Chromosomal Integration of *Klebsiella* Nitrogen Fixation Genes in *Escherichia coli*

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SUMMARY

Escherichia coli, C-M7, a His⁺Nif⁺ hybrid obtained by intergeneric mating with a *Klebsiella pneumoniae* donor strain, also inherited the unselected markers *gnd* and *rfb*. The R factor, R144drd3, which had been used to confer fertility on the donor, was present, detectable as covalently closed circular DNA of molecular weight 69×10^6 daltons. No other species of supercoiled DNA were isolated and the elimination of R144drd3 did not result in the loss of *Klebsiella* genes. Segregation analysis of donor markers indicated that the *Klebsiella* DNA was integrated at the *his* region of the *E. coli* chromosome in the probable order *his-gnd-nif-rfb*. Strain C-M7 produced a nitrogenase physiologically identical to that of *K. pneumoniae*, but synthesized a heteromeric species of gluconate-6-phosphate dehydrogenase.

INTRODUCTION

Intergeneric transfer of nitrogen fixation genes in matings between *Klebsiella pneumoniae* and an *Escherichia coli* C recipient, which does not naturally fix nitrogen, was reported by Dixon & Postgate (1972). The resulting His⁺Nif⁺ hybrids were unequivocally identified as strains of *E. coli* by their cultural and immunological characteristics. In a hybrid designated strain C-M7, *K. pneumoniae* *nif* genes remained stable after two successive subcultures on nutrient agar, whereas in other hybrids spontaneous Nif⁻ segregants arose at high frequency.

We now report a more extensive study of the stable hybrid C-M7, including physico-chemical studies on its DNA, its phage sensitivity, and its inheritance of non-selected *Klebsiella* markers, which shows that a segment of *Klebsiella* DNA, which includes the *nif* operon, was integrated in the chromosome of this hybrid. A culture of a prototrophic revertant of the hybrid (strain C-M74, below) has been deposited with the National Collection of Industrial Bacteria with the number 11023.

METHODS

Bacteria and bacteriophages. Details of bacterial strains, R factors and phages are given in Table 1.

Media. Nutrient broth (Oxoid no. 2) was used as complete medium. Solid medium was prepared by adding 1.5% agar (Difco, Bactoagar). The composition of NH₄⁺-free medium was: MgSO₄, 0.1 g; Na₂MoO₄.2H₂O, 25 mg; FeSO₄.7H₂O, 25 mg; K₂HPO₄, 12.06 g; KH₂PO₄, 3.4 g; glucose, 20 g; in 1 l H₂O. Phosphate solution was sterilized separately and subsequently added aseptically. Minimal media used were: DM mineral salts (Davis &

Table 1. *Bacterial strains, R factors and bacteriophages*

		Bacteria	
Strain	Genotype	Other properties	Origin
<i>Klebsiella pneumoniae</i>			
HF3	—	Prototrophic, Nif ⁺ ; derivative of M5a1 (R144drd3)	Dixon & Postgate (1972)
<i>Escherichia coli</i> K12			
J53	<i>pro met</i>	—	E. Meynell
J62-I	<i>nal pro his trp lac str</i>	—	N. Datta
W1166(Col V)		Colicin I indicator	E. Meynell
CR34 (Col E1)	<i>thy thr leu thi</i>	Carries colicin E1	D. Sheratt
<i>E. coli</i> C			
C603	<i>his arg str</i>	—	G. Bertani
C1463	<i>his-5 met G str-8 rha-2</i>	—	G. Bertani
C-M7	<i>arg str</i>	His ⁺ Nif ⁺ ; carries R144drd3	Dixon & Postgate (1972)
R factors			
Factor	Determinants	Type	Origin
R144drd3	Km Col I	I	E. Meynell
'AST'	Am Sm Tc	I	N. Datta
R1drd19	Km Am Cm Sm Su	F	E. Meynell
Bacteriophages			
Phage	Source	Phage	Origin
λvir	W. Brammar	P1vir	N. Willetts
λh ⁸⁰	N. Murray	EC1	This paper
φX 174	C. E. Dowell	If ₂	E. Meynell

Genetic symbols for chromosomal markers are those of Taylor & Trotter (1972). 'Nif' denotes nitrogen fixation genes in accordance with the terminology of Streicher, Gurney & Valentine (1971). Symbols for drug resistance determinants are as follows: Km, kanamycin; Am, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Su, sulphonamide.

Mingioli, 1950), E mineral salts (Vogel & Bonner, 1956) and M9 medium (Clowes & Hayes, 1968). Supplements and drugs were added at the following concentrations: L-amino acids, 25 µg/ml; kanamycin, 30 µg/ml; chloramphenicol, 30 µg/ml; streptomycin, 250 µg/ml; and naladixic acid, 25 µg/ml.

Growth conditions and tests for nitrogen fixation. Cultures on NH₄⁺-free agar plates were incubated at 30 °C under an atmosphere of N₂ (99 %) and CO₂ (1 %) in nylon bags (Hill, 1973). Growth in NH₄⁺-free liquid medium and ability to reduce acetylene were tested by the Pankhurst tube technique (Campbell & Evans, 1969) as modified by Postgate (1972).

Cell-free extracts of *Escherichia coli* C-M74 were prepared and assayed for dithionite-supported acetylene reduction by the method of Eady, Smith, Cook & Postgate (1972).

Bacterial mating. Overnight cultures of donor and recipient strains were grown in static nutrient broth at 37 °C. A mid-log phase culture (1 to 4 × 10⁸ c.f.u./ml) of the donor strain was prepared by a 1:10 dilution in prewarmed broth and incubation continued for 2 h at 37 °C without shaking. Cultures were mixed in a donor-to-recipient ratio of 1:10 and incubated for 4 h at 37 °C. The bacteria were then centrifuged, resuspended in buffer and plated on selective media to determine the number of donor bacteria in the mating mixture, the number of recombinants formed, and the number of recipients which had acquired drug resistance. Plates were incubated at 37 °C for 48 h.

Colicin I production. Chloroform-treated bacteria in saline phosphate buffer (NaCl, 8.5;

K_2HPO_4 , 7.0; KH_2PO_4 , 3.0 g/l H_2O ; pH 7.2) or culture supernatant were tested for Col I activity by spotting drops on to soft agar (0.7%) overlays containing 0.1 ml of *Escherichia coli* indicator strain, W1166 (Col V).

Phage sensitivity tests. Soft agar overlays containing the bacteria to be tested were spotted with drops of high-titre phage preparations. Sensitivity to male specific phage was tested by the phage multiplication method of Grindley & Anderson (1971).

Phage adsorption tests. Broth cultures (3×10^8 c.f.u./ml) were washed and resuspended in starvation buffer (Denhardt & Sinsheimer, 1965). Phage $\phi X174$ was added at a multiplicity of infection of 1 and unadsorbed phage in samples, removed after 0, 20 and 40 min, was assayed on *Escherichia coli* C by the procedure of Denhardt & Sinsheimer (1965).

Isolation of bacteriophage EC1. The supernatant from 100 ml of raw sewage, which was centrifuged at 6000 g for 10 min, was mixed with 100 ml of double-strength nutrient broth and 100 ml of an overnight culture of *Escherichia coli* C-M7. The mixture was centrifuged at 6000 g for 10 min after 18 h of agitation at 37 °C. A portion of the supernatant was then filtered through a 0.45 μm 'Millipore' filter and the filtrate assayed for the presence of bacteriophages which would lyse C-M7. A phage, derived from one of several plaque types observed, was purified by two successive single plaque isolations and designated EC1.

Stocks of the phage were prepared by propagation on C-M7 broth cultures. Since C-M7 carried R144drd3, which specified kanamycin resistance and production of Col I, phage suspensions prepared in this way were contaminated with Col I. Precipitation with polyethylene glycol followed by banding in a CsCl gradient freed the phage of colicin (Yamamoto *et al.* 1970).

Isolation of phage-resistant clones. A standard plaque-assay of phage EC1 was done on soft agar overlays of phage-sensitive bacteria which were grown in the absence of fixed nitrogen. Resistant colonies were picked from plaques after 24 h incubation and purified by two successive single colony isolations. The nutritional requirements of all resistant clones were checked to confirm their identity.

Gluconate-6-phosphate dehydrogenase (gnd). The *gnd* product was assayed by the colorimetric method of Peyru & Fraenkel (1968). It was extracted from organisms by sonication and characterized electrophoretically in polyacrylamide gels as described by Lew & Roth (1971).

Preparation of anti-serum to the Mo-Fe protein of Klebsiella pneumoniae. Mo-Fe protein, purified from *K. pneumoniae* M5a1 (Eady *et al.* 1972), was dialysed anaerobically to remove sodium dithionite, diluted anaerobically to 50 μg protein/ml with pyrogen-free NaCl (0.85%) and stored as six portions (0.2 ml each) under N_2 at -20 °C. A rabbit, free of natural reaction to the protein, was injected intramuscularly with a sample every 2 to 4 days followed by two similarly spaced intravenous injections. Three days after the last injection, 15 ml of serum were prepared from blood collected from the rabbit and used for conventional Ouchterlony plate tests in NaCl set with 2% 'Ion agar' (Oxoid Ltd).

DNA buoyant density determination. DNA preparation and CsCl density-gradient centrifugation were done according to the procedure of Rownd (1969). DNA was extracted from 10 ml cultures, which were grown in nutrient broth at a concentration of 2 to 4×10^8 c.f.u./ml, and finally dissolved in 1.0 ml of 0.1 SSC (0.015 M-NaCl, 0.0015 M-trisodium citrate, pH 7.0). Samples for centrifugation contained approximately 10 μg of test DNA, 3 μg of *Micrococcus lysodeikticus* DNA (Sigma, London) dissolved in 0.1 SSC and 0.9 ml CsCl (B.D.H. Ltd, Godalming, Surrey, optical grade, density = 1.79 g cm⁻³); the final volume was adjusted to approximately 1.0 ml with 0.1 SSC and the density to 1.71 g cm⁻³. These solutions were centrifuged in a Spinco model E analytical ultracentrifuge at 44 000 rev./min at 25 °C for 16 h. Ultraviolet light photographs were taken on Kodak commercial film

at the end of each run and traced with a Joyce-Loebl mark II microdensitometer. Buoyant densities were calculated as described by Schildkraut, Marmur & Doty (1962), using *M. lysodeikticus* DNA (density 1.731 g cm^{-3}) as a reference density.

Growth and DNA labelling conditions. An overnight culture was diluted 1:100 in 10 ml of pre-warmed nutrient broth. After 3 h, 0.1 ml of [^3H]thymidine (1 mc/ml) and 0.25 ml of deoxyadenosine (10 mg/ml) were added and incubation continued until a concentration of 2 to 4×10^8 c.f.u./ml was reached. Cultures were agitated throughout the incubation period and harvested by centrifugation at 0 to 2°C .

Isolation and purification of supercoiled DNA. Bacteria were lysed by a lysozyme-triton X-100 procedure (D. J. Sherratt, private communication). After centrifugation, cultures were resuspended in 0.33 ml of cold 25 % sucrose in 0.05 M-tris-HCl (pH 8.0). To this suspension, which was kept on ice, 0.07 ml of lysozyme solution (5 mg/ml in 0.25 M-tris-HCl, pH 8.0) was added and the mixture gently swirled. After 5 min, 0.15 ml of 0.25 M- Na_2EDTA (pH 8.0) was added and 5 min later, 0.53 ml of triton X-100 lysing mixture was added while the suspension was being well mixed. Triton X-100 lysing mixture consisted of 2.5 ml of 20 % triton X-100 in 0.1 M-tris-HCl (pH 8.0), 6.25 ml of 0.25 M- Na_2EDTA (pH 8.0), 1.25 ml of 1.0 M-tris-HCl (pH 8.0) and 15 ml H_2O . Lysis was continued on ice for 20 min and the crude lysate was then centrifuged at 16 000 g and 0 to 2°C for 12 min. The supernatant, which was carefully decanted, contained most of the supercoiled DNA and was designated 'cleared lysate'. Covalently closed circular (CCC) DNA in the cleared lysates was isolated by ethidium bromide-caesium chloride density gradient centrifugation (Radloff, Bauer & Vinograd, 1967). A mixture containing 1.0 ml of cleared lysate, 2.5 ml of TES-1 buffer (0.5 M-NaCl, 0.05 M-tris-HCl, 0.005 M- Na_2EDTA , pH 8.0), 0.5 ml of ethidium bromide solution (1 mg/ml in 0.1 M-sodium phosphate buffer, pH 7.0) and 3.8 g CsCl ('Analar' grade) was added to a 10 ml polypropylene centrifuge tube and overlaid with mineral oil. The mixture was centrifuged for about 16 h at 126 000 g and 0 to 2°C in an MSE Superspeed 50 preparative ultracentrifuge. Gradients were fractionated by drop collection in styrene 'Microtiter' plates (10 drops/fraction, 30 to 32 fractions/gradient). Portions (0.01 ml) of each fraction were spotted on Whatman no. 1 filter discs (2×2 cm) and assayed for ^3H -radioactivity as described below. Fractions corresponding to the denser of the two DNA bands in the gradients were pooled and dialysed for 16 h against TES-2 buffer (0.01 M-tris-HCl, 0.001 M- Na_2EDTA , 0.1 M-NaCl, pH 8.0). The resulting dialysate was used for sucrose gradient centrifugation.

Preparation of ^{14}C -labelled colicin E1 DNA (D. J. Sherratt, private communication). An overnight culture of *Escherichia coli* CR 34(Col E1) in M9 medium, supplemented with Cas-amino acids (200 $\mu\text{g/ml}$), thiamine HCl (0.002 $\mu\text{g/ml}$) and thymine (1 $\mu\text{g/ml}$) was diluted (0.2 ml in 10 ml) in the same medium and incubated in a shaking water bath at 37°C until a density of 1×10^8 to 2×10^8 c.f.u./ml was reached. Chloramphenicol (100 $\mu\text{g/ml}$) was then added and incubation continued for 90 min, while cessation of chromosome replication occurred. Thereupon, 0.4 ml of [^{14}C]thymidine (50 $\mu\text{Ci/ml}$, 53 mCi/mmol) was added and incubation continued overnight. Replication of Col E1 DNA was not inhibited by chloramphenicol. Supercoiled DNA was isolated from the culture and dialysed as described above. Only one radioactive band was observed in ethidium bromide-CsCl gradients, which corresponded to supercoiled Col E1 DNA.

Sucrose gradient centrifugation. Six linear sucrose gradients (5 ml) were simultaneously prepared from solutions of 5 and 20 % sucrose in TES-2 buffer in 5 ml cellulose nitrate tubes. Test material (100 μl) and reference DNA (100 μl , Col E1) were carefully layered on the gradients which were immediately centrifuged for 45 min at 120 000 g in the 6×5 m

Table 2. Transfer characteristics of hybrid C-M7 carrying R144drd3 and R1drd19

Transfer frequencies are quoted per donor organism. Km, kanamycin; Cm, chloramphenicol.

Donor	Recipient					
	<i>Escherichia coli</i> C-1463				<i>E. coli</i> K12 J62-1	
	Km	Cm	<i>his</i>	<i>met</i> G	Km	<i>his</i>
C-M7 (R144drd3)	3×10^{-1}	—	8×10^{-7}	$< 1 \times 10^{-7}$	1×10^{-2}	$< 1 \times 10^{-7}$
C-M7 (R144drd3, R1drd19)	—	3×10^{-2}	$< 1 \times 10^{-7}$	$< 1 \times 10^{-7}$	—	—
C603 (R144drd3)	> 1	—	$< 1 \times 10^{-8}$	1×10^{-7}	—	—
C603 (R144drd3) (R1drd19)	—	1×10^{-1}	$< 1 \times 10^{-7}$	1×10^{-6}	—	—

swing-out rotor on a Christ ω II ultracentrifuge. After centrifugation the rotor was allowed to decelerate, unbraked.

Alkaline sucrose gradients were prepared from 5 and 20% sucrose in 0.3 M-NaOH, 0.001 M-Na₂ EDTA and 1.0 M-NaCl (pH 12.7). The material to be sedimented was carefully placed on the gradients and left for 5 to 10 min before centrifugation. Gradients were centrifuged as above for 20 or 30 min at 120 000 *g*. After centrifugation, gradients were kept on ice and fractionated by drop collection directly on to Whatman no. 3 filter-paper strips marked in 2 cm squares (seven drops/fraction, 30 fractions/gradient). Strips were treated with cold 10% trichloroacetic acid (TCA) and washed initially with 90% alcohol and finally with ether.

Counting of radioactive samples. Dried filter-paper discs, containing TCA-precipitable radioactive DNA, were placed in scintillation vials and 10 ml of 'NE 233' liquid scintillator (Nuclear Enterprises Ltd, Edinburgh) were added to each vial. Samples were counted in an NE 8310 liquid scintillation counter.

RESULTS

Morphology. Hybrid C-M7 formed round, translucent, white-to-cream non-mucoid colonies on nutrient agar resembling those of the parent strain, *Escherichia coli* C603. Under phase contrast it consisted of small, non-motile, almost coccoid rods with occasional large pleomorphs, a character shown by the parent strain. These pleomorphs became particularly abundant when the hybrid was grown in nitrogen-fixing conditions.

Buoyant density of C-M7 DNA. DNA from donor, recipient and hybrid strains showed unimodal distribution in CsCl gradients (Fig. 1). The buoyant density of hybrid C-M7 DNA (1.710 g cm⁻³) was identical to that of the parental *Escherichia coli* DNA and different from that of *Klebsiella pneumoniae* DNA (1.715 g cm⁻³).

R factor R144drd3 in hybrid C-M7. Hybrid C-M7 was resistant to kanamycin, produced Col I and was sensitive to the male-specific phage If₂, implying that this strain carried R144drd3 (the I-like R factor used to promote the transfer of *Klebsiella pneumoniae* genes to *Escherichia coli*). Although R144drd3 transferred efficiently from C-M7 to other strains of *E. coli*, it did not promote the transfer of either *nif*, *his* or *met* G. Nor did the F-like R factor R1drd19 mobilize *his* or *met* G in C-M7 (Table 2); this behaviour contrasts considerably with other hybrids (Cannon, Dixon, Postgate & Primrose, 1974).

Attempts to eliminate R144drd3 from C-M7 using sodium dodecyl sulphate treatment (Salisbury, Hedges & Datta, 1972) were unsuccessful. However, the R factor 'AST' which belongs to the same compatibility group as R144drd3 was introduced into hybrid C-M7. Superinfection and elimination of kanamycin resistance and Col I, the determinants characteristic

Fig. 1

Fig. 2

Fig. 1. DNA buoyant density profiles after analytical caesium chloride density gradient centrifugation at 44 000 rev./min for 16 h. The DNA peaks at density 1.731 g/cm³ are of *Micrococcus lysodeikticus*. (a) DNA from *Escherichia coli* c603; (b) DNA from hybrid c-m7; (c) DNA from *Klebsiella pneumoniae* HF3. DNA was extracted and purified as described in Methods.

Fig. 2. Profiles of labelled DNA in cleared lysates of *Escherichia coli* c603 (●), and hybrid c-m7 (○), after dye-buoyant density centrifugation at 126 000 g, 0 to 2 °C, for 16 h. Cleared lysates were prepared by a lysozyme-EDTA-triton X-100 procedure (see text). 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.

of R144drd3, took place in the three clones examined, but the hybrid retained the His⁺Nif⁺ phenotype. We conclude from these data that the *Klebsiella his* and *nif* determinants were not associated with R144drd3 in hybrid c-m7.

Supercoiled DNA in Escherichia coli c603 (R144drd3) and c-m7. A strain of the original recipient, *E. coli* c603, carrying the above R factor, was constructed. Both these strains and hybrid c-m7 were gently lysed by the lysozyme-triton X-100 procedure. More than 98 % of the acid-insoluble DNA was removed by low-speed centrifugation. The cleared lysates were immediately centrifuged in caesium chloride-ethidium bromide gradients. Fig. 2 shows a comparison of hybrid c-m7 with c603; covalently closed circular DNA from the hybrid separated as a denser band below the less dense open circular (OC) and linear DNA.

Fig. 3

Fig. 4

Fig. 3. Profiles of labelled DNA from *Escherichia coli* c603 (R144drd3), analysed by velocity centrifugation in neutral (a) and alkaline (b) 5 ml, 5 to 20% sucrose gradients. Fractions of the denser band from dye-buoyant density gradients were pooled and dialysed against TES-2 buffer. Portions (100 μl) of the pooled fractions, mixed with 100 μl of Col E1 DNA, were layered on gradients and centrifuged for 45 min (a) and 30 min (b) at 120 000 g in a 52 rotor at 20 $^{\circ}\text{C}$ using a Christ ωII ultracentrifuge. After centrifugation, seven-drop fractions were collected directly on filter discs and TCA-precipitable label assayed for ^3H -radioactivity.

Fig. 4. Profiles of DNA from hybrid c-M7, analysed by velocity centrifugation in neutral (a) and alkaline (b) 5 ml, 5 to 20% sucrose gradients. Supercoiled DNA, obtained from dye-buoyant density gradients, was dialysed against TES-2 buffer and portions (100 μl each of c-M7 DNA and Col E1 DNA) layered on sucrose gradients. Centrifugation was for 45 min (a) and 30 min (b) at 120 000 g in a 52 rotor at 20 $^{\circ}\text{C}$ using a Christ ωII ultracentrifuge. Gradients were fractionated by drop collection (7 drops) directly on to filter discs and TCA precipitate label assayed for ^3H -radioactivity.

A comparable CCC-DNA band was obtained from *E. coli* c603 (R144drd3). Under these conditions no CCC-DNA was isolated from c603. Pooled fractions of the denser bands of the hybrid and c603 (R144drd3) were dialysed against TES-2 buffer (pH 8.0) and analysed by velocity centrifugation in neutral and alkaline sucrose gradients using [^{14}C]Col E1 DNA as a sedimentation marker. The dialysed material from both strains sedimented in two peaks under neutral and alkaline conditions (Fig. 3, 4). The S values of the two peaks in neutral sucrose gradients for both strains, calculated from the S value of Col E1 CCC-DNA (23 S) and accepting a linear relationship between the S value and the distance sedimented,

Table 3. *Phage sensitivity patterns of hybrid C-M7 and parent strains*

Phage	Host		
	<i>Klebsiella pneumoniae</i> HF3	<i>Escherichia coli</i> c603	Hybrid C-M7
λ vir	R	S	R
λ h ⁸⁰	R	S	R
ϕ X174	R	S	R
P1 vir	S	S	R
EC1*	R	R	S

* For origin of EC1, see text.

S, confluent lysis of bacteria in soft agar overlays spotted with drops of high titre phage preparations; R, no effect.

Table 4. *Adsorption of phage ϕ X174 to recipient and hybrid strains*

Strain	For procedure, see text.			
	Unadsorbed phage (p.f.u./ml) at (min)			% remaining
	0	20	40	
<i>Escherichia coli</i> c603	8×10^6	1.5×10^6	4.8×10^5	6
Hybrid C-M7	6.8×10^6	8.2×10^6	7.1×10^6	100

were 77 S and 48 S. The ratio of the S values (1.6:1) suggested that the peaks represented the covalently closed circular and open circular forms respectively, of plasmid DNA. The presence of both molecular forms was confirmed by the alkaline sucrose profiles (Figs. 3*b*, 4*b*). If DNA is sedimented at a pH above that which denatures OC-DNA, covalently closed circles sediment three to four times faster than a single strand filament with a molecular weight one-half that of the circle. This relationship was shown by the S values of the peak (208 S, 54 S) in alkaline sucrose gradients, derived from the S value of Col E1 CCC-DNA (58 S) under alkaline conditions. The CCC-DNA from *E. coli* c-603 (R144*drd*₃) and hybrid C-M7 were thus indistinguishable by sucrose gradient analysis; hence, only one plasmid species, assignable to R144*drd*₃, could be detected in each strain.

The molecular weight of R144*drd*₃, calculated from an S value of 77 for the CCC-form using the data of Bazaral & Helinski (1968), was 69×10^6 daltons.

Phage sensitivity of parental and hybrid strains. Strains of *Klebsiella pneumoniae* HF3, *Escherichia coli* c603, *E. coli* c603 (R144*drd*₃) and hybrid C-M7 were tested for sensitivity to the phages listed in Table 3. Phage resistance in C-M7 was probably due to the inability of the hybrid to adsorb the phages, since ϕ X174 did not adsorb to C-M7 (Table 4). Since ϕ X174 is a rough-specific phage (Wilkinson, Gemski & Stocker, 1972), this result suggests that *Klebsiella rfb* (rough) genes have been transferred to C-M7. [The *som* locus of *Klebsiella*, which maps close to *his* (Matsumoto & Tazaki, 1971), is the most likely equivalent of the *rfb* locus in *Salmonella typhimurium* and *E. coli* K12].

Isolation of a hybrid-specific phage. A phage, termed EC1, which proved of value in isolating segregants of C-M7, was isolated from sewage; its purification and the preparation of Col I-free stocks on C-M7 are described under Methods. Electron microscopy (S. Primrose, unpublished) showed EC1 to be a long-tailed phage. EC1 plated on hybrid C-M7 but both parental strains were resistant. Some strains of *Salmonella typhimurium* and *Klebsiella pneumoniae* were sensitive to EC1 but all *Escherichia coli* strains tested were resistant. In several tests, no C-M7 strains lysogenic for EC1 were obtained.

Stability of C-M7. Dixon & Postgate (1972) reported that C-M7 showed no tendency to produce Nif⁻ segregants when subcultured anaerobically on NH₄⁺-free agar plates. Phage

Fig. 5. Frequency and extent of deletions in the *his-nif* region of five groups of EC1-resistant segregants of *Escherichia coli* hybrid C-M7. The deleted regions are shown in black. In group 5 the extent of the deletions beyond the loci indicated was not determined. The shaded regions may or may not be deleted. Markers are not drawn to scale.

EC1 provided a useful tool for selecting spontaneous segregants of hybrid C-M7. The frequency of appearance of EC1-resistant mutants in cultures of C-M7 taken from colonies subcultured several times on NH_4^+ -free agar was 4×10^{-3} ; however, a large proportion of these appeared unstable, in that phage resistance was not sustained on subculture.

Five groups were distinguishable among 50 stable EC1-resistant mutants (Fig. 5). The majority of these mutants were sensitive to phages λ , λh^{80} and ϕX174 . Such mutants were probably deletions of the *rfb* locus. Three mutants were *rfb-nif* deletions, two were *rfb-nif-gnd* deletions and ten were *rfb-nif-gnd-his* deletions. The remaining mutants, which were $\text{Rfb}^- \text{Nif}^- \text{Gnd}^-$, did not grow on minimal medium supplemented with histidine and arginine. The deletions in this group presumably extended beyond the four loci which were examined.

Map position of nif. The order of the loci, *his*, *gnd*, *rfb*, as shown in Fig. 5, has been established in *Escherichia coli* and *Salmonella typhimurium* (Taylor & Trotter, 1972; Sanderson, 1972), and the properties of the five groups of deletions cited in Fig. 5 are compatible with this order in C-M7. The properties of group 2 mutants suggest that the *nif* operon was adjacent to *rfb* and, since all *rfb-gnd* deletion mutants examined were also Nif^- , the *nif* operon may tentatively be placed between *rfb* and *gnd*.

Nitrogenase from C-M7. Dixon & Postgate (1972) reported that nitrogenase synthesis in C-M7 was repressed by ammonium ions as in the donor strain *Klebsiella pneumoniae* HF3 (R144drd3). In further studies, expression of *nif* in this strain showed the oxygen sensitivity typical of nitrogen-fixing Klebsiellae: growth occurred readily in air with NH_4^+ but only anaerobically in nitrogen-fixing conditions. It also showed a form of temperature sensitivity resembling that of the donor strain M5a1 (see Postgate, 1974): batch cultures grew readily at 30 or 37 °C with NH_4^+ but only at 30 °C in NH_4^+ -free medium under N_2 .

A spontaneous Arg^+ revertant, strain C-M74, was selected and thus a prototrophic nitrogen-fixing *Escherichia coli* was obtained. Bulk culture of this strain followed by extraction of a crude cell-free extract using the precautions described by Eady *et al.* (1972) gave active nitrogenase preparations containing a component immunologically related to the Mo-Fe protein of *Klebsiella pneumoniae* (Fig. 6). Anaerobic chromatography on DEAE-cellulose gave two fractions corresponding to the Mo-Fe and Fe proteins of the donor. They were not purified or studied in detail, but the presumptive Mo-Fe fraction cross-reacted with purified Fe protein from *K. pneumoniae*.

Gluconate-6-phosphate dehydrogenase in C-M7. Gluconate-6-phosphate dehydrogenase from parental strains *Escherichia coli* C603 and *Klebsiella pneumoniae* HF3 (R144drd3) had

Fig. 6. Ouchterlony plate of antiserum to *Klebsiella pneumoniae* Mo-Fe protein and a crude cell-free extract of *Escherichia coli* C-M74. Antiserum, prepared as in Methods, is in the centre well, purified *Klebsiella* protein in wells K, and extract of the prototrophic *E. coli* hybrid in wells E.

Table 5. *Electrophoretic R_F values of gluconate-6-phosphate dehydrogenase from various strains*

Strain	R_F
<i>Klebsiella pneumoniae</i> HF3	0.38
<i>Escherichia coli</i> C603	0.47
HF3 + C603	0.37 and 0.47
Hybrid C-M7	0.43

The enzyme in cell-free extracts was subjected to electrophoresis in polyacrylamide gels; for details see text.

different electrophoretic mobilities in polyacrylamide gels, and clear separation was obtained when enzymes from both strains were examined together in the same gels (Table 5). The enzyme from hybrid C-M7, however, gave a single homogeneous band with an R_F value intermediate between those of the parent strains. This situation contrasts with that in hybrid C-M9, where the fragment of *Klebsiella* DNA is maintained on a plasmid and three species of gluconate-6-phosphate dehydrogenase are detectable (Cannon *et al.* 1974).

DISCUSSION

In hybrid C-M7, we have shown that a segment of *Klebsiella* DNA which included the nitrogen-fixation genes was probably integrated into the *E. coli* chromosome. Only one species of CCC-DNA was isolated from C-M7 and this could be identified as the R factor, R144*drd3*. However, our physico-chemical studies do not exclude the possibility that the *Klebsiella* DNA could be in a molecular form which would be undetectable by the dye-buoyant density procedure (Leavitt *et al.* 1971). Certainly the *Klebsiella* DNA was not associated with R144*drd3*; the sedimentation coefficient of this R factor remained unchanged in C-M7 and the elimination of R144*drd3* did not result in loss of the His⁺Nif⁺ phenotype.

Further evidence for the integration of the *Klebsiella* DNA was obtained from segregation analysis of donor markers. In addition to the selection marker *his*, the non-selected markers *nif*, *rfb* and probably *gnd* were also inherited by C-M7. Since the recipient strain C603 was Gnd^+ , the isolation of $His^-Gnd^-Nif^-Rfb^-$ segregants strongly suggests that the *Klebsiella* DNA had become integrated into the *his-rfb* region of the C-M7 chromosome. Hybrid C-M7 thus differs from other *Klebsiella pneumoniae*-*Escherichia coli* hybrids in which the donor chromosomal segments were conserved as covalently closed circular molecules of DNA (Cannon *et al.* 1974).

Our results do not distinguish whether C-M7 is haploid for the *his-rfb* region due to recombination or whether the hybrid is diploid for this region, the *Klebsiella* genes being maintained perhaps by tandem insertion. Approximately half of the hybrids arising from *Escherichia coli*-*Salmonella typhosa* crosses are haploid recombinants; the length of the donor DNA incorporated in such recombinants is generally less than 4 min (Johnson, Easterling & Baron, 1970; Johnson, Alexeichik & Baron, 1972). About 25 % of *E. coli* - *S. typhimurium* Trp^+ hybrids are true recombinants, whereas approximately 50 % are merodiploid hybrids, the donor DNA being integrated at novel sites (Mojica-a & Middleton, 1972). The frequency of segregation of ECI-resistant mutants in C-M7 may indicate that this hybrid is diploid for at least some of the inherited markers, and the synthesis of heteromeric gluconate-6-phosphate dehydrogenase implies that the *gnd* alleles of both parents were present. The possibility of partial duplication in the *his-rfb* region raises the question of the relative orientation of loci in the duplicated region. Duplicated segments, which are *cis* and tandem to the original loci, are known to be unstable (Novick, 1969), in contrast to duplicated integrations which are stabilized by genetic inversion (Adelberg & Bergquist, 1972). The instability of the donor markers in C-M7 would suggest that the *his*, *gnd* and *rfb* loci on the *Klebsiella* segment, if they are duplicated, are probably *cis* and tandem to the corresponding *E. coli* loci in the order *his-gnd-rfb*.

Previous genetic studies of nitrogenase (Dixon & Postgate, 1971; Streicher, Gurney & Valentine, 1971, 1972) have revealed that the *nif* genes are clustered close to the *his* operon of *Klebsiella pneumoniae*. In *Azotobacter vinelandii* the structural *nif* genes coding for the Mo-Fe and Fe proteins are co-ordinately repressed by NH_4^+ (Shah, Davis & Brill, 1972). By selecting revertants of an *Azotobacter* mutant unable to synthesize any component of nitrogenase, Gordon & Brill (1972) isolated constitutive mutants, which produced nitrogenase in the presence of NH_4^+ . We have observed that the *Escherichia coli* Nif^- hybrid, C-M7, produces a nitrogenase with the same physiological properties as that of the *Klebsiella* donor strain. Moreover, nitrogen fixation in *E. coli* hybrids is subject to NH_4^+ repression; presumably, this repression is exerted at the level of mRNA transcription as in *Klebsiella* (Tubb & Postgate, 1973) and involves the regulation of at least three structural genes; one for the single subunit of the Fe protein and two types of subunits of the Mo-Fe protein (Eady *et al.* 1972). We therefore conclude that the structural and regulatory genes for nitrogenase are linked, forming a *nif* operon.

The order of the loci, *his-gnd-rfb*, which has been established in *Salmonella typhimurium* (Sanderson, 1972) and *Escherichia coli* (Taylor & Trotter, 1972) has been inferred for the *Klebsiella* markers in C-M7 on the basis that the deletions obtained were compatible with this order. Our deletion analysis indicates that the *nif* operon lies between *rfb* and *gnd*. Streicher *et al.* (1972) have observed that the frequency of P1 transduction between *his D* and most *nif* mutations is 30 to 80 %. This suggests that *his D* and the most distal gene of the *nif* operon are separated by approximately 0.5 *E. coli* time units (Taylor & Trotter, 1967). The distance between *his* and *gnd* in *E. coli* is 0.4 min (Sunshine & Kelly, 1971), whereas the

distance between *his* and *rfb* is 0.7 min (Taylor & Trotter, 1972). The transduction data obtained in *Klebsiella pneumoniae* thus support the map position of *nif* reported here.

Most strains of *Escherichia coli* lack the O-side chains of the somatic lipopolysaccharide core (LPS) and are phenotypically rough (Jones, Koeltzow & Stocker, 1972). The recipient strain c603 was sensitive to the rough specific phage ϕ X174, whereas hybrid c-M7 did not adsorb this phage. The expression of *Klebsiella rfb* genes in *E. coli* suggests that there is considerable homology between the enzymes involved in LPS biosynthesis in both genera and contrasts with *Salmonella-E. coli* hybrids in which the *his*-linked *Salmonella rfb* genes are not expressed in the absence of the *ilv*-linked *Salmonella rfa* genes. Apparently *E. coli* cannot attach *Salmonella* O-specific side chains to its LPS core (Jones *et al.* 1972).

Gluconate-6-phosphate dehydrogenase is a dimeric enzyme consisting of identical subunits (Lew & Roth, 1971). The existence of a single homogeneous species of this enzyme, in c-M7, with an R_f intermediate between that of the parental strains, suggests that both alleles may be present but non-random association of monomeric subunits, one derived from each gene, occurs after translation. A possible alternative explanation is that recombination within the *gnd* structural gene could have occurred, giving rise to hybrid monomers, although this event would be an extremely uncommon one.

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