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 \(69 \times 10^6\) 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 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 physicochemical 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 I. Bacterial strains, R factors and bacteriophages

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Genotype</th>
<th>Other properties</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella pneumoniae</td>
<td></td>
<td>Prototrophic, Nif⁺; derivative of M₅₅₁ (R₁₄₄ᵈʳᵈ₃)</td>
<td>Dixon &amp; Postgate (1972)</td>
</tr>
<tr>
<td>Hf₃</td>
<td>—</td>
<td>—</td>
<td>E. Meynell</td>
</tr>
<tr>
<td>Escherichia coli  K₁₂</td>
<td></td>
<td>—</td>
<td>N. Datta</td>
</tr>
<tr>
<td>135</td>
<td>pro met</td>
<td>—</td>
<td>E. Meynell</td>
</tr>
<tr>
<td>662⁻¹</td>
<td>nal pro his trp lac str</td>
<td>—</td>
<td>D. Sheratt</td>
</tr>
<tr>
<td>W₁₁₁₆₆(Col V)</td>
<td>thy thr leu thi</td>
<td>Carries colicin E₁</td>
<td></td>
</tr>
<tr>
<td>C₁₄₆₃</td>
<td>his-₅ met G str-₈ rha-₂</td>
<td>—</td>
<td>G. Bertani</td>
</tr>
<tr>
<td>C-M₇</td>
<td>arg str</td>
<td>His⁺ Nif⁺; carries R₁₄₄ᵈʳᵈ₃</td>
<td>Dixon &amp; Postgate (1972)</td>
</tr>
<tr>
<td>E. coli C</td>
<td></td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>C₆₀₃</td>
<td>his arg str</td>
<td>—</td>
<td>G. Bertani</td>
</tr>
<tr>
<td>C₁₄₆₃</td>
<td>his-₅ met G str-₈ rha-₂</td>
<td>—</td>
<td>G. Bertani</td>
</tr>
<tr>
<td>C-M₇</td>
<td>arg str</td>
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<td>G. Bertani</td>
</tr>
<tr>
<td>E. coli c</td>
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</tr>
<tr>
<td>R₁₄₄ᵈʳᵈ₃ ‘AST’</td>
<td>Km Col I</td>
<td>I</td>
<td>E. Meynell</td>
</tr>
<tr>
<td>R₁₄₄ᵈʳᵈ₁₉</td>
<td>Km Am Cm Sm Su</td>
<td>F</td>
<td>E. Meynell</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phage</th>
<th>Source</th>
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<th>Origin</th>
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</thead>
<tbody>
<tr>
<td>Phir</td>
<td>W. Brammar</td>
<td>Phivir</td>
<td>N. Willetts</td>
</tr>
<tr>
<td>Ah¹⁰</td>
<td>N. Murray</td>
<td>ECI</td>
<td>This paper</td>
</tr>
<tr>
<td>φX₁₇₄</td>
<td>C. E. Dowell</td>
<td>If₂</td>
<td>E. Meynell</td>
</tr>
</tbody>
</table>

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;
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K$_3$HPO$_4$, 7.0; KH$_2$PO$_4$, 3.0 g/l H$_2$O; 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, WI166 (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 ($3 \times 10^8$ c.f.u./ml) were washed and resuspended in starvation buffer (Denhardt & Sinsheimer, 1965). Phage φX174 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* 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 R$^{143drd3}$, 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* 59a1 (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 $4 \times 10^6$ 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 4×10⁸ 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-NaEDTA (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-NaEDTA (pH 8.0), 1.25 ml of 1.0 M-tris-HCl (pH 8.0) and 15 ml H₂O. 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–cesium 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-Na₂EDTA, 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 overlayed 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-Na₂EDTA, 0.1 M-NaCl, pH 8.0). The resulting dialysate was used for sucrose gradient centrifugation.

**Preparation of ¹⁴C-labelled colicin E₁ DNA** (D. J. Sherratt, private communication). An overnight culture of *Escherichia coli* CR 34(Col E₁) in M₉ medium, supplemented with Casamino acids (200 µg/ml), thiamine HCl (0.002 µg/ml) and thymine (1 µg/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 × 10⁸ to 2 × 10⁸ c.f.u./ml was reached. Chloramphenicol (100 µg/ml) was then added and incubation continued for 90 min, while cessation of chromosome replication occurred. Thereupon, 0.4 ml of [¹⁴C]thymidine (50 µCi/ml, 53 mCi/µmol) 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
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Table 2. Transfer characteristics of hybrid C-M7 carrying R144дрd3 and R1дрd19

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

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Km</th>
<th>Cm</th>
<th>his</th>
<th>met G</th>
<th>Km</th>
<th>his</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli C-1463</td>
<td>C-M7 (R144дрd3)</td>
<td>3 x 10^{-1}</td>
<td>—</td>
<td>8 x 10^{-7}</td>
<td>&lt; 1 x 10^{-2}</td>
<td>1 x 10^{-2}</td>
<td>&lt; 1 x 10^{-7}</td>
</tr>
<tr>
<td></td>
<td>C-M7 (R144дрd3, R1дрd19)</td>
<td>3 x 10^{-2}</td>
<td>8 x 10^{-7}</td>
<td>&lt; 1 x 10^{-7}</td>
<td>1 x 10^{-7}</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>c603 (R144дрd3)</td>
<td>&gt; 1</td>
<td>—</td>
<td>&lt; 1 x 10^{-8}</td>
<td>&lt; 1 x 10^{-7}</td>
<td>1 x 10^{-7}</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>c603 (R144дрd3) (R1дрd19)</td>
<td>1 x 10^{-1}</td>
<td>1 x 10^{-7}</td>
<td>1 x 10^{-10}</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Swing-out rotor on a Christ swing-out rotor on a Christ 011 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 R144дрd3 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 R144дрd3 (the I-like R factor used to promote the transfer of Klebsiella pneumoniae genes to Escherichia coli). Although R144дрd3 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 R1дрd19 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 R144дрd3 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 R144дрd3 was introduced into hybrid C-M7. Superinfection and elimination of kanamycin resistance and Col I, the determinants characteristic
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 1.73 g/cm$^3$ are of Micrococcus lysodeikticus. (a) DNA from Escherichia coli c603; (b) DNA from hybrid C-M7; (c) DNA from Klebsiella pneumoniae W3. 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 $^3$H-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.
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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 °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 °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 [14C]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

<table>
<thead>
<tr>
<th>Phage</th>
<th>Klebsiella pneumoniae HF3</th>
<th>Escherichia coli c603</th>
<th>Hybrid C-M7</th>
</tr>
</thead>
<tbody>
<tr>
<td>λvir</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>λH*</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>φX174</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>P4vir</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>ECI*</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

* For origin of ECI, 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 φX174 to recipient and hybrid strains

For procedure, see text.

<table>
<thead>
<tr>
<th>Strain</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>% remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli c603</td>
<td>8 × 10^6</td>
<td>1.5 × 10^6</td>
<td>4.8 × 10^6</td>
<td>6</td>
</tr>
<tr>
<td>Hybrid C-M7</td>
<td>6.8 × 10^6</td>
<td>8.2 × 10^6</td>
<td>7.1 × 10^6</td>
<td>100</td>
</tr>
</tbody>
</table>

Phage sensitivity of parental and hybrid strains. Strains of Klebsiella pneumoniae HF3, Escherichia coli c603, E. coli c603 (R144drd3) 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 φX174 did not adsorb to C-M7 (Table 4). Since φX174 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 ECI, 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 ECI to be a long-tailed phage. ECI plated on hybrid C-M7 but both parental strains were resistant. Some strains of Salmonella typhimurium and Klebsiella pneumoniae were sensitive to ECI but all Escherichia coli strains tested were resistant. In several tests, no C-M7 strains lysogenic for ECI were obtained.

Stability of C-M7. Dixon & Postgate (1972) reported that C-M7 showed no tendency to produce Nif^- segregants when subcultured anaerobically on NH_4^-free agar plates. Phage
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ECr 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 NH₄⁺-free agar was 4 x 10⁻⁶; 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, λh₈₀ 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 Rfb⁻·Nif⁻·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 NH₄⁺ 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 NH₄⁺ but only at 30 °C in NH₄⁺-free medium under N₂.

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*<sub>f</sub> values of gluconate-6-phosphate dehydrogenase from various strains

<table>
<thead>
<tr>
<th>Strain</th>
<th><em>R</em>&lt;sub&gt;f&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Klebsiella pneumoniae</em> HF3</td>
<td>0.38</td>
</tr>
<tr>
<td><em>Escherichia coli</em> c603</td>
<td>0.47</td>
</tr>
<tr>
<td>HF3+c603</td>
<td>0.37 and 0.47</td>
</tr>
<tr>
<td>Hybrid c-M7</td>
<td>0.43</td>
</tr>
</tbody>
</table>

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*<sub>f</sub> 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, R<sub>144drd3</sub>. 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 R<sub>144drd3</sub>; the sedimentation coefficient of this R factor remained unchanged in c-M7 and the elimination of R<sub>144drd3</sub> did not result in loss of the His<sup>+</sup>Nif<sup>+</sup> 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 φX174, whereas hybrid C-7 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-7, with an R 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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REFERENCES


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