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Properties of the R factor R144*drd*3 in *Klebsiella pneumoniae* strain M5a1

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SUMMARY

The R factor, R144*drd*3, mobilized chromosomal genes in *Klebsiella pneumoniae* strain M5a1 at similar frequencies to those observed for this R factor in *Escherichia coli* K12. In a derivative *K. pneumoniae* donor, strain HF3, R144 persisted in the autonomous state but now gave rise to polarized transfer of the chromosome in the order O *his*...*arg* 2...*leu*... *trp*. R144*drd*3 was unstable in *K. pneumoniae* M5a1; after successive subculture drug resistance, colicinogeny and transmissibility were lost. In strain HF3, transmissibility was preferentially lost, but determinants for drug resistance, colicinogeny and superinfection immunity were retained; 10–11 copies per chromosome of R144 were present in log phase cultures of both strains.

1. INTRODUCTION

Certain strains of *Klebsiella pneumoniae* are naturally fertile and bring about gene transfer by conjugation (Matsumoto & Tazaki, 1970). The genetic linkage map of *K. pneumoniae*, which resembles that of *Escherichia coli* and *Salmonella typhimurium*, has been described (Matsumoto & Tazaki, 1971). Since we have been unable to detect a naturally occurring sex factor in the nitrogen-fixing strain *K. pneumoniae* M5a1, we have used the R factor R144*drd*3, which belongs to the Iα compatibility group to promote chromosome mobilization in this organism (Dixon & Postgate, 1971). In *Escherichia coli* K12, chromosome transfer mediated by R144 is dependent on the *recA* product (Moody & Hayes, 1972), although integration of this R factor into the chromosome has not been demonstrated, even by the highly selective technique of integrative suppression (Moody & Runge, 1972; Nishimura & Caro, 1973). We have isolated a derivative strain of *K. pneumoniae* carrying R144*drd*3 which gives polarized transfer of the chromosome from a fixed origin; this strain has been used to transfer the histidine (*his*) and nitrogen fixation (*nif*) genes from *Klebsiella* to *E. coli* (Dixon & Postgate, 1972).

In this paper we describe genetic and molecular properties of R144 in our donor strains of *K. pneumoniae*.

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2. MATERIALS AND METHODS

(i) *Bacterial strains, R factors and phage.* Strains employed are described in Table 1. The R factors used were R144*drd*3 (determining resistance to kanamycin (Km) and colicin I (Col I) production) and R64*drd*11 (determining resistance to tetracycline, ampicillin and streptomycin); both are members of the Iα compatibility group (Hedges & Datta, 1973). The I-specific phage employed was If2. Due to the instability of these R factors in *Klebsiella*, donor strains were lyophilized and subcultured as infrequently as possible at room temperature, and always in the presence of the appropriate antibiotics.

Table 1. *Bacterial strains*

Strain	Characteristics	Source or reference
Escherichia coli K12		
2395 (R64-11)	Indicator for phage If2	Bickle & Arber (1969)
W1166 (Col V)	Colicin I indicator	E. Meynell
4K	*leu ser thi str hsp* ($r^-_k m^-_k$)	S. Glover
Klebsiella pneumoniae		
M5a1	Wild-type	P. W. Wilson
UNF9	*nif*-9	Dixon & Postgate (1971)
UNF922	*nif*-9 *his*-2 *ser*-1 *str*-1	IPMS mutagenesis of UNF9
UNF923	*nif*-9 *his*-2 *leu*-2 *str*-1	IPMS mutagenesis of UNF9
UNF925	*nif*-9 *his*-2 *arg*-1 *str*-1	IPMS mutagenesis of UNF9
UNF9231	as UNF923, also *lys*-1	IPMS mutagenesis of UNF9
UNF9232	as UNF9231, also *trp*-1	IPMS mutagenesis of UNF9
EDO31	*thy*-1 *his*-1 *str*-2	S. Primrose
EDO34	*cys*-1 *his*-1 *str*-2	S. Primrose
M5a109B	*his*-1 *str*-2	UV mutagenesis of M5a1
M5a122	*leu*-1 *str*-3	NTG mutagenesis of M5a1

(ii) *Media, mating conditions and phage sensitivity tests.* These have been described previously (Cannon *et al.* 1974).

(iii) *Colicin I production.* This was determined according to the method of Monk & Clowes (1964). The indicator strain was W1166 (Col V).

(iv) *Isolation of auxotrophic mutants.* Strains were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoquanidine (NTG) (Adelberg, Mandel & Chen, 1965) or with isopropyl methane sulphonate (IPMS) (Dixon & Postgate, 1971). Auxotrophs were enriched by penicillin selection (Roth, 1970). The isolation of *nif* mutants has been described previously (Dixon & Postgate, 1971).

(v) *Isolation of strain HF3.* M5a1 (R144*drd*3) was irradiated with ultraviolet light (UV) to give 1% survival, grown overnight in broth and then diluted and plated on nutrient agar to give approximately 200 colonies per plate. The plates were incubated for 16 h at 37 °C and were then replica plated on to a lawn of the recipient strain, spread on the selection medium. Plates were incubated for 48 h; colonies on the master plate which gave large recombinant colonies on the replica were picked and tested for donor ability. Although several auxotrophic recipients

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were tested, only donor clones giving rise to His^+ recombinants at high frequency were detected. One such clone, designated HF3, was used for further study.

(vi) *Interrupted mating on membrane filters*. The procedure of Matney & Achenbach (1962) was followed.

(vii) *Buoyant density characterization of DNA*. Late log phase cultures in 40 ml of nutrient broth were harvested by centrifugation at 0–2 °C and resuspended in 1·25 ml of cold saline EDTA (0·1 M-NaCl, 0·1 M Na_2 EDTA, pH 8·0). This suspension was incubated for 10 min on ice followed by 5 min at 37 °C after adding 0·25 ml of a solution of 10 mg lysozyme/ml in 0·25 M tris-HCl, pH 8·0. It was then transferred to a water bath at 48 °C and cells were lysed by adding 1·5 ml of pre-warmed 4% sodium dodecyl sulphate. After 20 min, 0·5 ml of pronase (Sigma, London) solution (7 mg/ml) was added and incubation continued at 48 °C for 2 h. The lysate was then transferred to ice and the DNA precipitated with 0·54 vol. of cold isopropanol. The DNA was collected on a glass rod, washed with a 6:4 mixture of isopropanol:SSC (0·15 M-NaCl, 0·015 M tri-sodium citrate, pH 7·0) and finally dissolved in *c*. 2·0 ml of SSC. Before storing at 4 °C the DNA solutions were heated at 65 °C for 30 min to inactivate any nucleases which might be present. Samples for centrifugation contained 1·0 ml CsCl (B.D.H. Ltd., Godalming, Surrey, optical grade, density = 1·79 g cm^{-3}), approximately 10 μg of test DNA and 3 μg of *Clostridium perfringens* DNA (Sigma, London) as referent (ρ = 1·691 g cm^{-3}); the final density was adjusted to approximately 1·71 g cm^{-3}. These solutions were centrifuged in an M.S.E. Centriscan 75 analytical ultracentrifuge at 44000 rev/min at 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).

3. RESULTS

(i) *Chromosome transfer mediated by R144*drd*3 in* Klebsiella pneumoniae. The recombination frequencies obtained in matings between M5a1 (R144*drd*3) and various derivatives of M5a1 are given in Table 2. Since the R factor was unstable in this strain (see below), the yield of recombinants in each cross was standardized against the efficiency of conjugation, taken as the frequency of drug resistance transfer. This gave an index of chromosome mobilization for each mating. The mobilization indices for *his* and *leu* were constant in several independent crosses. Recombinants for *trp* occurred at higher frequency than any of the other markers tested (Table 2, Expt 2). An analysis of unselected markers (Table 4*a*) showed that most of the alleles inherited were those of the recipient indicating that transfer occurred from R^+ to R^- cells and that only relatively short fragments of donor genes were inherited by recombinants. The close linkage of *his* and *nif* previously reported (Streicher, Gurney & Valentine, 1971; Dixon & Postgate, 1971) was confirmed.

(ii) *Polarized transfer in strain HF3*. This strain was isolated after UV irradiation of M5a1 (R144*drd*3) and selection for clones which would transfer chromosomal

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markers at high frequency (see Materials and Methods). HF3 had Col I activity, was kanamycin-resistant and was sensitive to I-specific phage If2. With this strain as a donor, mobilization indices for *his* and *nif* were high (0·05–0·22), those for *arg*-2 *cys*-1 and *lys*-1 were intermediate (0·01–0·02) and those for *ser*-1 *leu*-2 and *trp* were low (< 0·002) (Table 3). An analysis of unselected markers inherited by recombinants suggested that HF3 gave polarized transfer of the M5a1 chromosome (Table 4*b*). Amongst recombinants selected for Lys+, 65 out of 115 (57 %) were His+, whereas only 20 out of 175 (18 %) recombinants selected for His+ were Lys+. Similarly, 145 out of 193 (75%) of Arg+ recombinants were His+, whereas only 13 out of 134 (10 %) selected for His+ also inherited Arg+.

Table 2. *Recombination frequencies obtained with M5a1 (R144*drd*3)*

Expt no.	Recipient	*trp*	*his*	*nif*	*lys*	*leu*	Frequency of Km transfer
1	UNF923	—	1×10^{-5} (0·001)	—	—	2×10^{-5} (0·002)	1×10^{-2}
2	UNF9232	5×10^{-5} (0·016)	3×10^{-6} (0·001)	—	2×10^{-6} (0·0007)	6×10^{-6} (0·002)	3×10^{-3}
3	M5a1 O9B	—	1×10^{-5} (0·001)	—	—	—	1×10^{-2}
4	M5a1 22	—	—	—	—	2×10^{-5} (0·002)	1×10^{-2}
5	UNF9	—	—	9×10^{-6} (0·001)	—	—	8×10^{-3}

The numbers signify recombinants per donor organism. Figures in parentheses are the index of chromosome mobilization for each marker determined by the ratio: recombination frequency/Km transfer frequency.

The kinetics of transfer of *his*-2, *arg*-1 and Km in an interrupted mating between HF3 (R144*drd*3) and UNF 925 are shown in Fig. 1. The curve for Km transfer was characteristic of an R factor; the maximum number of Km^R recipients was obtained after 30 min. All Km^R recipients also inherited Col I. The kinetics of chromosome transfer were typical of an Hfr strain, the times of entry of *his*-2 and *arg*-1 were 6 and 25 min respectively.

We conclude from these data that R144*drd*3 persists autonomously in HF3 but also gives rise to polarized transfer of the M5a1 chromosome, *his*-2 being a leading marker with *arg*-1 distal to it. Assuming that the map of *K. pneumoniae* M5a1 is similar to that of *K. pneumoniae* K1-117 (Matsumoto & Tazaki, 1972) and *E. coli* (Taylor & Trotter, 1972), chromosome transfer in HF3 most probably occurs in an anticlockwise direction with the marker order O, *his*-2...*arg*-2...*leu*...*trp*.

The polarized transfer exhibited by HF3 could be due to the presence of an R-prime factor in this strain, analogous to chromosome mobilization promoted by an F′ (Scaife & Gross, 1963). Recombinants resulting from such mobilization should also acquire the R-prime which should be capable of promoting further high frequency chromosome mobilization. HF3 was mated with UNF 922 and His+

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Table 3. *Transfer characteristics of HF3* (*R144*drd3)

Expt no.	Recipient	*trp*	*his*	*nif*	*arg*	*cys*	*lys*	*ser*	*leu*	Frequency of Km transfer
1	M5a109B	—	5×10^{-4} (0·100)	—	—	—	—	—	—	5×10^{-3}
2	M5a122	—	—	—	—	—	—	—	3×10^{-6} (0·001)	3×10^{-3}
3	UNF923	—	8×10^{-5} (0·200)	—	—	—	—	—	6×10^{-7} (0·002)	4×10^{-4}
4	EDO31	—	1×10^{-5} (0·100)	—	—	—	—	—	—	1×10^{-4}
5	EDO34	—	1×10^{-4} (0·200)	—	—	7×10^{-6} (0·014)	—	—	—	5×10^{-4}
6	UNF925	—	4×10^{-5} (0·100)	—	5×10^{-6} (0·015)	—	—	—	—	4×10^{-4}
7	UNF922	—	3×10^{-5} (0·050)	—	—	—	—	1×10^{-6} (0·002)	—	6×10^{-4}
8	UNF9231	—	1×10^{-5} (0·100)	—	—	—	1×10^{-6} (0·010)	—	1×10^{-7} (0·001)	1×10^{-4}
9	UNF9232	4×10^{-6} (0·001)	9×10^{-4} (0·223)	—	—	—	8×10^{-5} (0·020)	—	6×10^{-6} (0·001)	4×10^{-3}
10	UNF9	—	—	3×10^{-4} (0·100)	—	—	—	—	—	3×10^{-3}

The numbers signify recombinants per donor cell. Figures in parentheses are the chromosome mobilization index for each marker determined by the ratio: recombination frequency/Km transfer frequency.

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recombinants were selected. Ten recombinants were purified on the selection medium; all carried R144*drd*3 and transferred *his* and *leu* to UNF923 at low frequency (mobilization indices of about 0·001), characteristic of the mobilization exhibited by M5a1 (R144*drd*3). Hence, polarized transfer in HF3 was a property of this donor and was not readily transmissible to recipients.

Table 4. *Genetic analysis of M5a1 recombinants*

(*a*) Donor: M5a1 (R144*drd*3)

Recipient	No. of recombinants inheriting:				
	trp	*his*	*nif*	*lys*	*leu*
UNF9232	**138**	3	0	0	1
UNF9232	10	**141**	139	16	0
UNF923	—	**88**	82	—	—
UNF27	—	**86**	72	—	—

(*b*) Donor: HF3 (R144*drd*3)

Recipient	No. of recombinants inheriting:					
	trp	*his*	*nif*	*arg*	*lys*	*leu*
UNF9231	—	65	66	—	**115**	0
UNF9231	—	**175**	160	—	29	0
UNF925	—	145	134	**193**	—	—
UNF925	—	**134**	129	13	—	—
UNF9232	**46**	0	2	—	—	—
UNF9232	0	**63**	59	—	—	—
UNF31	—	**127**	119	—	—	—
UNF124	—	**98**	90	—	—	—

The bold figures indicate the selected markers and are the numbers of recombinants tested.
—, Marker not tested.

(iii) *Stability of R144*drd3 *in M5a1.* M5a1 (R144*drd*3) and HF3 were serially subcultured in broth at 37 °C and tested for drug resistance, colicinogeny and sex factor activity. The results are plotted in Figs. 2 and 3. When subcultured in the absence of kanamycin, M5a1 (R144*drd*3) was extremely unstable and lost drug resistance so that by the eleventh subculture (77 generations) only 1 in 10^4 bacteria were drug-resistant. The transferability of the plasmid eventually decreased at a faster rate than loss of drug resistance indicating that sex factor activity could be lost independently of the Km determinant (Fig. 2*a*); the population also lost the ability to support multiplication of phage If2. When M5a1 (R144*drd*3) was subcultured in the presence of Km, all components of the R factor were stable (Fig. 2*b*). HF3 was more stable than M5a1 (R144*drd*3) in the absence of Km and did not segregate Km or Col I (Fig. 3*a*) but the population again lost the ability to transfer drug resistance (Fig. 3*a*, *b*). Both strains also lost the ability to donate chromosomal markers.

The stability of kanamycin resistance and colicinogeny in HF3 prompted us to examine the compatibility properties of populations which had lost sex factor activity. A clone of HF3 which had been subcultured several times at 37 °C and

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which gave no drug resistance transfer or sensitivity to phage If2 was tested for superinfection immunity with R64*drd*11, an R factor of the I compatibility group. The transfer frequency of R64*drd*11 from *E. coli* 2395 to this HF3 derivative was 1×10^{-7} compared with 2×10^{-4} for the cross between 2395 and M5a1 wild type.

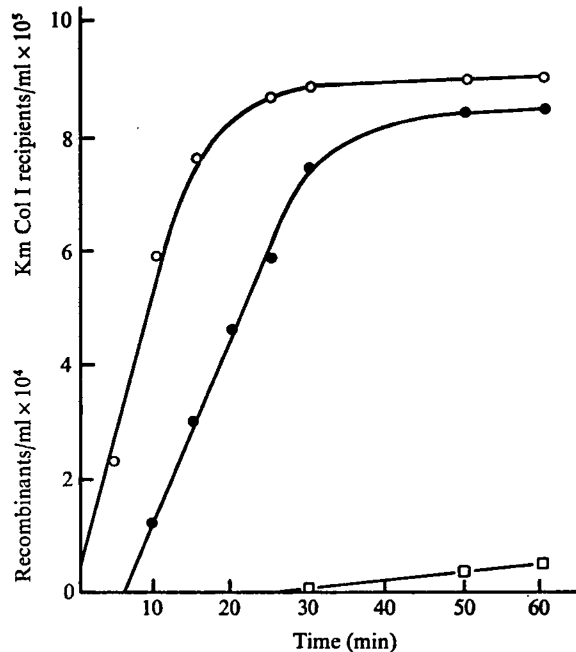

Fig. 1. Interrupted mating between HF3 and UNF925. One ml of donor at 2×10^8/ml and 9 ml of recipient at 5×10^8/ml, chilled to 4 °C, were mixed and 1 ml aliquots of mixture were impinged on membrane filters and washed twice with saline phosphate buffer prior to being transferred to cold minimal agar plates. Mating was initiated by rapidly transferring the membranes to pre-warmed minimal plates at 37 °C. At appropriate time intervals membranes were removed, placed in cold saline phosphate buffer and violently agitated on a 'Microid' shaker prior to plating on selective media. ○—○, Recipients inheriting Km Col I; ●—●, *his*-2 recombinants; □—□, *arg*-1 recombinants. Note change of scale for Km Col I recipients.

Hence, although the derivative of HF3 exhibited no sex-factor activity, it still retained surface exclusion. Kanamycin- and tetracycline-resistant clones of this derivative which rose from the mating with 2395, and which presumably carried both R144 and R64, were purified on nutrient agar containing both antibiotics. One clone was then grown overnight in the absence of drugs and then plated on nutrient agar containing tetracycline. The majority of tetracycline-resistant colonies (96 out of 100) had lost Km resistance and colicinogeny; R144 could therefore be eliminated from the HF3 derivative by superinfection.

After 20 generations, HF3 (R64-11) lost the ability to transfer drug resistance and was insensitive to phage If2, but retained tetracycline, ampicillin and streptomycin resistance. If the HF3 chromosome has specific affinity for R144, re-infection

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with this plasmid should result in the ability to promote high frequency chromosome mobilization. However, several attempts to superinfect HF3 (R64-11) with R144 failed; Km resistance was not transferable to this strain at a detectable frequency.

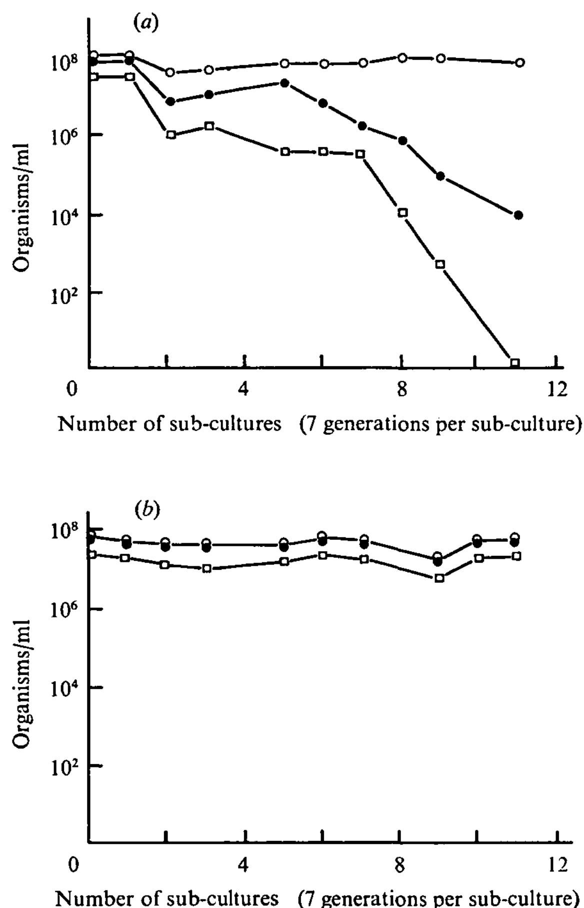

Fig. 2. Stability of R144*drd*3 in *K. pneumoniae* M5a1. M5a1 (R144*drd*3) was serially subcultured without aeration at 37 °C in (*a*) nutrient broth or (*b*) nutrient broth containing 20 μg kanamycin/ml. Each subculture was grown through approximately seven generations and screened for: ○—○, viable count; ●—●, kanamycin-resistant bacteria; □—□, number of *E. coli* K12 4K recipients receiving Km after 1 h mating with 1 ml of the population at 37 °C.

(iv) *Multiple copies of R144 in M5a1 and HF3.* The number of copies per chromosome of R144 in M5a1 and HF3 was estimated from profiles of DNA in analytical CsCl density gradients. A buoyant density of 1·715 g cm^{-3} for *Klebsiella pneumoniae* DNA was confirmed (Cannon *et al.* 1974) and Fig. 4 also shows that a satellite DNA band of buoyant density 1·710 g cm^{-3}, which is characteristic of R144 (Falkow *et al.* 1974), was present in strains M5a1 (R144*drd*3) and HF3 (R144*drd*3). This band, quantified by peak area, equalled 23 % of the main band

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and represented 10–11 copies per chromosome, assuming a molecular weight of 65×10^6 daltons for R144 (Cannon *et al.* 1974) and 3×10^9 daltons for the *Klebsiella* chromosome (which has a 20% larger genome than *E. coli*; S. Falkow, private communication).

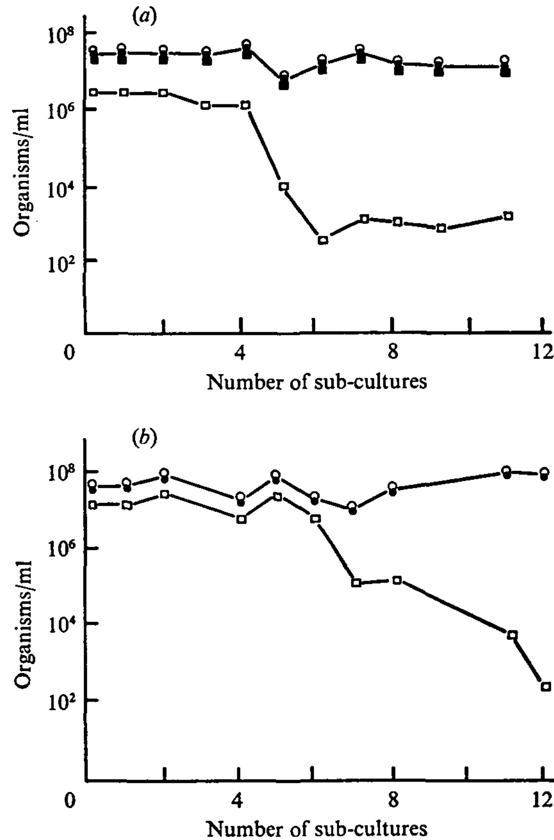

Fig. 3. Stability of R144 in HF3. HF3 was serially subcultured without aeration at 37 °C in (*a*) nutrient broth and (*b*) nutrient broth containing 20 μg kanamycin/ml. Each subculture was grown through about seven generations and screened for: ○—○, viable count; ●—●, kanamycin-resistant bacteria; ■—■, colicinogenic bacteria; □—□, number of *E. coli* K12 4K recipients receiving Km after 1 h mating with 1 ml population at 37 °C.

4. DISCUSSION

The I-like depressed R factor, R144*drd*3 mediated the production of chromosomal recombinants in *K. pneumoniae* at slightly lower frequencies than those observed with this R factor in *E. coli* K12 (Cooke & Meynell, 1969). The recombinant frequencies obtained were similar for several different markers tested, although slightly higher numbers of *trp* recombinants were observed, which parallels the behaviour of R144*drd*3 in K12. However, chromosome mobilization was more efficient in *K. pneumoniae* than in *E. coli* since plasmid transfer occurred

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at much lower frequency; approximately 100-fold less than in *E. coli* (Datta & Hedges, 1972). This increased efficiency of chromosome mobilization could possibly be related to the number of copies of R144 in *K. pneumoniae*. We have observed that there are 10–11 copies of R144 per *Klebsiella* chromosome compared with 1–3 copies for *E. coli* (Falkow *et al.* 1974). The additional copies of R144 in *K. pneumoniae* could increase plasmid-chromosome interactions, giving rise to a higher frequency of chromosome mobilization.

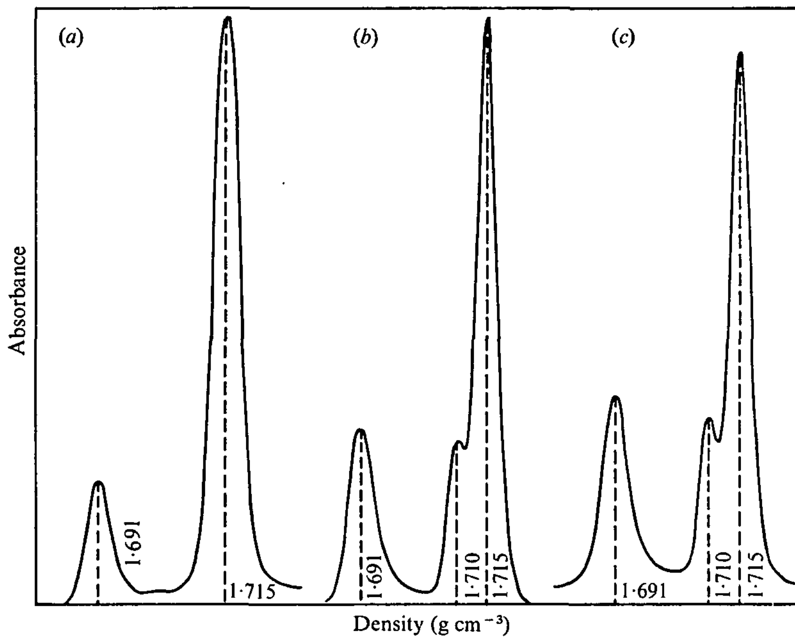

Fig. 4. DNA buoyant density profiles. The analytical caesium chloride density gradient centrifugations were at 44000 rev/min for 16 h. The peaks at density 1·691 g/cm^3 are of *Clostridium perfringens*. (*a*) DNA from *Klebsiella pneumoniae* M5a1. (*b*) DNA from *K. pneumoniae* M5a1 (R144*drd*3). (*c*) DNA from *K. pneumoniae* HF3 (R144*drd*3).

The derivative strain HF3 gave rise to polarized transfer of the *Klebsiella* chromosome in an anticlockwise direction *his* and *nif* being close to the transfer origin; the R factor, however, persisted in the autonomous state. Evidently, in HF3 there must be some specific interaction between the R factor and the chromosome in the *his* region. Since there are multiple copies of R144 in this strain, it is difficult to distinguish whether polarized transfer results from stable integration of a complete plasmid molecule or from transient integration of any one of the many R-factor copies due to increased homology. Superinfection of this strain with an R-factor of the same compatibility group resulted in the loss of kanamycin resistance and colicinogeny, the two known determinants carried on R144*drd*3, suggesting that if integration of the complete R factor molecule had occurred it was not stable.

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It is possible that the HF3 chromosome is unique and that some R144 DNA, perhaps only a short nucleotide sequence, has been integrated into the *Klebsiella* genome in the *his* region. The increased homology between the chromosome and R factor in this region would allow integration of the plasmid and subsequent chromosome mobilization to occur. The Richter ♀3 strain of *E. coli* K12 (Richter, 1961) has similar properties; this strain, which has lost F, retains a sex factor affinity locus close to *met* which allows high frequency mobilization of *met* if an R factor is introduced (Sugino & Hirota, 1962). Although it was possible to displace R144 from HF3 by superinfection with R64*drd*11, it was not possible to demonstrate specific homology between the chromosome and the infecting plasmid since sex factor activity of R64 was readily lost and superinfection of HF3(R64-11) with R144 could not be achieved.

The instability of R144 in *K. pneumoniae* compared with *E. coli* is not a surprising result of a change of host, since host-specific chromosomal determinants most probably influence plasmid stability. The spontaneous loss of Km resistance and colicinogeny from M5a1 (R144*drd*3) is possibly related to the mode of replication of this R factor in *K. pneumoniae*. Drug resistance and colicinogeny did not spontaneously segregate in HF3; these determinants are possibly stabilized by interaction with the host chromosome. However, transmissibility was lost both in the absence and presence of kanamycin. Although after successive subculture HF3 showed neither If2 sensitivity nor drug resistance transfer, it exhibited surface exclusion, indicating that at least some sex factor genes were still present. The loss of transferability of R144 in M5a1 and HF3 could be due to the occurrence of spontaneous phage-resistant mutants in the population. Such mutants, which do not produce sex-pili, would possibly have a selective advantage in *K. pneumoniae*. Comparable phage-resistant mutants occur spontaneously in stationary phase cultures of *E. coli* carrying R144*drd*3 (Salisbury, Hedges & Datta, 1972).

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