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Behavior of a Hybrid F'$_{ts114}$ *lac*$^+$, *his*$^+$ Factor (F42-400) in *Klebsiella pneumoniae* M5a1

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Episome F'$_{ts114}$ *lac*$^+$, *his*$^+$ (F42-400) was transferred from *Salmonella typhimurium* to *Klebsiella pneumoniae*. From the progeny, a strain of *K. pneumoniae* able to retransfer the episome was obtained. The His$^+$ phenotype in this strain is temperature sensitive. *Escherichia coli* female-specific phages φII, W31, and T3 were shown to plate on *K. pneumoniae*. From φII we obtained two derivatives; φIIK, which plates only on *K. pneumoniae*, and φIIE, which plates only on *E. coli*. Growth of phages T3 and φIIK was inhibited by F42-400 in *K. pneumoniae*. Growth in presence of acridine orange in a defined medium at 40 C resulted in a high level of curing. The frequency of His$^+$ cells after growth in acridine orange at 40 C was 0.001%. An extensive search to detect chromosome mobilization by F42-400 in *K. pneumoniae*, under different experimental conditions, was negative. We cannot exclude the possibility that the low transfer efficiencies prevented our detection of chromosome mobilization. A search among temperature-resistant, acridine orange-curing-resistant, or galactose-resistant derivatives of the *K. pneumoniae* donor strain failed to reveal any chromosome transfer. Our failure to detect Hfr's may be a result of: (i) the peculiarity of episome F42-400, (ii) the peculiarity of *K. pneumoniae* chromosome, or (iii) low transfer efficiency. *K. pneumoniae*-modified F42-400 and phage 424 were restricted by *E. coli* K-12. *E. coli* K-12-modified episome F42-400 and phage 424 were restricted by *K. pneumoniae*. *E. coli* C failed to restrict F42-400 modified with *K. pneumoniae* specificity. The ability of *K. pneumoniae* to accept F42-400 is less, by about a factor of 50, than that of *E. coli* C. As an explanation for the differences in the behavior of *E. coli* C and *K. pneumoniae* in ability to receive F42-400 it was suggested that recipient bacteria have specific sites for interaction with the F-pilus tip; these are present in *E. coli* C, leading to high transfer efficiency, whereas they may not be present (or if present, are not accessible) in *K. pneumoniae*, leading to low transfer efficiency.

Klebsiella pneumoniae M5a1 is one of the first *Enterobacteriaceae* strains isolated which is capable of converting atmospheric nitrogen to ammonia (43, 65). *K. aerogenes* and *K. pneumoniae* M5a1 strains possess the ability to use histidine or proline as a nitrogen source even in the presence of glucose (83; unpublished data). This is unlike the situation in *Salmonella typhimurium*, where utilization of histidine as a nitrogen source is subjected to catabolite repression in glucose media (83). It is desirable to have a genetic system to study the unique properties of *Klebsiella*.

Three different means of genetic transfer already exist in *Klebsiella*. *E. coli* generalized transducing phage P1 has been shown to mediate generalized transduction in *K. pneumoniae* M5a1 (100). Phage PW52 is known to mediate generalized transduction in *K. aerogenes* (63, 83). Methods are available to extend the host range of phage P1, and this technique has been employed in isolating P1-sensitive strains from P1-insensitive strains of *Klebsiella* (41, 101). Resistance transfer factors belonging to the I type (R144), when present in *K. pneumoniae* M5a1, can transfer chromosomal markers, at a low frequency, to other *K. pneumoniae* strains (33) or even to *E. coli* C (34). A strain of *K. pneumoniae* that is unable to fix nitrogen (33) is able to transfer chromosomal markers to other *K. pneumoniae* strains (67, 68) or to *Enterobacter aerogenes* (66). The nature of the sex factor in this strain is not known.

We are interested in the generation of *K. pneumoniae* M5a1 donors that can produce recombinants at high frequencies when mated

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with suitable recipients (similar to that produced by *E. coli* Hfr's). Several different kinds of episomes with sex factor activity, e.g., F, ColV, and R, are able to generate Hfr strains in *E. coli* (22, 40, 51, 53, 77, 79, 80, 118). R factors have wider host range than F factors (26), and R factors belonging to the F or I type are capable of chromosome mobilization (20, 33, 70). Stable integration of R factors giving rise to clones that can transfer chromosomal markers at high frequency is not common (71). However, under special selective conditions (integrative suppression of chromosome replication), F-type R factors, but not I-type R factors, have been found to integrate into the *E. coli* chromosome, generating Hfr strains (40, 77, 79).

Interaction of the F factor with the *E. coli* chromosome to generate Hfr derivatives and the generation of F' factors from Hfr strains have been studied extensively (14, 22, 61, 94). Mutants of the F' factor (e.g., F'$_{ts114}$ *lac*$^+$ = F42-114) have been isolated which can replicate and/or segregate (1) normally at 30 C but not at 42 C (25). When *E. coli lac*$^-$ cells carrying F42-114 are plated on indicator media at 42 C, majority of the colonies that come up are Lac$^-$ (50). However, a small proportion of Lac$^+$ clones does come up, and some of these have their F' integrated into the chromosome, resulting in Hfr formation (10). This method of integrative suppression of F' replication has been used successfully to isolate Hfr strains in which the F factor is integrated in *tonA* or *tonB* (10, 42), *tsx* (10), *gal* (48), or *bfe* (54) loci.

F factors from *E. coli* can be transferred to different bacterial genera: *Salmonella* (92), *Proteus* (36), *Pasteurella* (56, 57), *Erwinia* (17, 18), *Citrobacter* (30, 31), *Klebsiella* (12, 87), and *Rhizobium* (26). The F' factor has been reported to mobilize chromosomal markers in *S. typhimurium* (64, 92), *Pasteurella* (56), and *Erwinia* (17). With all of the above three genera it has been possible to obtain Hfr derivatives (18, 57, 92). Although F'-carrying *C. freundii* and *Klebsiella* can retransfer the F' factor, there has been no evidence for chromosome mobilization (30, 87). This probably indicates the absence or a low level of F-chromosome interaction in *C. freundii* and *Klebsiella*. Integrative suppression of F' replication offers a means by which selection pressure could be exerted to isolate clones where the F' factor has integrated into the chromosome. This method has been used in an attempt to isolate Hfr strains in *S. typhimurium* and in *C. freundii*, but without success (31, 64).

In the accompanying paper we describe the isolation and partial characterization of a hybrid F' factor carrying *ts114*, *lac*$^+$, and *his*$^+$ markers (F42-400) (84). While those studies were in progress we transferred F42-400 to *K. pneumoniae* M5a1. *K. pneumoniae* carrying F42-400 is capable of retransferring F42-400 to *K. pneumoniae* and *E. coli* strains. However, we have been unable to detect either chromosome mobilization or Hfr formation. During our studies we observed that *E. coli* C is a better recipient of F42-400 than *K. pneumoniae*. Our experiences with this system are described below.

MATERIALS AND METHODS

Strains. Bacterial strains and bacteriophage strains used in this study are listed in Tables 1 and 2.

Media. The liquid media used were: M9, T7.8, nutrient broth, and Luria broth.

The solid media used were M9 plates, enriched M9 plates, nutrient agar plates, tryptone yeast extract agar plates, eosin methylene blue plates, and tetrazolium plates. All of these are described in the accompanying paper (84). In addition, the following minimal media were used: C (99) and E (106). Carbon sources were used at 0.4% and amino acid supplements were used at 20 μg/ml; nicotinic acid (1 μg/ml), thiamine (1 μg/ml), and biotin (0.1 μg/ml) were added where necessary.

For scoring the Hut$^+$ phenotype (ability to use histidine as a nitrogen source), ammonium chloride in M9 medium was replaced by histidine and 0.4% sodium succinate was used as a carbon source. λ broth contained (per liter): tryptone (Difco), 10 g; sodium chloride, 5 g; maltose, 2 g; and magnesium sulfate, 2 g. λ soft agar is λ broth solidified with 0.6% Difco agar. Where necessary, $CaCl_2$ and $MgSO_4$ were added to the Luria broth to a final concentration of 5 and 10 mM, respectively. Phage lysates were stored in TMG buffer [10 mM $MgSO_4$, 10 mM tris(hydroxymethyl)-aminomethane-hydrochloride (pH 7.4), and 0.01% gelatin]. Streptomycin and nalidixic acid were made as described previously (84).

Culture conditions. Cultures were grown, essentially as described before (84), at 25, 37, or 40 C. For growing *Klebsiella* under selective growth conditions (84), we always used sucrose as the carbon source.

Phage assays and phage stocks. Phage assays were done by the soft-agar overlay technique (3) by plating an appropriate volume of the phage dilution with indicator bacteria in exponential or early stationary phase ($CaCl_2$ and/or $MgSO_4$ were added where necessary) on tryptone yeast extract agar plates. For plating phage 424, bacteria were grown in broth to early stationary phase, and the cells were washed and suspended in 0.5 volume of 10 mM $MgSO_4$. A 0.1-ml volume of phage suspension was mixed with 0.2 ml of the bacterial suspension, and adsorption was allowed to occur for 20 min at 25 C before the mixture was plated on tryptone yeast extract agar plates with λ soft agar. Plates were incubated at 25, 37, or 40 C. Phage stocks were made by the plate method (3) and stored either in Luria broth or in TMG at 4 C.

Lysates of φII, obtained from J. Beckwith and from

TABLE 1. *Bacterial strains*

Strain no.	Genotype/phenotype	Synonyms	Source and/or references
Klebsiella pneumoniae M5a1			
BK1	Wild type		J. R. Postgate; reference 33
BK3	*leu-2, trp-1, nif-9232, his-2, lys-1, str*^{R}		J. R. Postgate
BK5	Wild type		R. C. Valentine (100)
BK6	*hisD4*		R. C. Valentine (100)
BK32	F42-400 (F'$_{ts114}$ *lac*$^+$, *his*$^+$)/BK6[a]		BS217 + BK6 → His$^+$ selection[b]
BK38	F42-400/*hisD4*, Gal^S		Nitrosoguanidine mutagenesis of BK32
BK41	*hisΔ30*		K. Shanmugam (97)
BK63	*hisD4*, Gal^S		F$^-$ clone from BK38
BK66	*leu-2, (bio-hut)Δ 165, trp-1, nif-9232, his-2, lys-1, str*^{R}		Spontaneous Chl^R from BK3
BK69	*(nic-uvrB) Δ 154, hisD4, str*^{R}		Spontaneous Gal^R Chl^R from BK63, made streptomycin resistant
Escherichia coli C BE48	*thr-5, leu-5, his, xan, lac*^{c}	C1055	M. Sunshine; reference 115
Escherichia coli K-12 BE18	*gal, met,* $r_K^- m_K^-$ F$^+$	993	W. Arber
BE39	*his Δ 750, str*^{R}	SB1801	P. E. Hartman (39)
BE42	*his Δ 3153,* (*lac-pro*)$_{XIII}$, *thi*	SB2201	P. E. Hartman; reference 107
BE72	*thi, thr, leu, lac*	C600	F. Jacob; reference 6
BE76	*his Δ 3153,* (*lac-pro*)$_{XIII}$, *thi, nalA*		Ethyl methane sulfonate mutagenesis of BE42
BE77	F42-400/BE76		BS217 + BE76 → His$^+$ selection
Salmonella typhimurium LT2			
BS217	F42-400/(*his-gnd-rfb*)*Δ515, met*		Reference 84
BS231	F57 (F' *his*$^+$)/*his, ser*	TA1	B. Ames

[a] F-prime number/host strain number (61).

[b] Donor (BS217) mated with (+) the recipient (BK6), and progeny was selected for (→) histidine independence (His$^+$).

[c] In our laboratory we have observed this strain to be lactose negative.

G. Meynell, gave plaques when plated on strain BK6. They were plaque purified and grown on strain BK6. At that time it was observed that these phages (called φIIK) would not grow on *E. coli* BE42. Plaque-purified φII grown on strain BE42 (called φIIE) did not grow on *K. pneumoniae* BK6. This may be similar to the alterations in host range of φII observed by Monner and Boman (73, 74). Plaque-purified T3 did not exhibit this peculiarity. For experiments described in Table 4, φII obtained from J. Beckwith and T3 obtained from G. Meynell were used (114). Phages that were plaque purified and grown on strain BK6 were used for platings on *K. pneumoniae*. Phages that were plaque purified and grown on strain BE42 were used for platings on *E. coli*.

Bacterial mating. Replica mating, spot mating, plate mating, filter mating, and liquid mating were done as described previously (84). Wherever *K. pneumoniae* strains were used as recipients, sucrose was used as the carbon source.

Phage sensitivity tests. Phage sensitivity tests were done by the cross-streak method (102).

Mutagenesis of bacteria. *N'*-methyl-*N*-nitro-*N*-nitrosoguanidine and ethyl methane sulfonate were used as described previously (72, 90).

Isolation of strain BK32. *S. typhimurium* BS217 was filter mated with strain BK6 for 2 h at 25 C. The mating mixture was plated on sucrose plates and incubated at 25 C for 3 days. The presence of sucrose and absence of methionine counterselected the donor strain, and the absence of histidine selected for His$^+$ progeny. His$^+$ progeny appeared at a frequency of 7.3×10^{-6} (700 times the unmated control value). One hundred clones were purified and checked for growth on sucrose plates with and without histidine at 25 and 40 C. All 100 clones grew on sucrose plates supplemented with histidine either at 25 or 40 C, on sucrose plates at 25 C, but not on sucrose plates at 40 C, i.e., they had a temperature-sensitive His$^+$ phenotype. This kind of temperature sensitivity is expected if the His$^+$ progeny have acquired the episome F42-400. In addition, all of the 100 clones, like the parental strain BK6, were sensitive to *K. pneumoniae* phage TW3 but not to *S. typhimurium* phage P22. Donor ability

TABLE 2. *Bacteriophage strains*

Strain	Source and/or references
TW3	R. C. Valentine
MS2	R. S. Gupta
f2	C. V. S. Raj
fd	W. Arber
ϕII	J. Beckwith; reference 24
ϕII	G. Meynell (114)
T3	G. Meynell (114)
W31	G. Meynell (114)
T7	G. Meynell (114)
T1	P. E. Hartman
P22	M. Levine (59)
424 *CIts*[a]	S. Kumar; reference 51

[a] Temperature inducible.

of these 100 clones was checked by replica mating at 25 C. Even though the replica mating did not give satisfactory results, one clone was isolated that produced His$^+$(Str^R) progeny when mated with strain BK3. By the above criteria this strain (BK32) was identified as *K. pneumoniae* M5a1 carrying the episome F42-400. Strain BK32 is Scr$^+$, TW3^S, P22^R. When 10^4 cells per ml were grown at 25 C in nutrient broth to 10^8 cells per ml, His$^+$ cells appeared at a frequency of 8×10^{-1}. When a similar experiment was done at 40 C, His$^+$ cells appeared at a frequency of 1.3×10^{-4}. The segregants that failed to grow on sucrose plates grew on sucrose plates supplemented with histidine, i.e., the segregants are His$^-$. From this we conclude that growth at 40 C in nutrient broth leads to an efficient conversion of His$^+$ to the His$^-$ phenotype. Acridine orange (AO) is known to cure F factors (45). We have shown that F42-400 can be cured in a defined medium, T7.8, in *S. typhimurium* (84). When strain BK32 was grown in presence of 5 μg of AO per ml, the frequency of His$^-$ cells increased from 0.1 to 75%. Efficiency of plating of strain BK32 (all comparisons were made with the titer on sucrose plates incubated at 25 C) on histidine-supplemented sucrose plates at 40 C was 4×10^{-1}, whereas on sucrose plates (without histidine) at 40 C it dropped to 3×10^{-4}.

Isolation of strain BK38. Strain BK32 was mutagenized with nitrosoguanidine, segregated in sucrose medium at 25 C, and plated for single colonies on sucrose plates at 25 C. Galactose sensitivity was identified by replicating the colonies onto tetrazolium-galactose plates. One clone, sensitive to galactose in defined sucrose media, was purified and checked for its donor ability (BK38). When strain BK38 was plated on glucose-galactose plates, the efficiency of plating was 4.7×10^{-1}, whereas on sucrose-galactose or succinate-galactose plates it decreased to 3×10^{-5}. When strain BK38 was grown in nutrient broth at 40 C, starting from an inoculum of 10^8 cells per ml to about 10^9 cells per ml, the frequency of His$^-$ cells was greater than 98%. When strain BK38 was grown in T7.8 with 5 μg of AO per ml at 25 C, starting from an inoculum of 10^8 cells per ml to about 10^9 cells per ml, the frequency of His$^-$ cells was 10%. Strain BK38 when mated with strain BK3 produced His$^+$ (Str^R) progeny. Strain BK38 is Scr$^+$, TW3^S, P22^S like its predecessor BK32.

Curing. Temperature-curing and AO curing in broth and in T7.8 minimal medium were done as described previously (84).

Nomenclature. Recommendations of Demerec et al. (32) were followed throughout. Standard abbreviations were adopted (91, 103). Sucrose was abbreviated as Scr. The nomenclature of Arber (7, 8) was followed for restriction and modification. Phage 424 grown on *K. pneumoniae* M5a1 was abbreviated as 424.M5a1.

RESULTS

Donor ability of strain BK32 and BK38. Data on donor ability are presented in Table 3. Transfer efficiency is low because the matings were done at 25 C (116). The progeny were checked for temperature sensitivity of the His$^+$ phenotype and segregation of His$^-$ cells. By these criteria, strains BK32 and BK38 were shown to carry the F'$_{ts114}$ *his* episome and to transfer this to other *K. pneumoniae* strains. His$^+$ progeny obtained by mating strain BK38 with strain BE48 became Lac$^+$, and this phenotype was temperature sensitive. This suggests that the episome F'$_{ts114}$ *lac*, *his* is carried by strain BK38.

Plating efficiency of sex-specific phages. The F factor is responsible for the production of sex pilus, and several phages (f1, fd, f2, MS2, and R17) specifically adsorb to sex pilus, leading to productive infection (13, 16, 21). These phages have been used as probes for the presence of sex pilus in *E. coli* (1) and *S. typhimurium* (92). Spot tests of phages f2 and fd on strain BK32 at 25 C did not indicate any sign of lysis. When MS2 phage was plated on strain BK32 at 25 C, no plaques appeared. When the same MS2 lysate was plated on *E. coli* HfrH under identical plating conditions, the titer was 10^{11} plaque-forming units per ml. We are aware that these experiments are inconclusive.

We have found that *E. coli* female-specific phages ϕII (24), W31 (109), and T3 (96), but not T7, plate on *K. pneumoniae* M5a1. Plating behavior of ϕIIK, a derivative of ϕII, and T3 on *K. pneumoniae* donors (Table 4) indicates a reduction in efficiency of plating associated with faint, irregular-shaped plaques, compared with that of the females. Similar differences in

TABLE 3. *Donor ability of strains BK32 and BK38*[a]

Donor	Recipient	Frequency of His$^+$(Str^R) progeny/10^8 donor cells
BK32	BK3	9.4
BK38	BK3	20.0

[a] Uninterrupted matings were done at 25 C for 3 h with exponential-phase donor cells and early-stationary-phase recipient cells in a 1:5 proportion.

TABLE 4. *Efficiency of plating of female-specific phages*[a]

Bacterial strain	Sex factor status	Efficiency of plating of phage:		
		T3	φIIK	φIIE
BK6	F⁻	1	1	$<10^{-9}$
BK32	F′	1.5×10^{-1}	6.5×10^{-1}	$<10^{-9}$
BK38	F′	1.0×10^{-1}	3.6×10^{-1}	$<10^{-9}$
BE42	F⁻	1	$<10^{-9}$	1
BE77	F′	4.4×10^{-1}	$<10^{-9}$	2.4×10^{-1}

[a] Indicator bacteria were grown in minimally defined media at 25 C to exponential phase. About 10^7 cells were used for plating the phage on nutrient agar plates. The plates were incubated at 25 C for 2 days before they were counted.

plating efficiency and plaque morphology were observed when φIIE, a derivative of φII, and T3 were plated on an *E. coli* strain carrying F42-400. Data in Table 4 also indicate that φIIK is unable to form plaques on *E. coli* and φIIE is unable to form plaques on *K. pneumoniae*.

Transfer efficiency at 40 C. Efficiency of plating of *K. pneumoniae* on a completely defined medium is decreased to $\sim 10^{-2}$ if the platings are done at 42 C, whereas at 40 C the efficiency of plating is 5×10^{-1}. For this reason, we chose 40 C as the nonpermissive temperature in our experiments. F42-400 does not replicate at 40 C (see Table 6), although it is likely that the severity of inhibition of replication at 40 C may be less than that at 42 C.

Transfer efficiencies were studied both in *K. pneumoniae* and *E. coli*. When we mixed donors grown at 25 C with recipients grown at 37 C and incubated the mating mixture at 40 C, we observed a gradual increase in the $His^+(Str^R)$ progeny frequency (Table 5). In both cases, the frequency of $His^+(Str^R)$ progeny was increased by about 40-fold when matings were done at 40 C instead of at 25 C. During the period of mating, there was no increase in the donor or recipient titer. Transfer replication itself would not have been inhibited at 40 C, for even at 42 C the *ts114* mutation does not inhibit transfer replication (112). At temperatures of 30 C or below, F pili are not formed well, and this may account for the decreased fertility (81, 116). The slow increase in $His^+(Str^R)$ progeny may owe its origins to the synthesis of F pili on shifting the cells from 25 to 40 C. Maximum transfer efficiency of F42-400 observed in *K. pneumoniae* × *K. pneumoniae* crosses was 10^{-3} and in *E. coli* × *E. coli* crosses was 3×10^{-2}.

Attempts to increase the transfer efficiency in K. pneumoniae-K. pneumoniae matings. The above experiments suggested that matings in *K. pneumoniae* are not as efficient as in *E. coli*. Since this could obscure any chromosome mobilization that may occur and prevent detection of any Hfr's that might be formed, several different approaches were tried to determine whether the transfer efficiency could be increased to the level found in *E. coli*.

TABLE 5. *Fertility of matings at 25 and 40 C*[a]

Donor	Recipient	Mating conditions		Frequency of $His^+(Str^R)$ progeny/10^8 donor cells
		Duration (h)	Temp (C)	
BK38	BK3	3	25	18
BK38	BK3	1	40	23
BK38	BK3	2	40	310
BK38	BK3	3	40	750
BK38	BK3	4	40	1,000
BK38	BK3	5	40	340
BE77	BE39	3	25	480
BE77	BE39	1	40	700
BE77	BE39	2	40	10,000
BE77	BE39	3	40	16,000
BE77	BE39	4	40	26,000
BE77	BE39	5	40	22,000

[a] Uninterrupted matings were done as described in the text, for different time periods.

Several media were used to grow donor and recipient strains: M9, C, and E; a medium with and without yeast extract and Casamino Acids; nutrient broth; and Luria broth. Strains were also grown by the plate method (67). Mating was done at 25 or 40 C. None of the above methods improved transfer efficiency.

Strain BK38 was mutagenized with nitrosoguanidine and segregated, and about 10,000 clones were examined for increased donor ability. None was found. *Klebsiella* strains are known to harbor a variety of plasmids (12, 19, 27, 44, 49, 85–87). There is no evidence for extrachrosomal deoxyribonucleic acid (DNA) in *K. pneumoniae* M5a1 (15), and our strains are not resistant to 10 μg of chloramphenicol per ml. However, we cannot completely eliminate the possibility that *K. pneumoniae* M5a1 harbors a plasmid.

If *K. pneumoniae* were to harbor a plasmid that is surface exclusion positive (Sex$^+$) (58) with regard to F, it would result in a low transfer efficiency. Stationary-phase cells of *E. coli* carrying Sex$^+$ plasmids are phenotypically Sex$^-$ (113). Strain BK3 grown to exponential phase at 37 C or for 16, 24, and 48 h at 40 C were used in matings with donor strain BK38. His$^+$(Str^R) progeny appeared in all the cases at a frequency of 1.2×10^{-4} to 3.6×10^{-4}. Cells grown to stationary phase did not have increased recipient ability when compared with exponential cells.

If *K. pneumoniae* were to harbor a plasmid that is not only Sex$^+$ but also incompatible with F (1), then F$^-$ segregants from *K. pneumoniae* carrying episome F42-400 would likely to have increased recipient abilities. Strain BK38 was cured of its episome, and a series of Gal^R Chl^R clones was isolated. One of them was made resistant to streptomycin (strain BK69) and another to nalidixic acid. These strains were used as recipients in a mating with donor strain BK38, selecting for His$^+$(Str^R) or His$^+$(Nal^R) progeny. The progeny appeared at a frequency of 3.6×10^{-4} to 4.4×10^{-4}. A control mating with strain BK3 gave a frequency of 10^{-4}. These differences are small, and we conclude that cured clones from strain BK38 do not have better recipient abilities.

Strain BK66, a derivative of strain BK3 with similar recipient ability, was put through sodium dodecyl sulfate curing conditions (2). Cells growing in a medium with a 10% sodium dodecyl sulfate concentration were washed free of sodium dodecyl sulfate and mated with strain BK38 selecting for His$^+$(Str^R) progeny. Progeny were streaked on tryptone yeast extract agar plates at 40 C. One hundred cured clones were isolated and mated in liquid with strain BK38 selecting again for His$^+$(Str^R) progeny. A small variation in the transfer efficiency was observed. Clones giving rise to a higher number of progeny were selected. Even after two cycles of F42-400 transfer and elimination, no significant improvement in the transfer efficiency was observed.

Replica mating of strain BK38 with strain BK3 or BK66 at 25 C, with selections for His$^+$(Str^R) progeny, does not produce recombinant patches. The situation remains the same even if the replicated plates are immediately incubated at 40 C for 4 h and later shifted to 25 C. About 1,000 mutagenized clones from strain BK66 were checked to see if we could detect recombinant patches, on replication to strain BK38. None produced recombinant patches. All the experiments done to increase the transfer efficiency in *K. pneumoniae* × *K. pneumoniae* matings were unsuccessful.

Chromosome mobilization. In the accompanying paper we show that F42-400 is capable of mobilizing the *S. typhimurium* chromosome (84). Does it mobilize chromosomal markers from *K. pneumoniae*? When strain BK38 was mated with strain BK3 for 3 h, His$^+$(Str^R) progeny appeared at a frequency of 8.6×10^{-4}. Under the same conditions there was no increase of Leu$^+$(Str^R) or Trp$^+$(Str^R) progeny over the control value (2×10^{-7}). The lysine marker in the strain reverts at a relatively high frequency (6×10^{-6}), making it an unsuitable marker for these studies. We conclude that, if chromosome mobilization of *trp* or *leu* markers occurs, then its frequency must be less than 10^{-3} of that of the episome transfer itself.

Irradiation with ultraviolet light increases chromosome mobilization in *E. coli* (35). Similar experiments were done in matings between strains BK38 and BK3 under various conditions: 3.7×10^{-1} to 4×10^{-3} survival; matings at 25 or 40 C for a period of 3 to 4 h. Under none of the experimental conditions used could we detect any mobilization of the *trp* or *leu* marker.

Curing. In our earlier experiments on curing in *S. typhimurium*, we used T7.8 medium (84). It was observed that *K. pneumoniae* grows in this medium at 25 C but not at 40 C. *K. pneumoniae* grows in M9 medium (pH 7.0) or in nutrient broth adjusted to pH 7.8 at 40 C. Addition of thiamine, threonine, and glutamic acid to T7.8 medium allowed the cells to grow at 40 C. It would appear that some biosynthetic pathway (thiamine, threonine, or glutamic acid) becomes temperature sensitive in *K. pneumoniae* at pH 7.8 but not at pH 7.0. We have not investigated this further. In all of our curing experiments with *K. pneumoniae* in T7.8 medium we routinely supplemented the medium with thiamine, threonine, glutamic acid, and methionine (89, 93).

Growth of strain BK38 in T7.8 medium at 40 C with AO leads to very low levels of His$^+$ cells. The majority of the cells are His$^-$ as a result of curing. Data in Table 6 show that at 25 C in the absence of AO the episome is stable. Growth in the presence of 5 μg of AO per ml at 25 C decreased the frequency of His$^+$ cells to about 2%. When cells were grown at 40 C without AO, the frequency of His$^+$ cells decreased to less than 1%. Growth of cells at 40 C with 10 μg of AO per ml decreased the frequency of His$^+$ cells to 0.001%. Curing is not as efficient as in *S. typhimurium*, where under similar conditions of AO treatment the frequency of His$^+$ cells dropped to 0.0001% (84). We equate the loss of

TABLE 6. *Curing in strain BK38*[a]

AO (μg/ml)	Incubation temp (C)	Duration of incubation (h)	Total CFU/ml	Fraction of CFU that are His$^+$	His$^+$ phenotype of picked colonies
0	25	48	5.0×10^8	1.0×10^0	200/200
0	25	72	9.6×10^8	9.8×10^{-1}	97/97
5	25	72	8.2×10^8	1.7×10^{-2}	1/99
0	40	48	4.0×10^8	7.5×10^{-3}	0/38
10	40	48	7.2×10^6	1.5×10^{-5}	0/98
10	40	72	1.0×10^7	6.0×10^{-6}	0/100

[a] T7.8 sucrose medium supplemented with histidine was used for curing at 25 C. For incubations at 40 C, the above medium contained in addition: methionine, thiamine, threonine, and glutamic acid (89, 93). Other details have been described previously (84). Colonies from sucrose plates supplemented with histidine were picked to test for the His$^+$ phenotype. CFU, colony-forming units.

the His$^+$ phenotype with the loss of the episome F42-400 and conclude that F42-400 is lost efficiently from the cells grown at 40 C in the presence of AO. These curing conditions were used in the search for "Hfr" strains.

Search for Hfr strains. In the accompanying paper we describe experiments in which F42-400 was successfully used to obtain Hfr's in *S. typhimurium* under special conditions. We isolated a large number of temperature-resistant clones, with and without ultraviolet light treatment, and with and without AO curing treatment, from strains BK32 and BK38. A total of about 15,000 clones was checked to determine whether they would produce Trp$^+$(Str^R) progeny on replica mating with strain BK3 or BK66. One hundred presumtive donors were tested further in liquid matings with strain BK66. No donors were found.

Strain BK38 is sensitive to galactose. In *E. coli*, *galE* mutants accumulate galactose-phosphate, which is lethal to the cell (38, 117). Galactose-resistant mutants isolated from *galE* strains contain a class in which inactivation of *galT* prevented the accumulation of galactose-phosphate (4). This general philosophy had been used to integrate F42-114 or λ into the *gal* operon of *E. coli* (48, 98). If the galactose sensitivity of strain BK38 is similar to that of *E. coli galE* mutants, it may be possible to isolate Gal^R clones from strain BK38 that are similar to those isolated by Ippen et al. (48) in *E. coli*. We have isolated Gal^R derivatives of strain BK38 at 25 and 40 C with and without AO treatment. About 100 clones were checked to determine whether they would produce Leu$^+$(Str^R) or Trp$^+$(Str^R) progeny by liquid mating with strain BK3. No donors were found. Interpretation of this is made difficult because nothing is known about the nature of galactose sensitivity in strain BK38 and the ability of F42-400 to integrate in the *gal* region of *E. coli* or *S. typhimurium*.

Restriction between K. pneumoniae and E. coli. If DNA from one bacterial strain is introduced into another related bacterial strain, it is likely to get destroyed by restriction nucleases. This would result in a decreased number of recombinants in heterospecific crosses (9). It has been assumed that *E. coli* K-12, but not *E. coli* C, would restrict DNA from *K. pneumoniae* (34). We present evidence that this is indeed the case. The data on heterogeneric transfers of F42-400 are presented in Table 7. There was no difference in the His$^+$(Str^R) progeny in 25 C matings between *K. pneumoniae* donors and *K. pneumoniae* or *E. coli* C recipients (rows 1, 2, 3, and 5). When the recipient was *E. coli* K-12, the His$^+$(Str^R) progeny was reduced to 3% of that obtained with *K. pneumoniae* (compare row 4 with 3). When the same matings were done at 40 C, a different situation was revealed. As expected, the His$^+$(Str^R) progeny in *K. pneumoniae* matings was increased in 40 C matings compared with that of the 25 C matings by about 45-fold (row 3). However, the temperature effect on transfer efficiency was not uniform with all the matings studied; for *K. pneumoniae-E. coli* K-12 it was about 200-fold (row 4) and for *K. pneumoniae-E. coli* C it was about 1,000-fold (row 5). When the recipient was a K-12 strain and mating was done at 40 C, the frequency of His$^+$(Str^R) progeny was reduced to about 13% (row 4) of that obtained with the *K. pneumoniae* recipient (row 3). There is a smaller reduction (13% instead of 3%) in 40 C matings compared with 25 C matings. A dramatic situation was revealed in *K. pneumoniae-E. coli* C matings at 40 C, where the frequency of progeny obtained was about 60-fold higher (row 5) than if the recipient was *K. pneumoniae*. In different experiments, the frequency of His$^+$(Str^R) progeny in 40 C matings between strains BK38 and BE48 varied from 2 to 6%. These frequencies are comparable to those obtained in *E. coli-E. coli* matings (Table

5). It is clear from the above that *E. coli* K-12, but not *E. coli* C, restricts F42-400 modified by *K. pneumoniae*. This restriction seems to be less in matings done at 40 C than in matings done at 25 C.

In matings at 25 C between *E. coli* K-12 as donor and *E. coli* K-12 or C as recipient, the frequency of His+(Str^R) progeny was the same (rows 7 and 8). When the recipient was *K. pneumoniae*, the frequency of His+(Str^R) progeny decreased to 7% (row 6) of that obtained with *E. coli*. This suggests that *K. pneumoniae* possess the ability to restrict F42-400 DNA modified by *E. coli* K-12.

E. coli phage 424 (51) is capable of growth in *K. pneumoniae* M5al (101). We have used this phage to demonstrate restriction in the recipient strains used in the above experiment. Data in Table 8 show that 424.K12 is restricted by *K. penumoniae* (10^{-4}). Phage 424.M5a1 is restricted by *E. coli* K-12 (2×10^{-3}). Restriction as measured by phage platings was more severe than that measured by F' transfers. Similar results have been obtained for *E. coli* K-12 (P1) restriction (9). A strain of *E. coli* K-12 deficient in its restriction and modification ability did not restrict 424.M5a1. Phage 424.O was restricted by both *E. coli* K-12 and *K. pneumoniae* strains, although to slightly lesser extents. We were unable to detect plaques when phage 424.K12 was plated on strain BE48. However, we did find phage 424.M5a1 plating on strain BE48 at a low efficiency. Morphology of these plaques was different from those of phage 424 on *E. coli* K-12 or on *K. pneumoniae*, suggesting that these may be some kind of host range variants. This matter is being investigated at the present time.

TABLE 7. *Restriction in matings between K. pneumoniae and E. coli*[a]

Donor	Recipient	Frequency of His+(Str^R) progeny/10^8 donor cells	
		25 C mating	40 C mating
BK32	BK3	20	
BK32	BE48	26[b]	
BK38	BK3	11	480[c]
BK38	BE39	0.31	65[d]
BK38	BE48	28[e]	30,000[f]
BE77	BK3	12[g]	
BE77	BE39	170	
BE77	BE48	170	

[a] Matings in rows 1 and 2 were done for 0.5 h; all the other matings were done for 3 h. Progeny from several of the matings were scored for the presence of unselected markers.

[b] 38/38 were Leu- Thr- Xan- (*lac* marker was not scored).

[c] 100/100 were Leu- Trp- Lys-, and the His+ phenotype was temperature sensitive.

[d] 100/100 showed the temperature-sensitive His+ phenotype.

[e] 40/40 were Leu- Thr- Xan- Lac+, and the Lac+ phenotype was temperature sensitive.

[f] 100/100 were Leu- Thr- Xan- Lac+.

[g] 50/50 were Leu- Trp- Lys-, and the His+ phenotype was temperature sensitive.

DISCUSSION

Isolation of F' donors. The presence of plasmid DNA in strains of *Enterobacteriaceae* is not uncommon. In fact, several of the *Klebsiella* strains studied have been shown to harbor plasmid DNA (12, 19, 27, 44, 49, 85, 86, 87). A class of R factors has been shown to possess the ability to inhibit their own transfer and that of other transfer-proficient plasmids (fertility inhibition, Fin+) (111). It is possible to visualize situations in which some of the enterobacterial plasmids exhibit the Fin+ phenotype. In an extreme case, Fin+ determinants might become chromosomal. *K. aerogenes* harbors a plasmid that confers resistance to streptomycin and chloramphenicol and is Fin+. Introduction of an F' factor into *K. aerogenes* does not result in strains that can retransfer the F' factor. However, removal of the native plasmid results in the retransfer of the F' factor (12).

K. pneumoniae is sensitive to 10 μg of chloramphenicol per ml (unpublished data) and is reported not to harbor plasmids (15). We have been able to obtain strain BK32, which can

TABLE 8. *Restriction of phage 424 in K. pneumoniae and in E. coli*[a]

Phage	Efficiency of plating on strains:					
	BK5	BK3	BE72	BE39	BE18	BE48
424.M5a1	1	1.1×10^{0}	2.2×10^{-3}	2.4×10^{-3}	1	4.3×10^{-5}[b]
424.K12	8.5×10^{-5}	1.9×10^{-4}	1	9.7×10^{-1}	9.7×10^{-1}	1×10^{-10}
424.0		7.9×10^{-4}	6.5×10^{-3}	7.9×10^{-3}	1	

[a] Phage 424.0 was grown on strain BE18.

[b] Plaque morphology is different from the others. It may denote the occurrence of phage variants.

transfer the episome F42-400, without recourse to special methods. These observations are consistent with *K. pneumoniae* M5a1 being Fin$^-$. When R1*drd*19 was transferred to *K. pneumoniae* M5a1, no retransfer of the R factor was observed (33). This was interpreted as suggesting dissociation of the transfer element from the resistance element (33). However, one cannot exclude the possibility that the cells may be Fin$^+$, thus inhibiting retransfer. When strain BS231 (which carries F′ *his*$^+$, F57) was mated with strain BK41 (which has a histidine deletion) and the resulting His$^+$ progeny clones were checked for retransfer ability, none was found. This behavior is analogous to that with R1*drd*19. The reasons for failure to observe retransfer ability in the two situations may not be similar. We conclude that not all F′ transfers produce strains with retransfer abilities.

Sex-specific phage platings. We have not been able to detect lysis of strain BK32 by male-specific MS2 phages or f2 or fd phages. Since these phages adsorb onto the F pilus, any condition that would inhibit F pilus formation should decrease the phage adsorption and hence its multiplication. Situations have been reported in which F-specific phages do not produce plaques on *S. typhimurium* males, yet these strains can support the multiplication of phage in broth. This has been attributed to the poor development of F pili (92). Growth in liquid media is a more sensitive assay than plaque formation for the presence of sex pilus. Strain BK32 may be poor in its F pili development, resulting in its inability to plate F-specific phages (92).

From ϕII we obtained two derivatives, ϕIIK, capable of growth only in *K. pneumoniae*, and ϕIIE, capable of growth only in *E. coli*. This reminds one of Monner and Boman's (73, 74) observation of increased host range of Wollman's ϕII by growth in *E. coli* K-12. We have not observed similar variations with T3. The plating behavior of ϕIIK and ϕIIE phages is similarly affected by the presence of F42-400 in *K. pneumoniae* and *E. coli*, respectively. The plating behavior of T3 is similarly affected by F42-400 in both *K. pneumoniae* and *E. coli*. The ability of F to inhibit female-specific phage T7 is manifested at the level of translation and requires the functioning of at least two genes on F, *pif*A and *pif*B (78). Phage ϕII is a close relative of T7 (46, 47), and the mechanism of growth inhibition of phages ϕII and T7 by the F factor may be similar (60). Even though phage T3 is not a very close relative of T7 or ϕII (28, 46, 47), the mechanism of inhibition of growth of T3 by the F factor may also be similar to that of T7 and ϕII.

Strains BK32 and BK38 can maintain and retransfer F42-400 to other strains; they restrict the growth of phages ϕII and T3. All of those genes necessary for F replication, maintenance, transfer, and inhibition of growth of phages ϕII and T3 must be present and functional on F42-400. We wish to stress that we have no data on the identity of F42-400 in *S. typhimurium* and *K. pneumoniae*.

Chromosome mobilization. Chromosome mobilization is one of the manifestations of several transferable plasmids. Chromosome mobilization may require physical association between the transferable plasmid and the chromosome, brought out by legitimate or illegitimate recombination (37, 76). Not all of these associations need result in the formation of Hfr's (22). One of the steps leading to DNA transfer involves cutting of a specific sequence of DNA. Such a cut might be introduced into the bacterial chromosome by the transferable plasmid without any need for the physical association of the plasmid and the chromosome (5, 111). F factors and F-type R factors, but not I-type R factors, have been shown to integrate into *E. coli* chromosome (77, 79, 118). I-type R factor, but not F-type R factor, has been shown to interact with the *K. pneumoniae* M5a1 chromosome (33, 34) as measured by chromosome mobilization.

In *S. typhimurium* we have observed F42-400 to mobilize Ade$^+$ at 10^{-2}, Ilv$^+$ at about 10^{-3}, and Pro$^+$ at about 10^{-4} of that of the episome transfer frequency itself. Even with the increased transfer efficiency at 40 C we were unable to detect chromosome mobilization in *K. pneumoniae*. Inability of F to mobilize the *K. pneumoniae* chromosome had been reported earlier (87). R114*drd*3 is transferred at a frequency of 1 and mobilized the chromosomal histidine region at a frequency of 9×10^{-5} in *E. coli* (20). R144*drd*3 is transferred at a frequency of 10^{-2} (R. A. Dixon, personal communication) and mobilized the chromosomal histidine region at a frequency of 2×10^{-5} to 10^{-5} in *K. pneumoniae* (33). When we normalize the chromosome mobilization frequencies with that of the R factor, the chromosome mobilization frequency in *E. coli* is 10^{-4} and in *K. pneumoniae* is 2×10^{-3} to 10^{-3}. The sensitivity of our experiments in detecting chromosome mobilization is 10^{-3}. These considerations make it difficult to distinguish between the two interpretations of our inability to observe chromosome mobilization. (i) The F factor is unable to mobilize the *K. pneumoniae* chromosome. (ii) The low sensitivity of our experiments precludes detection of chromosome mobilization. It remains to be shown whether there is any

relationship between the inability of a plasmid to mobilize *K. pneumoniae* chromosome and its belonging to the F type.

Search for Hfr's. Certain kinds of physical interactions between the transmissible plasmid and the chromosome result in chromosome mobilization (22, 35, 76). Occurrence of F-mediated chromosome mobilization does not necessarily mean that Hfr's can be isolated (23, 64, 84). Physical interaction does not appear to be essential for chromosome mobilization (5, 111). We have looked for, and failed to detect, chromosome mobilization by F42-400. It is possible that our choice of *leu* and *trp* markers and the low transfer efficiency caused our failure to detect chromosome mobilization. We wish to emphasize that transfer functions of F42-400 are expressed normally in *K. pneumoniae*. We hoped that the high-curing conditions offered us greater selection pressure to integrate F42-400 into *K. pneumoniae* chromosome leading to Hfr formation. We were unsuccessful in our attempts. The ability of F42-400 to generate Hfr's in *S. typhimurium* required the duplication of the histidine region (84). Such a requirement seems to be absent in *E. coli* (unpublished data). Similar failures have been reported with the F'$_{ts114}$ *lac*$^+$ episome in *S. typhimurium* (64) and with the F'$_{ts114}$ *gal*$^+$ episome in *Citrobacter freundii* (30). Our evidence does not allow a choice among the different explanations offered below to explain our failure.

(i) Although *K. pneumoniae* is proficient in transductional and conjugational recombination (33, 100), it may be deficient in episome-chromosome recombination. This idea is not far fetched: λ recombination in A-to-J region by the RecE pathway is independent of *recA* and *recF* functions, whereas bacterial recombination is absolutely dependent upon the *recA* function (A. J. Clark, personal communication, 1974).

(ii) F42-400 may be deficient in the F sites necessary for integration into the *K. pneumoniae* chromosome (84).

(iii) F42-400 integration into the chromosome may result in lethality at 40 C or above. Many of the *E. coli* C Hfr's grow poorly on defined media at 37 C and do not grow at 42 C (93). Although such gross abnormalities have not been reported for *E. coli* K-12 Hfr's there are reports of decreased colony sizes of Hfr's compared with those of F$^-$ cells (14, 62). In the absence of knowledge about how these growth abnormalities are generated, one could visualize a similar situation operating in *K. pneumoniae*. Since a search for Hfr's at 25 C also proved negative, one would have to extend the idea of temperature sensitivity and say that F42-400 integrations result in unconditional lethality.

(iv) F42-400 integrations into the chromosome are viable, but transfer functions are not expressed (23).

(v) Low transfer efficiency obscured our detecting Hfr's.

Bacteriophage Mu-1 is able to join two circular duplex DNA molecules in a random fashion in *E. coli* RecA$^-$ cells (104, 105). When one of the circular DNA molecules is F and the other is a bacterial chromosome, such a joining is equivalent to the integration of F into chromosome. In fact, many of such Mu-promoted F-chromosome interactions lead to Hfr formation (105). Mu-1 infections have been used successfully to isolate Hfr strains in *C. freundii* (29), where integrative suppression of F replication was not successful (30). We have screened our collection of *Klebsiella* strains for Mu sensitivity and found only one strain, *K. aerogenes* W52, to be sensitive. However, this strain is not known to fix nitrogen. None of the known nitrogen-fixing *Klebsiella* strains was sensitive to Mu-1 (unpublished data; K. T. Shanmugam, personal communication).

Recipient control of transfer efficiency. The transfer efficiency of F42-400 in *E. coli* was lower (6%) than that reported for F42-114 in *E. coli* (36%) (25). This may be a result of differences in strains and/or mating conditions. The transfer efficiency of F42-400 in *K. pneumoniae* was only 0.1%. However, when the same donor was mated with *E. coli* C at 40 C, transfer efficiencies of up to 6% were obtained. The simplest interpretation is that low recipient ability of *K. pneumoniae* is the cause of the low transfer efficiencies observed in *K. pneumoniae* matings. We do not consider that the decreased transfer efficiency in *K. pneumoniae* is a result of the absence of homology between the leading end of the F' factor and the *K. pneumoniae* chromosome (22), or of surface exclusion in *K. pneumoniae* (58).

Conjugation is initiated by the interaction of the sex pili with the recipient cell surface (82). The sex pilus tip is different from the sex pilus shaft (16, 21, 69), and it is likely that the interaction between donor and recipient bacteria is initiated by the sex pilus tip. Several kinds of evidence exist that support the idea of a specific interaction between donor and recipient strains.

Rough strains of *Salmonella* have increased recipient ability for R factors compared with their parental smooth strains (52). Removal of *O* side chains of the *S. typhimurium* cell wall lipopolysaccharide increased the recipient ability. However, when a greater part of the lipopolysaccharide, including the more proximal portion of the R core, was removed, both donor

and recipient abilities were decreased (108). Similar observations were made with *E. coli* strains (110). *E. coli* mutants resistant to ampicillin (75 to 100 μg/ml, class III) were poor in their recipient ability, were sensitive to female-specific phage φW, and had altered lipopolysaccharide. Selection of phage-φW resistant mutants, from the above strains, resulted in decreased resistance to ampicillin, and some of these mutants had increased recipient ability (11, 75). In another study it was observed that about 5% of the *E. coli* mutants resistant to phage ST-1 were poor in their recipient ability (88). All of these observations are in agreement with the idea that recipient bacteria possess specific receptors involved in interaction with sex pilus leading to the establishment of mating pairs (75, 88, 108).

Bacteria carrying an F factor are poor recipients (surface exclusion) in conjugation (58). Mating of HfrC with F^+ bacteria is inefficient because of surface exclusion. However, when an HfrC carrying a derepressed R factor was mated with F^+ bacteria, surface exclusion was not operative. The sex pili produced by the donor had both F pilin and R pilin. These observations were interpreted as suggesting that surface exclusion depends upon the specificity of the pilus that interacts with the recipient bacteria (55).

Decreased recipient ability of *E. coli* mutants resistant to ST-1 was observed with F-mediated transfers but not with those mediated by an I-type R factor (88). In general, R factors have a wider host range than F factors (26). All of these observations are compatible with the idea that recipient bacteria have different specific receptors on their surface for interaction with different kinds of sex pili.

We have interpreted our observations solely in terms of the probability of occurrence of a surface interaction. Isolation of *E. coli* mutants (*pel*) that can adsorb λ but do not allow the entry of DNA (95) raises the possibility that the defect in *K. pneumoniae* is at the DNA entry stage.

In conclusion, our work has shown the following. (i) An F'-carrying *K. pneumoniae* M5a1 strain is able to retransfer the episome to other *K. pneumoniae* and *E. coli* strains. (ii) Phages φII and T3, female-specific phages of *E. coli*, grow in *K. pneumoniae* and their growth is inhibited by an F factor in *K. pneumoniae*. (iii) A restriction-modification system is present in *K. pneumoniae*, which affects both conjugal transfer and phage 424 platings. (iv) Transfer efficiency in *K. pneumoniae* is less than that in *E. coli* because of poor recipient ability of *K. pneumoniae* females.

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