test tube as described by Merrick *et al.* (1982).

DNA isolation and hybridization techniques

Small scale preparations of total DNA were prepared according to Dhaese *et al.* (1979). Large plasmid DNA isolation was performed as described by Labigne-Roussel *et al.* (1981). Mu DNA was prepared according to Bukhari and Ljungquist (1977). Restriction endonucleases were from Biolabs. Digestions were performed according to the manufacturers recommendations. Horizontal gel electrophoresis was carried out as previously described by Elmerich *et al.* (1978) except that SDS was omitted from the buffer. Restriction fragments were recovered from the gels by electroelution. DNA probes were labelled with [α-^{32}P]dATP (Amersham) by nick-translation (Rigby *et al.*, 1977). Hybridization was performed as described by Southern (1975).

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