Derivation and Properties of F-prime Factors in *Escherichia coli* Carrying Nitrogen Fixation Genes from *Klebsiella pneumoniae*

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SUMMARY

A His⁺ Nif⁺ *Escherichia coli* K12, Hfr strain (UNF43) was constructed by an intergeneric mating between a *Klebsiella pneumoniae* donor strain (HF3) and a His⁻, Hfr *E. coli* strain (SB1824) which transfers *his* as an early marker. An F-prime *nif* plasmid, FN39, carrying genes which correspond to the *E. coli* chromosomal region, *metG gnd his shiA*, but excluding *purF* and *aroD*, was isolated from UNF43. Translocation of carbenicillin resistance genes from a P-type R-factor, R68, to FN39 increased the stability of *his* and *nif* on the derivative F-prime, FN68.

Sedimentation analysis of both F-primes in sucrose gradients revealed four covalently closed circular (CCC) DNA species of molecular weights 279 ± 9 , 136 ± 3 , 90 ± 1 and 44 ± 1 megadaltons. It is suggested that the two smallest CCC-DNA species are component replicons of the composite F-primes of molecular weight 136 ± 3 megadaltons, and that the molecules of 279 ± 9 megadaltons are CCC-dimers.

FN68 was transferable in intergeneric matings to *Klebsiella aerogenes*, *K. pneumoniae* and *Salmonella typhimurium* but not to *Proteus mirabilis*; only carbenicillin resistance and sex factor activity were transferred to *Erwinia herbicola*. *nif* genes on FN68 were expressed in a Nif⁻ mutant of *K. pneumoniae* and also in *S. typhimurium*, which in conventional tests is naturally non-nitrogen-fixing; expression of the *his* determinant of FN68 became temperature-sensitive in *S. typhimurium*.

INTRODUCTION

In the enteric bacterium *Klebsiella pneumoniae*, genes determining nitrogen fixation (*nif*) are clustered close to the histidine operon (Streicher, Gurney & Valentine, 1971; Dixon & Postgate, 1971). Most *nif* mutations are 30 to 80 % co-transducible with *hisD* on the *Klebsiella* chromosome, (Streicher, Gurney & Valentine, 1972). A deletion analysis of the *nif* region indicates that *nif* genes are located between *his* and *shiA* in *Klebsiella* (Shanmugam, Loo & Valentine, 1974).

nif genes have been transferred from *K. pneumoniae* to *Escherichia coli* strain c (Dixon & Postgate, 1972). In a stable hybrid c-M7, *nif* genes were integrated into the *his* region of the *E. coli* chromosome (Cannon *et al.*, 1974 *a*) while in two less stable hybrids, c-M9 and c-L4, there was evidence that *nif* genes were carried on plasmids (Cannon *et al.*, 1974 *b*). Although these plasmids were mobilized by R factors they were not self-transmissible. Sex-factor plasmids containing *nif* genes would facilitate both diploid analysis and further genetic transfer of *nif*; a preliminary report of the construction of such F-prime plasmids was presented by Dixon (1974). We describe here the isolation and properties of F-prime factors carrying the nitrogen fixation genes from *K. pneumoniae*.

Table 1. *Bacteria, plasmids and bacteriophages*

Bacterial strain	Markers	Source or reference	
<i>Klebsiella pneumoniae</i>			
HF3	Prototroph, Nif ⁺	Dixon, Cannon & Postgate (1975)	
UNF124	<i>nif-12 his-4 str-1</i>	This laboratory	
UNF925	<i>nif-9 his-2 arg-1 str-1</i>	Dixon <i>et al.</i> (1975)	
<i>Escherichia coli</i> K12			
Prototroph	—	S. B. Primrose	
AB311	Hfr <i>thr leu pro str</i>	Taylor & Adelberg (1960)	
SB1824	<i>hisD</i> derivative of AB311	Blumenthal (1972)	
SB1801	<i>his-750</i> (deletion covering <i>his gnd</i> RHA-2A) <i>str</i> λ^R <i>ara gal malA xyl mtl</i> (λ) [−]	Garrick-Silversmith & Hartman (1970)	
AB2463	<i>thi arg his-4 pro thr leu str recA lac gal ara xyl mtl</i>	Willetts, Clark & Low (1969)	
UNF502	<i>thi arg his-4 pro thr leu str lac gal ara xyl mtl nal</i>	<i>nal</i> ^R derivative of AB1157 (Willetts <i>et al.</i> , 1969)	
AB396	<i>purFl trp-1 argH1 thr-1 leu-6 mtl-2 xyl-7 gal-6 lac tonA2 thi-1</i>	Voll (1972)	
AB2880	<i>his pro arg aroD362 shiA352 xyl str</i>	Pittard & Wallace (1966)	
JCS466	<i>trp recA56 his spc</i>	N. Willetts	
J62-1	<i>lac pro his trp nal</i>	<i>nal</i> ^R mutant of J62 (N. Datta)	
J62-2	<i>lac pro his trp spc</i>	<i>spc</i> ^R mutant of J62 (N. Datta)	
<i>Escherichia coli</i> C			
C1463	<i>his-5 metG str-8 rha-2</i>	G. Bertani	
C603	<i>his arg str</i>	G. Bertani	
<i>Salmonella typhimurium</i> LT7			
1201	<i>his str</i>	S. B. Primrose	
UNF602	<i>str</i>	LT7-1201 (FN68); this laboratory	
<i>Erwinia herbicola</i>			
YC74	Prototroph	M. P. Starr	
<i>Proteus mirabilis</i>			
5006-1	<i>nic his nal str</i>	J. N. Coetzee	
Plasmids	Markers	Compatibility group	Source or reference
FN39	<i>metG gnd his nif shiA</i>	F	This paper
FN68	Carb ^R derivative of FN39	F	This paper
FCm	Cm ^R	F	S. B. Primrose
R68 (R6886)	Km ^R Carb ^R Tc ^R	P	Holloway & Richmond (1973)
R751	Trim	P	Jobanputra & Datta (1974)
Bacteriophage		Plasmid specificity	Source
If2		I	E. Meynell
MS2		F	S. B. Primrose
R17		F	C. Kennedy
PRR1		P	R. M. Olsen

Symbols for drug resistance determinants are: Km, kanamycin; Carb, carbenicillin; Tc, tetracycline; Trim, trimethoprim; Cm, chloramphenicol. Genetic symbols for chromosomal markers are those of Taylor & Trotter (1972).

METHODS

Bacterial strains, plasmids and bacteriophages. These are listed in Table 1.

Media. Complete medium was nutrient broth (Oxoid No. 2) solidified with 1.5 % Davis New Zealand agar. NH_4^+ -free medium was NFDM (Cannon *et al.*, 1974 a) and was solidified

with 1.5 % Serva high gel strength agar. Minimal medium was Davis & Mingioli (1950) mineral salts. Cultures for DNA isolation were grown in minimal medium C, containing: K_2HPO_4 buffer pH 7.2, 60 mM; $(NH_4)_2SO_4$, 15 mM; $MgSO_4 \cdot 7H_2O$, 0.8 mM; NaCl, 0.17 mM; $CaCl_2 \cdot 2H_2O$, 6.8 μ M; glucose, 0.2 % (w/v). The buffer concentration of this medium was lowered to 20 mM when cultures were grown for transformation.

Antibiotics. These were added to media as freshly-prepared solutions sterilized by filtration. The following final concentrations (μ g/ml) were added to select plasmid-mediated drug resistance in *E. coli*: kanamycin sulphate, 30; tetracycline-HCl, 15; carbenicillin, 200; trimethoprim lactate, 50; chloramphenicol, 30. Chromosomal resistance markers were selected at the following final concentrations (μ g/ml): streptomycin, 250; spectinomycin 100; nalidixic acid, 25. With *Proteus mirabilis*, carbenicillin was used at 50 μ g/ml; with *Salmonella typhimurium*, carbenicillin was used at 400 μ g/ml. Carbenicillin was a gift from Beecham Research Laboratories, Brockham Park, Betchworth, Surrey; trimethoprim lactate was kindly supplied by Burroughs Wellcome & Co., The Wellcome Foundation, Berkhamsted, Hertfordshire, and spectinomycin was a gift from the Upjohn Co., Kalamazoo, Michigan, U.S.A.

Conjugation. For broth matings, donor and recipient were grown overnight in static nutrient broth and then diluted into prewarmed broth and incubated with slow shaking at 37 °C until donor cultures contained about 2×10^8 bacteria/ml and recipient cultures about 5×10^8 bacteria/ml. Cultures were mixed at a donor-recipient ratio of 1:10 (1:1 for intergeneric matings) and incubated for 2 h (16 h for intergeneric matings) at 37 °C without shaking. Mixtures were resuspended in saline phosphate buffer (Cannon *et al.*, 1974 *a*) and plated on selective media; for intergeneric matings selective plates were incubated for 3 to 5 days and then transferred to room temperature for up to 7 days, because exconjugants sometimes showed long delays in colony formation.

Transfer of P-group plasmids occurred more efficiently when mating mixtures were incubated on an agar surface. A 0.1 ml sample of late-exponential-phase culture of the donor was mixed with 0.1 ml of a late-exponential-phase recipient culture, spread on nutrient agar and incubated for 6 h at 37 °C. Bacteria were scraped off into saline phosphate buffer and plated on selective media. All matings employing P-group plasmids were performed in this fashion except where otherwise stated; for *Erwinia herbicola* a similar procedure, with 16 h incubation, was used with an F-group plasmid.

Nitrogenase assays. Acetylene reduction was the usual measure of nitrogenase activity. Either Pankhurst tubes were used (Postgate, 1972) or 0.1 ml of an overnight nutrient broth culture was added to 5 ml NFD, containing casein hydrolysate (100 μ g/ml) and tryptophan (25 μ g/ml) for Trp⁻ strains, in a sterile 7 ml bijou bottle. Cultures were capped with Suba-seal closures (William Freeman Ltd, Barnsley, Yorkshire) equilibrated to atmospheric pressure with a sterile needle and incubated at 30 °C. After 16 to 20 h, when visible growth had occurred, 1 ml acetylene was injected.

When specific activities were required, cultures (5 ml) were grown anaerobically in conical flasks (25 ml) then flushed with argon and 2 ml acetylene was injected. With *S. typhimurium* 1 ml samples were removed aseptically and anaerobically from a growing Pankhurst tube culture and injected into a Suba-sealed 18 ml Warburg flask containing argon + 0.1 atm C_2H_2 . Alkaline pyrogallol supported on cotton wool was present in its side-arm. Tests of specific activity lasted for 0.5 to 3 h at 30 °C.

Ethylene production was measured by injecting 0.5 ml gas samples into a Pye 104 gas-liquid chromatograph fitted with a 45 cm Porapak R column (1 mm internal diameter) at 37 °C, with N_2 as carrier gas at a flow rate of 7 ml/min.

When it was necessary to screen many colonies for nitrogen fixation, clones were grown on NFDM agar at 30 °C under 0.99 atm N₂+0.01 atm CO₂ in nylon bags (Hill, 1973). Nitrogen-fixing colonies of *K. pneumoniae* are brown on NFDM plates. Nif⁺ *E. coli* colonies do not have such a distinctive colour, but are larger than Nif⁻ colonies. With a derivative of *S. typhimurium*, ¹⁵N₂ incorporation was assessed as described by Dixon & Postgate (1972).

Gluconate 6-phosphate dehydrogenase. This was estimated by the colorimetric method of Peyru & Fraenkel (1968).

ColI production. Colicin I production was determined by the method of Monk & Clowes (1964). The indicator was *E. coli* K12 W1166 (ColV).

Segregation and acridine orange curing. These techniques were performed according to Willetts & Bastarrachea (1972).

Partial purification of plasmid DNA. An overnight culture of host bacteria was diluted 2:100 in 1.6 l of minimal medium C, grown to 2×10^8 to 4×10^8 cells/ml and harvested by centrifugation at 0 to 2 °C. The cells were resuspended in 56 ml cold 25 % sucrose in 50 mM-tris-HCl pH 8.0 and dispensed as 7 ml portions in 8 centrifuge tubes kept on ice. A 1.4 ml portion of freshly-prepared cold lysozyme solution (5 mg/ml in 0.25 M-tris-HCl pH 8.0) was added to each tube followed in 5 min by 2.8 ml cold 0.25 M-Na₂EDTA (pH 8.0) and in another 5 min by 11.2 ml 2 % (w/v) sodium dodecyl sulphate (SDS) in 50 mM-HCl pH 8.0 and 62.5 mM-Na₂EDTA. Complete lysis occurred immediately and 5.6 ml of 5 M-NaCl was added to each tube. After overnight incubation on ice the crude cell lysates were centrifuged at 28000 g and 2 °C for 20 min. The supernatant fluid was transferred to a 1.5 l beaker on ice and the DNA precipitated by slowly adding 0.54 vol. cold isopropanol. The precipitate was collected by centrifugation at 1000 g and 2 °C for 10 min. The DNA was finally dissolved in approximately 3.0 ml of TEN-C buffer (tris-HCl, pH 8.0, 20 mM; Na₂EDTA, 1 mM; NaCl, 20 mM; CaCl₂.2H₂O, 30 mM) and insoluble material removed by centrifugation at 20000 g and 2 °C for 20 min. DNA solutions were stored at 4 °C over a few drops of chloroform. Typical 230:260:280 ratios for DNA prepared by this procedure were 0.489:1.051:1, but examination by analytical CsCl centrifugation using the MSE Centriscan 75 showed that only 1 to 2 % of material absorbing at 257 nm was DNA.

DNA-buoyant density determination. Solutions for centrifugation contained 1.0 ml CsCl (BDH; optical grade, density 1.79 g/cm³), approximately 3 µg of FN68 DNA prepared as above, and 3 µg of *Clostridium perfringens* DNA (Sigma; density 1.691 g/cm³) as referent; the final density was adjusted with water to approximately 1.71 g/cm³. These solutions were centrifuged in an MSE Centriscan 75 analytical ultracentrifuge at 44000 rev./min and 25 °C, and after 16 h the extinction profiles at 254 nm of the resulting gradients were recorded. Buoyant densities were calculated as described by Schildkraut, Marmur & Doty (1962).

Transformation. The procedure of Cohen, Chang & Hsu (1972) was used for transformation except that the low-phosphate medium C described above was used instead of their medium H1 for growing bacteria. CaCl₂-treated cultures were exposed to FN68 DNA prepared as above, diluted tenfold in L-broth and incubated at 37 °C for 90 min before plating.

Other procedures. Cannon *et al.* (1974 a) gave details for labelling, extraction, centrifugation and counting of DNA. The S values of peaks in sucrose gradients were calculated from the S values of ColE1 CCC-DNA (23S in neutral and 58S in alkaline sucrose gradients) assuming a linear relationship between the S value and the distance sedimented. Molecular weights were calculated from S values in neutral sucrose gradients using the data of Bazaral & Helinski (1968).

Fig. 1. Simplified map of *E. coli* K12 (Taylor & Trotter, 1972), showing the point of origin and direction of chromosomal transfer by the donor strain, SB1824, and the location of *metG* (Blumenthal, 1972). Numbers inside the circle refer to chromosomal transfer time in minutes. The outer arrow indicates the origin and direction of transfer of chromosomal genes by the *K. pneumoniae* strain, HF3. The gene symbol for nitrogen fixation is *nif* (Streicher, Gurney & Valentine, 1971).

RESULTS

Construction of a Nif⁺ Hfr strain of E. coli K12

A nitrogen-fixing hybrid of an Hfr strain of *E. coli* was obtained by performing an intergeneric mating with a donor strain of *K. pneumoniae*. This donor, HF3 (R144*drd*3) has been used previously to construct Nif⁺ hybrids of *E. coli* C (Dixon & Postgate, 1972); it normally carries the R factor R144*drd*3 and gives polarized transfer of chromosomal markers, with *his* and *nif* close to the transfer origin (Dixon, Cannon & Postgate, 1975). The recipient used was SB1824, a His⁻ Hfr strain of *E. coli* with F integrated close to *his* (Fig. 1). Plasmid DNA in the supercoiled configuration was not observed in this strain (Fig. 2). His⁺ exconjugants were selected and screened for inheritance of *nif*. Nine His⁺ colonies were obtained and purified on the selection medium; only two were Nif⁺. Both His⁺Nif⁺ isolates were sensitive to F-specific phages MS2 and R17, but neither was resistant to kanamycin, produced colicin I or was sensitive to I-specific phage, If2, and therefore neither hybrid carried R144*drd*3. This conclusion was confirmed by the absence of CCC-DNA from the one of these strains examined, UNF43 (Fig. 2). This result was in striking contrast to the Nif⁺ hybrids of *E. coli* C derived from what was apparently the same Klebsiella donor, which harbour this R factor (Cannon *et al.*, 1974*a, b*).

One of the Nif⁺ isolates, designated UNF43, was used for further studies. In interrupted mating experiments between this strain and J62-1, a Trp⁻His⁻ recipient, the time of entry of *his* and *trp* were 5 and 20 min respectively. Twenty His⁺ exconjugants obtained after 10 min of mating were also Nif⁺, indicating that *nif* was also transferred as an early marker. Similar curves for *his* and *trp* transfer were obtained for the donor AB311, the strain from which SB1824 was derived, although the number of recombinants produced was ten-fold higher. The low number of recombinants obtained with UNF43 probably reflects the presence of heterologous Klebsiella DNA in the donor, which could reduce pairing between the donor and recipient chromosomes (Curtiss *et al.*, 1968).

Fig. 2

Fig. 3

Fig. 2. Profiles of labelled DNA in cleared lysates of *E. coli* SB1824 (●), and UNF43 (○), after dye-buoyant density centrifugation at 126000 *g* and 15 °C for 16 h. Cleared lysates of 10 ml cultures were prepared by a lysozyme-EDTA-Triton X-100 procedure (Cannon *et al.*, 1974 *a*). After centrifugation, fractions (10 drops) were collected on filter discs and assayed for ³H-radioactivity.

Fig. 3. Profiles of labelled DNA in cleared lysates of *E. coli* JC5466 (○), and JC5466(FN39) (●), after dye-buoyant density centrifugation at 126000 *g* and 15 °C for 16 h. Cleared lysates of 30 ml cultures were prepared by a lysozyme-EDTA-Triton X-100 procedure (Cannon *et al.* 1974 *a*). After centrifugation, fractions (10 drops) were collected in styrene 'Microtiter' plates. A portion (0.01 ml) of each fraction was spotted on a filter disc and assayed for ³H-radioactivity.

Isolation of an F'nif

Exponential-phase cultures of the donor strain UNF43 and a His⁻ *recA nalA* recipient JC5466 were mixed at a donor-recipient ratio of 1:5 and incubated for 60 min. The mating was stopped by adding 25 µg nalidixic acid/ml mating mixture. His⁺ exconjugants were selected on a minimal medium containing tryptophan, spectinomycin and nalidixic acid. Forty His⁺ isolates were purified on the selection medium; 12 were Nif⁺ and sensitive to F-specific phage MS2. One Nif⁺ isolate, carrying a presumptive F'*nif* factor designated FN39, was studied in detail. In a qualitative test on a u.v.-irradiated spread plate, this strain was as sensitive to u.v. irradiation as its parent JC5466 and was therefore presumably *recA*. It transferred *his* at high frequency to AB2463, a *recA* strain, and to SB1801, a *his* deletion strain of K12 (Table 2, Expts 1 and 2). Twenty exconjugants purified from each cross were Nif⁺, MS2-sensitive and re-transferred *nif* and *his* when replica-plated on to a lawn

Table 2. *Transfer properties of nif plasmids*

Expt.	Donor	Recipient	Selection	Transfer frequency	Exconjugant phenotype
1	JC5466(FN39)	AB2463	His ⁺	2×10^{-1}	20/20 Nif ⁺ MS2 ^S
2	JC5466(FN39)	SB1801	His ⁺	3×10^{-1}	10/10 Nif ⁺ MS2 ^S
3	SB1801(FN39)(R68)	UNF502	His ⁺	7×10^{-1}	—
			Km	1×10^{-4}	—
			Carb	7×10^{-2}	—
			Carb His ⁺	3×10^{-4}	7/10 His ⁺ Nif ⁺ Carb ^R Tc ^S Km ^S MS2 ^S
4	JC5466(FN68)	AB2463	His ⁺	3×10^{-1}	135/135 Carb ^R Nif ⁺
			Carb	3×10^{-1}	103/103 His ⁺ Nif ⁺
			Carb His ⁺	3×10^{-1}	—
5	JC5466(FN68)	SB1801	Carb	3×10^{-1}	10/10 His ⁺ Nif ⁺
			His ⁺	2×10^{-1}	10/10 Nif ⁺ Carb ^R

of JC5466 spread on selection medium. The presence of a plasmid in JC5466 (FN39) was confirmed by comparing the buoyant density profiles of DNA from this strain and its parent strain JC5466 in CsCl-ethidium bromide gradients (Fig. 3).

Interaction between FN39 and the R factor, R68

Translocation of carbenicillin resistance from plasmids of the P compatibility group to other plasmids has been demonstrated (Beringer, 1974; Hedges & Jacob, 1974). We followed a similar procedure to introduce carbenicillin resistance into the F'*nif*, FN39. The R factor, R68, a member of the P compatibility group (Holloway & Richmond, 1973) conferring resistance to ampicillin-carbenicillin, kanamycin and tetracycline, was introduced into SB1801 carrying FN39. A broth mating was carried out between SB1801 (FN39) (R68) and a His-Rec⁺ recipient, UNF502 (Table 2, Expt 3). Under these conditions, transfer of the F-prime (selection for His⁺) occurred at a much higher frequency than kanamycin resistance, a marker on R68. Transfer of carbenicillin (Carb), however, occurred at an intermediate frequency. Selection for co-transfer of both Carb and *his* should therefore give a considerable enrichment for F-primes to which carbenicillin resistance has been translocated. Indeed, 7 out of 10 exconjugants selected for inheritance of both markers appeared to contain a recombinant plasmid; they were sensitive to kanamycin and tetracycline, were His⁺ Nif⁺ and were lysed by MS2 phage. The remaining three exconjugants apparently retained both plasmids, they were resistant to kanamycin and tetracycline and transferred *his* and Carb independently. However, strains carrying the recombinant plasmid, designated FN68, gave co-transfer of *his nif* and carbenicillin resistance (Table 3, Expts 4 and 5).

*Compatibility properties of F'*nif* plasmids*

Plasmids of the P compatibility group show mutual exclusion and cannot co-exist in the same host. Hence, a reduction in transfer frequency, indicative of surface exclusion, was observed when a strain carrying R751 was mated with a recipient carrying R68 (Table 3, Expts 1 and 2). FN68 did not exclude entry of R751 and both plasmids co-existed stably (Table 3 Expt 3) indicating that FN68 does not retain P compatibility. Strains carrying FN68 and FN39 did exclude an F' factor F'Cm, indicating that both plasmids are members of the F compatibility group (Table 3, Expts 4, 5 and 6). However, occasional super-infection of the resident plasmid occurred since, in the majority of exconjugants, markers on both

Table 3. *Compatibility properties of F'nif plasmids*

Expt. no.	Donor	Recipient	Selection	Transfer frequency*	Exconjugant phenotype
1	J62-2(R751)	SB1801	Trim	> 1	—
2	J62-2(R751)	SB1801(R68)	Trim	2×10^{-4}	—
3	J62-2(R751)	SB1801(FN68)	Trim	6×10^{-1}	20/20 Trim ^R Carb ^R His ⁻ Nif ⁺
4	Prototrophic K12 (F'Cm)	SB1801	Cm	7×10^{-1}	—
5	Prototrophic K12 (F'Cm)	SN1801(FN39)	Cm	2×10^{-4}	28/30 Cm ^R His ⁺ Nif ⁺ 2/30 Cm ^R His ⁻ Nif ⁻
6	Prototrophic K12 (F'Cm)	SB1801(FN68)	Cm	4×10^{-5}	20/21 Cm ^R Carb ^R His ⁺ Nif ⁺ 1/21 Cm ^R Carb ^R His ⁻ Nif ⁻

* Matings with R751 were performed by the surface mating procedure described in Methods.

plasmids co-existed stably (Table 3, Expts 5 and 6). In some clones, this was probably the result of F' fusion; since one out of 30 carrying both FN39 and F'Cm and six out of 21 clones carrying FN68 and F'Cm co-transferred *his* and Cm^R to JC5466 at high frequency.

Stability of F'nif plasmids

In the *recA* strain, JC5466, the F-prime FN39 gave rise to spontaneous His⁻ Nif⁻ segregants at a frequency of 10 % and was cured at a 100 % efficiency by acridine orange. In one cured clone examined no CCC-DNA was detected in CsCl-ethidium bromide gradients. FN68 did not give spontaneous segregants at high frequency; although *his* and *nif* were cured 100 % efficiently by acridine orange treatment, carbenicillin resistance was extremely stable. Presumably Carb became stabilized by integration into the host chromosome (Hedges & Jacob, 1974).

Other markers carried by F'nif plasmids

Derivatives of JC5466 carrying FN39 or FN68 were tested for their ability to donate other determinants located close to the *his* operon; transfer of *purF*, *metG*, *shiA* and *aroD* was tested (see Fig. 1). Presumably, neither FN39 nor FN68 carried *purF*, since transfer of this determinant was not detected either by direct selection or as an unselected marker in Carb^R exconjugants (Table 4, Expts 1 and 2). Both F' factors carried *metG*, since *his met* co-transfer was detected in crosses with C1463, a *metG* derivative of *E. coli* (Table 4, Expts 3 and 4). Thirty His⁺ exconjugants derived from a mating with FN68 inherited *metG*, and Met⁺ exconjugants inherited *his* (Table 4, Expt 4). When FN68 or FN39 was transferred to AB2880, a His⁻ *shiA aroD* recipient, His⁺ exconjugants from each mating inherited *shiA*, a marker located close to *his*, but not *aroD*, and no co-transfer of *shiA* and *aroD* could be detected (Table 4, Expts 5 and 6).

Both plasmids most probably carry a determinant for gluconate 6-phosphate dehydrogenase (*gnd*). Derivatives of strain SB1801, a *gnd* deletion background, carrying each plasmid gave a positive reaction for gluconate 6-phosphate dehydrogenase when tested by the rapid colorimetric assay for this enzyme (Peyru & Fraenkel, 1968), whereas SB1801 itself was clearly negative in this assay. The nitrogenase activity of plasmid-containing strains *in vivo* as measured by acetylene reduction and assimilation of ¹⁵N₂ is shown in Table 5. The specific activity for acetylene reduction of *E. coli* strains carrying *nif* plasmids was equivalent to that shown by the natural nitrogen-fixers, *K. pneumoniae*.

From the above results we conclude that both plasmids carry *nif* genes and probably a

Table 4. Other markers carried on F'*nif* plasmids

Expt. no.	Donor	Recipient	Selection	Transfer frequency	Exconjugant phenotype
1	JC5466(FN39)	AB396*	PurF ⁺	$< 1 \times 10^{-7}$	—
2	JC5466(FN68)	AB396*	PurF ⁺ Carb	$< 1 \times 10^{-7}$ 5×10^{-1}	— 0/30 PurF ⁺
3	JC5466(FN39)	C1463	His ⁺ His ⁺ MetG ⁺	1×10^{-1} 1×10^{-1}	— —
4	JC5466(FN68)	C1463	His ⁺ MetG ⁺	2×10^{-1} 2×10^{-1}	30/30 MetG ⁺ 30/30 His ⁺
5	JC5466(FN39)	AB2880*	His ⁺ ShiA ⁺ AroD ⁺ ShiA	3×10^{-1} 3×10^{-1} 5×10^{-7}	— 0/10 AroD ⁺ —
6	JC5466(FN68)	AB2880*	His ⁺ ShiA ⁺ AroD ⁺ ShiA ⁺	5×10^{-1} 4×10^{-4} 1×10^{-7}	— 0/10 AroD ⁺ —

* Streptomycin-resistant derivatives of the strains indicated.

Table 5. Nitrogenase activity in strains carrying F'*nif* plasmids

Strain	Ethylene produced (nmol/mg protein/min)	Atom % ¹⁵ N excess*
<i>Escherichia coli</i> K12		
JC5466	< 0.1	NT
JC5466(FN68)	40	1.20
JC5466(FN39)	57	NT
<i>Salmonella typhimurium</i>		
LT7-1201	< 0.01	0.02
UNF602	2-20	0.61
<i>Klebsiella pneumoniae</i>		
HF3	63	1.88

* Measured in 3 to 4-day cultures; see Methods. NT, not tested.

chromosomal region corresponding to the *metG gnd his shiA* region of the *E. coli* chromosome, but not including the *purF* and *aroD* genes.

Intergeneric transfer of *nif* plasmids

Tubb (1974) used FN68 to transfer *nif* genes to *gln* mutants of *Klebsiella aerogenes*, providing evidence for the involvement of glutamine synthetase in the ammonia regulation of nitrogenase. Transfer of FN68 from *E. coli* to a His⁻ Nif⁻ *K. pneumoniae* occurred at low frequency and resulted in segregation of carbenicillin resistance from *his* and *nif*. Using the solid surface mating procedure, only one out of 150 His⁺ exconjugants was carbenicillin-resistant whereas 87 out of 96 Carb^R exconjugants inherited both *his* and ability to fix nitrogen (Table 6, Expt 1). Five of the His⁺ Carb^R exconjugants tested were not sensitive to MS2 phage and re-transfer of these determinants to *E. coli* could not be detected. This is characteristic of the behaviour of F-like plasmids in *K. pneumoniae* M5a1 (Dixon & Postgate, 1971).

FN68 could be transferred to *S. typhimurium* at low frequency but without expression of F as sensitivity to the F-specific MS2 phage (Table 6, Expt 2). Segregation again occurred, but in this case transfer of Carb^R preponderated about 20-fold over *his nif* transfer. Although

Table 6. *Intergeneric matings with FN68*

Expt. no.*	Donor	Recipient	Selection	Transfer frequency	Exconjugant phenotype	
1	<i>Escherichia coli</i> K12 JC5466(FN68)	<i>Klebsiella pneumoniae</i> UNF124	His ⁺	5×10^{-4}	149/150	His ⁺ Nif ⁺ Carb ^R
			Carb ^R	1×10^{-5}	87/96 9/96	His ⁺ Nif ⁺ Carb ^R His ⁻ Nif ⁻ Carb ^R
2	JC5466(FN68)	<i>Salmonella typhimurium</i> LT7-1201	His ⁺	1×10^{-7}	5/5	Nif ⁺ Carb ^R MS2 ^R
			Carb ^R	2×10^{-6}	5/5	His ⁻ Nif ⁻ Carb ^R MS2 ^R
3	JC5466(FN68)	<i>Erwinia herbicola</i> YC74	Carb ^R	1×10^{-7}	5/12	Nif ⁻ Carb ^R MS2 ^S
			—	—	7/12	Nif ⁻ Carb ^R MS2 ^R

* Matings 1 and 3 were performed using the agar surface mating procedure; mating 2 was performed in broth.

His⁺ exconjugants grew readily in complex medium at 37 °C, growth on minimal medium occurred at 30 °C but not at 37 °C, suggesting that expression of the FN68 *his* genes was temperature-sensitive in this host. Nitrogenase activity was also only detectable at 30 °C, but synthesis of this enzyme is naturally temperature-sensitive in this strain of *K. pneumoniae* (see Postgate, 1974). One Nif⁺ exconjugant, UNF602, was checked for fixation with ¹⁵N₂ (Table 5) and its identity as *S. typhimurium* was reconfirmed using the API system for identification of Enterobacteriaceae (Hughes & Hughes Ltd, Brentwood, Essex). The low levels of acetylene-reducing activity (Table 5) probably signify low levels of *nif* expression, because the normal activity of the parent strain varies over only a twofold range according to age and test conditions. It was not due to extra oxygen sensitivity, because the technique of anaerobic handling of samples recorded in Methods was used. Acetylene reduction by this strain was repressed in cultures with 1 mg (NH₄)₂SO₄/ml medium.

Transfer of carbenicillin resistance at low frequency occurred in surface, but not broth, matings with *Er. herbicola*. Five out of 12 Carb^R exconjugants tested were MS2 sensitive; none of 12 Carb^R exconjugants tested expressed nitrogen-fixing ability (Table 6, Expt 3). One F⁺ Carb^R derivative was mated with *E. coli* C603 overnight in broth at 37 °C; Carb^R progeny were obtained at about 10⁻⁴/donor cell but none of 15 clones tested was His⁺ or Nif⁺.

Neither *his* nor carbenicillin resistance transfer was detected in surface or liquid matings with the His⁻ strain of *P. mirabilis*.

Buoyant density of FN68 DNA

Partially-purified FN68 DNA from *E. coli* JC5466 separated into two species of buoyant densities 1.710 and 1.714 g/cm³ on analytical CsCl gradients (Fig. 4). The peak at buoyant density 1.710 g/cm³ which is identical to that of *E. coli* DNA is most likely JC5466 chromosomal DNA, and since FN68 was constructed of *E. coli* and *Klebsiella* DNA, the shoulder at the intermediate buoyant density of 1.714 g/cm³ is probably FN68 DNA. This shoulder represents 11.5 % of the total peak area.

*Sedimentation analysis of F' *nif* factors*

Closed circular DNA obtained by CsCl-ethidium bromide centrifugation from *E. coli* JC5466 carrying FN39 or FN68 was analysed by velocity centrifugation in neutral and alkaline sucrose gradients. Four molecular species of S values 140, 103, 86 and 64 in neutral

Fig. 4

Fig. 5

Fig. 6

Fig. 4. Buoyant density profile of DNA from a cleared lysate of JC5466(FN68) after analytical CsCl centrifugation in an MSE Centriscan 75 at 44000 rev./min and 25 °C for 16 h. Cleared lysate DNA was purified as described in the text. The peak at density 1.691 g/cm³ is of *Clostridium perfringens* DNA.

Fig. 5. Profiles of ³H-labelled DNA from *E. coli* JC5466(FN39), analysed by velocity centrifugation in neutral (*top*) and alkaline (*bottom*) 5 to 20 % sucrose gradients (5 ml). Supercoiled DNA, obtained from dye-buoyant density gradients, was dialysed against TES buffer (10 mM-tris-HCl, 1 mM-Na₃EDTA, 100 mM-NaCl, pH 8.0). Portions (150 µl) of the pooled fractions, mixed with 50 µl of ¹⁴C-labelled ColEI CCC-DNA, were layered on gradients and centrifuged for 36.5 min (*top*) and 20 min (*bottom*) at 120000 g in a 52 rotor at 20 °C using a Christ ω II ultracentrifuge. Gradients were fractionated by drop collection (7 drops/fraction) directly on to filter discs and trichloroacetic acid precipitate label assayed for ³H-radioactivity.

Fig. 6. Profiles of ³H-labelled DNA from *E. coli* JC5466(FN68), analysed by velocity centrifugation in neutral (*top*) and alkaline (*bottom*) 5 to 20 % sucrose gradients (5 ml). For details see Fig. 5.

sucrose gradients of either FN39 or FN68 (Figs. 5, 6, *top*) were CCC-DNA because two- to threefold increases occurred in these respective S values in alkaline sucrose (Figs. 5, 6, *bottom*). The DNA profiles were reproducible on 18 gradients of each type. Molecular weights of 279 ± 9 , 136 ± 3 , 90 ± 1 and 44 ± 1 megadaltons correspond to these S values. The largest molecules were thus twice the size of the next largest, the molecular weight of which (136 megadaltons) was approximately the sum of those of the two smaller plasmids. Although carbenicillin resistance genes were carried on FN68 but not on FN39, the molecular weights of both F-primes were indistinguishable by velocity centrifugation in sucrose gradients. A comparable analysis of closed circular DNA from *E. coli* SB1801 carrying FN68 revealed four comparable CCC species.

Transformation of *E. coli* and *Klebsiella* with F' nif DNA

Transformations of *E. coli* c603 and *K. pneumoniae* with plasmid-enriched DNA from two different hosts carrying FN68 are shown in Table 7. All His⁺ transformants of c603 using FN68 DNA from JC5466 were Nif⁻ Carb^s but produced F-type sex-pili, indicating

Table 7. Transformation of *E. coli* and *K. pneumoniae* by FN68 DNA

Transformation mixtures (0.3 ml) which contained 0.075 µg DNA (see Methods) were diluted to 3.0 ml with L-broth and incubated for 90 min at 37 °C before plating. Total cell counts were 5×10^8 to 6×10^8 /ml. Carbenicillin concentrations used for selection were 100 µg/ml for c603 and 200 µg/ml for UNF925.

Origin of FN68 DNA	Recipient	Selection	Transformants /µg DNA	Phenotype of transformants
JC5466(FN68)	c603	His ⁺	9.3×10^3	10/10 MS2 ^s Nif ⁻ Carb ^s
JC5466(FN68)	c603	Carb ^R	0	—
SB1801(FN68)	UNF925	His ⁺	2.5×10^3	8/8 Nif ⁺ Carb ^R
SB1801(FN68)	UNF925	Carb ^R	3.0×10^3	8/56 His ⁺ Nif ⁺

co-transformation of sex factor activity. No Carb^R transformants were obtained by direct selection in three repetitions of this experiment. In contrast, when FN68 DNA from SB1801 was used and the recipient was UNF925, all His⁺ transformants were Nif⁺ Carb^R while eight out of the 56 Carb^R transformants were His⁺ Nif⁺. The results can be interpreted best if the dissociation of FN68 into component replicons, suggested by the sucrose gradient analysis, led to plasmids which functioned more readily in transformation than the parent F-primes, because of their smaller size. Although the frequency with FN68-enriched DNA was lower than that reported by Cohen *et al.* (1972) for highly purified small plasmids in *E. coli*, the numbers of Carb^R transformants increased with DNA concentration, reaching a maximum at 0.75 µg/ml.

DISCUSSION

We have constructed an F-prime *nif* factor by a procedure routinely used for the isolation of F' factors (Low, 1968). A drug-resistant derivative of the F' was constructed by translocation of carbenicillin resistance genes from a P plasmid. Similar genetic techniques to those described here could be employed to construct F-prime factors carrying other regions of the *K. pneumoniae* chromosome.

The DNA of the F-prime factors was derived from diverse origins; it included F-factor DNA, probably both *E. coli* and *Klebsiella* chromosomal genes and, in the case of FN68, P plasmid DNA. The molecular stability of the F-primes could be affected by their heterologous composition; hence the complex molecular pattern observed. The molecular state of some plasmids varies with their hosts (Clowes, 1972). R1, for example, has been isolated from *E. coli* as a circular molecule of molecular weight 68 megadaltons while in *Proteus* it dissociates into component replicons of molecular weight 58 and 10 megadaltons. Recently, Oliver, Morris & Whitfield (1974) have shown that an F'*his gnd* and a cryptic plasmid were present in *S. typhimurium* as a composite molecule with a molecular weight equal to the sum of its precursors' molecular weights. However, in an *E. coli* host three CCC-species were detected with molecular weights corresponding to those of the composite and precursor plasmids.

Dissociation of FN68 gave rise to different progeny in gene transfer experiments. In conjugational transfer of FN68 from JC5466 to *Er. herbicola*, F⁺ Carb^R progeny were obtained which did not carry *his* or *nif*, according to further transfer to c603. With *K. pneumoniae*, *his* and *nif* apparently separated from Carb^R and were transferred at a higher frequency; with *S. typhimurium* a similar separation of determinants occurred but the relative transfer frequencies were reversed (Table 7). In transformation experiments with FN68 DNA prepared from SB1801 a rather similar separation occurred, but with plasmid DNA from JC5466

a new combination appeared regularly: the recipient c603 became F⁺His⁺ Nif⁻ (Table 7). These observations show that several marker combinations may result from dissociation of FN68; one of these separates F and Carb from *Klebsiella his* and *nif*, another splits the *Klebsiella* DNA so as to separate *nif* from F and *his*, the latter being together on a plasmid which transforms c603 particularly readily.

Translocation of carbenicillin resistance from a P plasmid to the F'*nif* factor not only provided a useful selectable marker but also increased the stability of the *Klebsiella* determinants. The insertion of carbenicillin resistance into a plasmid should increase its molecular weight by 1 to 4 megadaltons (Hedges & Jacob, 1974). We observed no difference in molecular weight estimated from sedimentation profiles of FN68 and FN39; our sucrose gradient technique was not sufficiently sensitive to detect such a small change in molecular weight. However, using FN68 DNA we obtained His⁺Nif⁺Carb^u transformants of *K. pneumoniae*, indicating that all three determinants are located on the same molecule.

Although FN68 was used for transformation of *E. coli* and *Klebsiella* the frequency was low. This was due in part to the presence of chromosomal DNA in the preparation, and obviously the large size of the plasmid, making it readily degradable by mechanical shear, lowered the transformation frequency. The unwieldy size of FN68 made the large-scale isolation of purified and intact plasmid molecules, necessary for efficient transformation, practically impossible. The apparent component replicons of FN68 observed on sucrose gradients were present in amounts too small to allow preparative isolation.

An F'*nif* factor should provide a useful genetic tool for the study of the nitrogen-fixation genes, their expression and their regulation, since all the mutants of *E. coli* which have become available to molecular biologists in the last two decades are now potential hosts for *nif* genes. The plasmids can be used for intergeneric transfer of *nif*, but the restricted host range of F, the large size of the plasmids and their tendency to dissociate limit their value for intergeneric *nif* transfer. In our hands, transfer and expression of *his* and *nif* on FN68 was limited to three out of five enteric genera tested: *Klebsiella*, *E. coli* and *S. typhimurium*.

Finally, we recognize that these studies provide only circumstantial evidence that the *nif* genes on the plasmids are in fact structural genes determining nitrogenase synthesis. It would be possible to interpret all our findings, and earlier conjugational *nif* gene transfers, if cryptic *nif* genes were present in the recipient *Escherichia*, *Klebsiella* and *Salmonella* species and if the plasmids carried only a regulatory determinant which allowed expression of cryptic *nif*. Though this is an unlikely prospect, a precedent for the existence of cryptic *nif* in microbes is presented by those strains of *Rhizobium* which do not fix nitrogen away from the host plant yet which have now been shown unequivocally to carry *nif* genes (Child, 1975; Scowcroft & Gibson, 1975). Experiments are being undertaken to demonstrate the presence of *nif* structural genes on these plasmids.

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