Expression of *Klebsiella nif* and *his* Genes in *Salmonella typhimurium*

By J. R. POSTGATE AND V. KRISHNAPILLAI*

Agricultural Research Council, Unit of Nitrogen Fixation,
University of Sussex, Brighton BN1 9QJ

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SUMMARY

Derivatives of *Salmonella typhimurium* carrying F prime or P prime plasmids with *Klebsiella nif* and *his* genes had specific nitrogenase activities similar to *Klebsiella* in selective conditions, even to showing 'hyperinduction' under argon. No evidence was obtained for catabolite repression of normal *nif* expression but dibutyl cyclic AMP often augmented 'hyperinduction'. In non-selective conditions the *Klebsiella his nif* determinants were rapidly lost from the plasmids; the low levels of *nif* expression and temperature-sensitive *his* expression previously reported were probably due to ready loss of *his nif* in the test conditions used.

INTRODUCTION

The ability of *Salmonella typhimurium* to accept and express plasmids carrying the *nif* genes of *Klebsiella pneumoniae* was reported briefly by Cannon, Dixon & Postgate (1976). The ability is consistent with the substantial evidence that expression of nitrogen fixation (*nif*) genes is regulated by a mechanism which has much in common with a system for regulation of utilization of histidine studied by Magasanik and his colleagues. This system, which is present in *K. aerogenes*, *S. typhimurium* and *Escherichia coli*, regulates the use of several other sources of fixed nitrogen (proline, urea, nitrate, etc.); it is based on the ability of the enzyme glutamine synthetase to act as an activator of the genes for histidine utilization (*hut*) as well as performing its physiological function as an enzyme, provided it is in its deadenylylated form (see Magasanik *et al.*, 1974).

Certain details of the regulation of *hut* by fixed nitrogen differ between *S. typhimurium* and *K. aerogenes*. In both organisms, gene expression is repressed by ammonium ions and glucose, but in *K. aerogenes* w-70, starvation of fixed nitrogen releases *hut* from both catabolite repression and NH_4^+ repression, whereas it is not released from catabolite repression in *S. typhimurium* strain 15-59 in comparable conditions (Brill & Magasanik, 1969). Catabolite repression of *hut* in *K. aerogenes* can be overcome by adding cyclic AMP or by use of non-repressing substrates. After *S. typhimurium hut* genes have been transferred to *K. aerogenes* on an F prime plasmid, they follow the *K. aerogenes* pattern of regulation (Prival & Magasanik, 1971). The possibility therefore arises that expression of *K. pneumoniae nif* in *S. typhimurium* may also follow a changed regulatory pattern; a precedent for changed expression characteristics in the new genetic background seemed also to arise since histidine biosynthesis governed by *K. pneumoniae his* genes apparently became temperature-sensitive in *S. typhimurium* (Cannon *et al.*, 1976). This paper reports further experiments on the expression of *Klebsiella nif* and *his* genes in *Salmonella*.

* Present address: Department of Genetics, Monash University, Clayton, Victoria 3168, Australia.

Table 1. *Bacteria and plasmids*

Bacterial strain	Markers	Source or reference	
<i>Klebsiella pneumoniae</i> M5a1	Prototrophic	P. W. Wilson	
<i>Escherichia coli</i> JC5466	<i>trp recA56 his spc</i>	N. Willetts	
<i>Salmonella typhimurium</i> LT7-1201	<i>hisΔ str</i>	S. B. Primrose	
UNF602	<i>str</i>	LT7-1201 (FN68); Cannon <i>et al.</i> (1976)	
VKSI to 4	<i>str</i>	LT7-1201 (RP41); this laboratory	
Plasmids	Markers	Compatibility group	Source or reference
FN68	<i>metG rfb gnd his nif shiA</i> Cb	F	Cannon <i>et al.</i> (1976)
RP41	<i>rfb gnd his nif shiA</i> Cb Km Tc	P	Dixon, Cannon & Kondorosi (1976)

Symbols for drug resistance determinants: Km, kanamycin; Tc, tetracycline; Cb, Carbenicillin. Symbols for chromosomal markers are those of Taylor & Trotter (1972).

METHODS

Bacterial strains and plasmids are listed in Table 1. Complete medium was Oxoid no. 2 nutrient broth supplemented where necessary with 2 % (w/v) Difco Bacto-agar. Minimal medium was Davis & Mingioli (1950) mineral salts; NH_4^+ -free medium used for culture in nitrogen-fixing conditions was NFDM (Cannon *et al.*, 1974) containing Casamino acids ($100 \mu\text{g ml}^{-1}$). Antibiotics and supplements used were prescribed by Cannon *et al.* (1976) except that spectinomycin, used to counterselect salmonella in certain mating experiments, was used at $600 \mu\text{g ml}^{-1}$. Cannon *et al.* (1976) also described the procedures for conjugation using F prime and P prime plasmids. Viable counts were performed by diluting cultures in saline phosphate buffer, spreading on agar plates and incubating to constant count. The plating efficiency of the salmonella strain used here was always 20 to 40 % lower on minimal agar than on nutrient agar; counts on nutrient agar were systematically about 20 % lower at 30 °C than at 37 °C (see Table 4, expt 1).

Nitrogenase activity. Cannon *et al.* (1976) described the detection of nitrogenase by the acetylene test using Pankhurst tubes and the special procedure used with *S. typhimurium* to assess specific activities. Protein was estimated with the Folin-Ciocalteu reagent (Lowry *et al.*, 1951) with bovine serum albumin as standard, but for most tests, protein content was judged from absorbance using a curve relating E_{540} to bacterial protein.

Reagents. Sodium $N^6, O^{2'}$ -dibutyryl adenosine 3':5'-cyclic monophosphate (dibutyl cyclic AMP) was obtained from Sigma.

RESULTS

Nitrogen-fixing salmonellae

Salmonella typhimurium UNF602 was a derivative of LT7-1201 carrying the F' *his nif* plasmid FN68. The preparation of UNF602 and its ability to fix acetylene and $^{15}\text{N}_2$ was reported by Cannon *et al.* (1976). *Salmonella typhimurium* VKSI was one of four His⁺ trans-conjugants from a surface mating of *E. coli* JC5466 (RP41) with *S. typhimurium* LT7-1201, all of which showed positive acetylene-reducing activity in qualitative tests in Pankhurst tubes. Unlike UNF602, in which the fertility determinants of the F prime plasmid were repressed, VKSI retained donor activity and transferred RP41 readily to *E. coli* JC5466 in surface matings. Irradiation of the parent strain LT7-1201 with u.v.-light or treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine gave no Nif⁺ derivatives on anaerobic plates or in

Table 2. *Specific acetylene-reducing activities of nitrogen-fixing Salmonella typhimurium UNF602*

For full test procedure, see Cannon *et al.* (1975). Cultures from minimal glucose agar plates were inoculated into Pankhurst tubes containing minimal medium with 100 µg Casamino acids ml⁻¹ as sole nitrogen source and carbon source at 1 % (w/v). Samples were removed for assay aseptically and anaerobically after 18 to 24 h at 30 °C for acetylene reduction assay lasting 60 to 120 min at 30 °C. Specific activities are expressed as nmol C₂H₄ formed (mg bacterial protein)⁻¹ min⁻¹. *Klebsiella pneumoniae* gave specific activities of 30 to 60 in comparable conditions with glucose.

Growth substrate	Specific activity
Glucose	29–60
Mannitol	59
Rhamnose	33
Arabinose	60

No growth was observed with glycerol, xylose, sodium gluconate or sodium lactate in Pankhurst tubes.

Table 3. '*Hyperinduction*' of nitrogenase activity of *S. typhimurium* UNF602 under argon and the effect of cyclic AMP

Samples (1 ml) from a Pankhurst tube culture (18 to 24 h) in glucose NFDM medium were injected into 18 ml Warburg manometer flasks containing 1 ml fresh medium with or without 0.8 to 1 µmol dibutyl cyclic AMP under argon with a pyrogallol plug in the side arm. After pre-incubating for up to 3.7 h at 30 °C, acetylene (2 ml) was injected and samples were taken for analysis by gas chromatography at intervals up to 120 min at 30 °C. Nitrogenase activities are expressed as nmol C₂H₄ (mg bacterial protein)⁻¹ min⁻¹.

Expt	Pre-incubation period (h)	Cyclic AMP	Nitrogenase activity
1	3.7	+	136
		—	70
2	3.5	+	195
		—	154
3	2	+	119
		—	119
4	1	+	98
		—	74.5
5	0	—	30–60
6*	3.5	+	75
		—	75

+, Present; —, absent.

* Control experiment in N₂. Growth from 25 to 44 µg bacterial nitrogen ml⁻¹ took place during pre-incubation.

Pankhurst tubes with 20 µg histidine ml⁻¹ as sole nitrogen source, confirming the unlikelihood of cryptic *nif* genes existing in this microbe.

Specific nitrogenase activity. Early experiments had given relatively low and variable specific activities in UNF602 (Cannon *et al.*, 1976) and comparable tests with VKSI also gave low values. However, extremely low activity of organisms stored on nutrient agar alerted us to the possibility that the medium on which the parent population had been grown might influence the apparent activity. Table 2 records specific activities for populations of UNF602 taken from Pankhurst tubes which had been inoculated from cultures on minimal agar: the values were comparable to those in *K. pneumoniae* and little affected by change from glucose to other carbon substrates.

Effect of cyclic AMP. The equivalence of the specific activities of cultures with glucose or other carbon substrates suggests that *K. pneumoniae nif* genes are not subject to catabolite repression in *S. typhimurium*. The experiments of Streicher, Gurney & Valentine (1972) with

Fig. 1. Loss of His⁺ phenotype in non-selective conditions from *S. typhimurium* LT7-1201 carrying plasmids with *K. pneumoniae* *his* and *nif* genes. Serial subcultures of *S. typhimurium* strains UNF602 and VKSI in nutrient broth at 30 and 37 °C were counted on non-selective (nutrient agar) and His⁺-selective (glucose/salts) plates at 30 °C over six passages. Initial populations counted contained 4×10^8 to 7×10^8 viable cells ml⁻¹; each subculture was an approximately 1/10³ dilution of the parent. ○, UNF602 at 37 °C; △, UNF602 at 30 °C; ●, VKSI at 37 °C; ▲, VKSI at 30 °C. Dashed and continuous lines distinguish temperatures.

adenylcyclase mutants show that *nif* expression in *K. pneumoniae* does not involve cyclic AMP. Nevertheless, cyclic AMP can relieve catabolite repression of *hut* in nitrogen-limited *K. aerogenes* so it was of interest to test for an effect of added cyclic AMP on *nif* expression in the new genetic background. The concentration of dibutyl cyclic AMP chosen was that effective in *K. aerogenes* (Calcott & Postgate, 1972). Samples (1 ml) from a Pankhurst tube were injected into two Warburg vessels set up under argon without acetylene but with 1 ml of fresh medium; one vessel contained in addition 0.8 to 1 µmol dibutyl cyclic AMP. After incubation for up to 3.7 h to allow further derepression of *nif*, acetylene was injected and samples were taken for analysis by gas chromatography at intervals for a further 120 min. Table 3 records the results of several such experiments. Incubation of the suspensions under argon caused augmentation of nitrogenase activity; similar augmentation was observed previously in nitrogen-limited continuous cultures of nitrogen-fixing bacteria under argon (Daesch & Mortensen, 1968, with *Clostridium pasteurianum*; Dalton & Postgate, 1969, with *Azotobacter chroococcum*). This 'hyperinduction' of nitrogenase activity was also observed with *K. pneumoniae* M5a1 in the conditions used here to test *S. typhimurium*. A specific activity in the region of 180 to 200 µmol C₂H₄ mg⁻¹ min⁻¹ seemed to be the maximum level of *nif* expression in both *K. pneumoniae* and *S. typhimurium*; dibutyl cyclic AMP did, in most experiments, augment sub-maximal nitrogenase activities, but the effect was not sufficiently consistent to permit the conclusion that catabolite repression was operating in *nif* expression.

Stability of nif plasmids in S. typhimurium

Six successive cultures in nutrient broth of strains UNF602 and VKSI were counted on nutrient agar and minimal glucose medium (the latter selects for the *his* determinant on the

Table 4. Apparent temperature sensitivity of *K. pneumoniae* *his* genes in *S. typhimurium*

Plate counts on flask cultures of strains of *S. typhimurium*, mostly carrying *his nif* plasmids (for details, see text) were made at 30 and 37 °C on nutrient agar (NA), glucose minimal agar (MM) and sometimes glucose minimal agar plus 20 µg L-histidine ml⁻¹ (MM-his). LT7-1201 is the parent strain without plasmids; VKSI-1 is a derivative of VKSI picked after growth on MM at 37 °C.

Expt	Strain	10 ⁻⁸ × Viable count					
		At 30 °C			At 37 °C		
		NA	MM	MM-his	NA	MM	MM-his
1	LT7-1201*	8.8	—	7.2	9.5	—	6.5
2	UNF602	7.25	3.5	—	7	0.16	—
3	UNF602	7.8	1.5	2.6	—	—	—
4	VKSI	2.85	0.52	—	3.05	0.17	—
5	VKSI*	2.95	2.05	—	2.76	0.01	—
6	VKSI	1.75	0.14	0.95	—	—	—
7	VKSI-1	—	0.75	1.81	2.76	< 0.01	—

* Parent cultures from nutrient broth; all others were from liquid MM.

two *his nif* plasmids). Figure 1 records the ratio of the selected to unselected counts for broth cultures at 30 and 37 °C: the *his nif* genes of both plasmids were rapidly lost in *S. typhimurium*. On the sixth passage, counts were also made on media selecting for the drug resistance markers of the two plasmids: carbenicillin for FN68; carbenicillin and kanamycin for RP41. The resistance determinants of RP4, the parent plasmid of RP41, were stably conserved over six passages at either temperature but that of FN68 was partially lost: by a factor of 8 at 30 °C and a factor of 33 at 37 °C.

Temperature sensitivity of *K. pneumoniae* *his* genes in *S. typhimurium*

Strain UNF602 (and other derivatives carrying FN68) expressed *his* at 30 °C but not at 37 °C when tested from a parent stock in nutrient broth, and no His⁺ transconjugants were observed in *E. coli* × *S. typhimurium* matings if selection was at 37 °C instead of 30 °C (Cannon *et al.*, 1976). Further study of temperature sensitivity in both UNF602 and VKSI showed that it was less pronounced if the parent stock was maintained on minimal medium but was still observed. It was quantitative: counts on a minimal medium at 37 °C were lower by 4- to 200-fold than at 30 °C (Table 4, expts 2, 4, 5).

The temperature sensitivity could be observed qualitatively in streaks spread by a conventional loop from a given population and incubated at the two temperatures. Since apparently temperature-tolerant strains appeared, 10 such derivatives of VKSI were isolated. All 10 showed qualitative evidence of temperature sensitivity as streaks on minimal agar; one, VKSI-1, was checked quantitatively and was as temperature-sensitive as VKSI (Table 4, expt 7).

If apparent temperature sensitivity is due to rapid loss of *his nif* determinants from the plasmid at 37 °C, comparable loss might be expected at 30 °C, at which temperature counts on plates of minimal medium with added histidine ought to be greater than on plates without histidine. Histidine augmented the plate counts of UNF602, VKSI and VKSI-1 at 30 °C (Table 4, expts 3, 6, 7) though not, as with the parent strain, to the value obtained on nutrient agar (expts 1, 3, 6).

DISCUSSION

Salmonella typhimurium LT7-1201 expressed plasmid-borne *K. pneumoniae* M5a1 *nif* genes as effectively as does the parent *K. pneumoniae* according to the acetylene reduction test. Ammonium repression was displayed (Cannon *et al.*, 1976), and so was 'hyperinduction' under argon. Earlier reports that the level of *nif* expression is low in *Salmonella* must be attributed to ready dissociation and loss of the *K. pneumoniae* component of the plasmid DNA in the non-selective conditions used to maintain the parent stock. If selection for His⁺ was maintained throughout, low levels of *nif* expression were not observed.

Though the regulation of *nif* has much in common with that of *hut*, and *hut* shows catabolite repression in *S. typhimurium* 15-59 (Brill & Magasanik, 1969), no comparable repression was observed with *K. pneumoniae nif* in *S. typhimurium* LT7. An effect of cyclic AMP sometimes occurred during 'hyperinduction' but was not consistently observed. 'Hyperinduction' of nitrogenase expression complicates the interpretation of derepression experiments with intact, growing bacteria since one does not know whether it reflects a true derepression (i.e. increased nitrogenase syntheses) or simply more effective mobilization of ATP or reductant; the phenomenon needs more detailed study in *Klebsiella* before its significance in *Salmonella* can be usefully discussed.

It seems, however, that *Klebsiella nif* genes are fully expressed and show all the phenotypic characters in *S. typhimurium* LT7 that they do in the parent *K. pneumoniae*. A preliminary report (Bloom, Streicher & Tyler, 1976) that the *hut* system of *S. typhimurium* 15-59 does not respond to *Salmonella* glutamine synthetase, though *Salmonella* glutamine synthetase can activate *hut* in *Klebsiella*, raises the question of whether the expression of *Klebsiella nif* genes in *Salmonella* indicates response to *Salmonella* glutamine synthetase or changed promoter specificity. Tests for expression of *nif* in strain 15-59 would be interesting in this context; the relation of the regulation pattern of the *hut* system in 15-59 to that in LT7, the strain used here, is not known, but it is different from that in LT2 (Meiss, Brill & Magasanik, 1969).

The apparent temperature sensitivity of the expression of *Klebsiella his* genes in *S. typhimurium* LT7 can also be explained in terms of dissociation of *Klebsiella* DNA from the plasmids. Loss of *his* was clearly demonstrated in non-selective conditions (Fig. 1); loss of drug resistance determinants from the plasmids did not occur with RP41 and was considerably less than the loss of *his nif* from FN68. Augmentation of counts on minimal media by histidine provides evidence for loss of *his* at the seemingly permissive temperature even in selective conditions. Figure 1 does not permit any generalization to be made about the relative rates of loss of *Klebsiella* determinants at the two temperatures tested, nor about the stability of the *Klebsiella* component in the two plasmids. It is possible, however, that the initial rate of loss was higher at 37 °C and that this temperature thus appeared 'non-permissive' for the bulk of the population and could, in primary selection for His⁺ salmonellae, lead to loss of all positive transconjugants. The fact that presumptive temperature-tolerant variants showed unchanged responses to temperature suggests an operational explanation along these lines rather than a new mode of expression of *Klebsiella* genes in the *Salmonella* genetic background.

The specific instability of *Klebsiella his nif* determinants when the plasmids are in *Salmonella* is a curious phenomenon: why should the *Klebsiella* DNA dissociate particularly easily? This has a bearing on the use of constructed plasmids for other intergeneric transfers: for practical reasons, positive selection for His or Nif is desirable and incubation for transconjugants or transformants should be at a lower temperature than would be necessary if

drug resistance were the selected marker. For more stable nitrogen-fixing transconjugants, means may be needed to induce chromosomal integration of *his nif* such as occurred in *E. coli* strain CM7 (Cannon *et al.*, 1974).

Salmonella typhimurium LT7-1201 is mildly pathogenic and has been the cause of laboratory infections (G. Humphries, personal communication). Because the combination of drug resistance determinants on RP41 includes two agents effective against salmonellosis, all derivatives carrying this plasmid have now been destroyed.

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