Genet. Res., Camb. (1973), **21**, *pp.* 47–55
With 1 *text figure*
Printed in Great Britain

Genetic recombination between *Klebsiella pneumoniae* and *Enterobacter aerogenes*

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(*Received* 3 *August*, 1972)

SUMMARY

Intergeneric recombination of chromosomal genes between strains of *Klebsiella pneumoniae* and *Enterobacter aerogenes* has been observed. In these experiments a male strain of *K. pneumoniae* acted as donor and *E. aerogenes* acted as recipient. Closely linked genes were co-transferred with very high frequency, and transfer of a large fragment carrying several separated genes was not uncommon. Subcultures of a hybrid which had received a large fragment carrying five markers were tested for genetic stability; no spontaneous segregants were found among 34000 colonies tested. These results suggest that the transferred fragment had been integrated, and indicate the close genetic relationship between *K. pneumoniae* and *E. aerogenes*. This is in agreement with their very similar biochemical properties.

1. INTRODUCTION

The occurrence of conjugation and genetic recombination between chromosomal genes of strains belonging to different genera of the family *Enterobacteriaceae* has been reported previously. These include *Escherichia coli* and *Salmonella* (Baron, Spilman & Carey, 1959; Miyake & Demerec, 1959), *E. coli* and *Shigella* (Luria & Burrous, 1957) and *E. coli* and *Proteus mirabilis* (Gemski, Wohlhieter & Baron, 1967). However, intergeneric chromosomal recombination has not been looked for among strains of the *Klebsiella–Enterobacter* group (Jones & Sneath, 1970). We have recently reported genetic recombination in *Klebsiella pneumoniae*, and described its genetic linkage map (Matsumoto & Tazaki, 1970, 1971). So, using the *Klebsiella* strains, we have attempted to obtain hybrids between strains of *K. pneumoniae* and *Enterobacter aerogenes*, two bacterial species which have long been considered to be very closely related, as judged by the usual cultural and biochemical tests. The results of these experiments show that the two species are, indeed, also closely related genetically.

2. MATERIALS AND METHODS

Bacterial strains. The genetic characteristics of the *K. pneumoniae* and *E. aerogenes* strains are described in Table 1. The genetic nomenclature used is that recommended by Demerec *et al.* (1966). The *K. pneumoniae* strain has been described

https://doi.org/10.1017/S0016672300013203 Published online by Cambridge University Press

previously (Matsumoto & Tazaki, 1970, 1971). The sex factor carried by the *K. pneumoniae* strain Kl-117 and its subline D8 has not yet been characterized. Among five *E. aerogenes* strains tested for their fertility in crosses with this male strain of *K. pneumoniae*, two were able to act as recipients, and one of these, strain Ea-144, was chosen for further study. This *E. aerogenes* strain was isolated by the author in 1967 from the stool of a human adult and has the following cultural and biochemical characteristics, which are typical of *E. aerogenes* (Edwards & Ewing, 1962; Fife, Ewing & Davis, 1965). Ea-144 is a Gram-negative rod, motile in semisolid agar. It is not encapsulated, as shown by the indian-ink method (Edwards & Ewing, 1962) though it forms mucoid colonies on nutrient agar. The strain does not produce indole, gives a negative methyl-red reaction and a positive Voges–Proskauer reaction, and grows on Simmons's citrate agar. It reduces nitrate to nitrite, and decarboxylates lysine and ornithine, but does not hydrolyse arginine. Gelatine is liquefied slowly; urea is not decomposed on Christensen's urea agar. The strain ferments glucose rapidly with abundant gas production; it also ferments raffinose, lactose, sucrose, trehalose, galactose, mannose, rhamnose, mannitol, sorbitol, arabinose, xylose, adonitol, inositol and salicine within 24 h. Dulcitol is not attacked during 14 days of incubation. The *E. aerogenes* strain does not share any somatic antigen with the *K. pneumoniae* strain, as judged by cross-agglutination tests. The strain was sensitive to various antibiotics in current use.

The *K. pneumoniae* strain employed probably corresponds to the *Klebsiella aerogenes* of Cowan *et al.* (1960). The taxonomic name *E. aerogenes* used here is not necessarily synonymous with another familiar name *Aerobacter aerogenes*, a species name which has been used by bacteriologists and biochemists for a long time but with some taxonomic ambiguities (Carpenter, 1970).

Auxotrophic mutants of Ea-144 were obtained after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis, followed by a combination of penicillin screening and replica plating for characterization as recommended by Glover (1968). All the *E. aerogenes* strains were highly resistant to benzylpenicillin, and aminobenzylpenicillin at a concentration of 200 μg/ml was used for penicillin screening. Quite often, auxotrophic mutants of *E. aerogenes* did not grow or grew very poorly at 37 °C on minimal medium supplemented with the growth factor(s) which they required, although they grow well at 27 °C on the same medium. Such plates were therefore always incubated at 27 °C.

Media. Complete medium was a nutrient broth or nutrient agar. Its composition was as follows; Bacto-peptone 1·0 %, Bacto-beef extract 0·7 %, Bacto-yeast extract 0·3 %, sodium chloride 0·5 %, and Bacto-agar (where necessary) 1·5 %. It was adjusted to pH 7·0. Minimal medium was that of Davis & Mingioli (1950) from which citrate had been omitted, and solidified where necessary by adding agar at a concentration of 15 g/l. Minimal medium was enriched with L-amino acid(s) and/or uracil at concentrations of 20 μg/ml and 5 μg/ml, respectively, as required. Streptomycin, when required, was incorporated at a concentration of 200 μg/ml.

Biochemical and serological methods. These recommended by Edwards & Ewing

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Table 1. *Genetic characteristics of strains of* K. pneumoniae *and* E. aerogenes

Subline	Genetic markers
Kl-117 (*K. pneumoniae*)	
D8	Prototrophic, Str^s, male*
Ea-144 (*E. aerogenes*)	
R114	*leu-1, isl-1, his-1, pro-2, ura-1,*† *thr-2, met-1, str-1*
R122‡	*leu-1, isl-1, his-1, pro-1, lys-3, thr-1, met-2, arg-5, str-1*
R123‡	*leu-1, isl-1, his-1, pro-1, lys-3, thr-1, met-12, arg-52, str-1*

leu, isl, his, pro, thr, met, lys and *arg* indicate a requirement for leucine, isoleucine, histidine, proline, threonine, methionine, lysine and arginine, respectively.
str-1 is a mutation resulting in high level resistance to streptomycin.
* The maleness of this strain was proven in intraspecies recombinations (Matsumoto & Tazaki, 1970).
† This mutation leads to a requirement for both uracil and arginine.
‡ For growth of R122 and R123, arginine cannot be replaced by citrullin, ornithine, or *N*-acetylglutamate. For growth of R122, methionine cannot be replaced by homocystine, cystathionine, or vitamin B_{12}; for R123, it can only be replaced by homocystine.

(1962) were used. The amino acid decarboxylase test was performed by Møller's method (Møller, 1954).

Ultraviolet (u.v.) irradiation. Log phase cells grown in nutrient broth were centrifuged, resuspended in saline at a concentration of 10^8 cells/ml, and then irradiated to about 10^{-4} survival. Dilutions of these irradiated cultures were plated on nutrient medium.

Acriflavin treatment. The method recommended by Hayes (1968) was followed, using nutrient broth adjusted to pH 7·6. About 10^3 cells were inoculated into each 10 ml of the nutrient broth containing various concentrations of acriflavin. Inoculations on to the master plates for replica plating were made from the culture containing the highest concentration of acriflavin that allowed visible growth.

Matings. These were performed as described for intraspecies crosses in *K. pneumoniae* (Matsumoto & Tazaki, 1970). Young agar cultures (usually after 5–8 h of incubation) to be crossed were suspended in saline at about 5×10^8 cells/ml, and donor and recipient cultures were mixed in a ratio 1:5. The mixture was kept at 27 °C for 30 min, and 0·1 ml samples were spread on selective plates. These were incubated for 48 h. Colonies appearing on these plates were purified on nutrient agar by single colony isolation, and then tested for their genetic characteristics. In parallel with these mating experiments control tests were carried out by inoculating the culture of *K. pneumoniae* or *E. aerogenes* alone on the same selective plates.

3. RESULTS

(i) *Evidence for the occurrence of genetic recombination between strains of* K. pneumoniae *and* E. aerogenes

K. pneumoniae was mated with three multiply auxotrophic mutants of *E. aerogenes*, recombinants for various loci were selected, and the frequency of inheritance

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Table 2. *Recombination frequencies in crosses between* K. pneumoniae *and* E. aerogenes

Donor	Recipient	Selected marker*	Recombination frequency†	No. of recombinants tested for its genotype‡
D8	R114	*met-1*+	$< 5 \times 10^{-8}$	0
		isl-1+	$3{\cdot}4 \times 10^{-4}$	100
		thr-2+	$1{\cdot}3 \times 10^{-5}$	102
		ura-1+	$6{\cdot}7 \times 10^{-6}$	102
		leu-1+	$3{\cdot}9 \times 10^{-6}$	75
		pro-2+	$1{\cdot}1 \times 10^{-6}$	93
		lys-3+	$< 5 \times 10^{-8}$	0
D8	R122	*isl-1*+	$5{\cdot}5 \times 10^{-4}$	102
		met-2+	$7{\cdot}7 \times 10^{-5}$	102
		arg-5+	$5{\cdot}1 \times 10^{-4}$	100
D8	R123	*isl-1*+	$4{\cdot}8 \times 10^{-4}$	102
		arg-52+	$7{\cdot}0 \times 10^{-4}$	100
		met-12+	$1{\cdot}6 \times 10^{-4}$	95

* The contraselecting agent was streptomycin.
† Number of colonies per donor cell.
‡ Genotypes of these recombinants are given in Tables 3, 4 or 5.

of unselected markers was measured. The results of these crosses are given in Tables 2–5.

The recombination frequencies in these intergeneric crosses (Table 2) were about ten times lower than in intraspecies crosses using the same donor (Matsumoto & Tazaki, 1970). Selections for *met-1*+ and *his-1*+ were unsuccessful – that is, no recombinants were obtainable on plates selective for these markers. Possibly this is related to the fact that the donor transfers the *his* and *lys* markers at a very low frequency in intraspecies crosses (Matsumoto & Tazaki, 1970).

Five of the loci of R 114, i.e. *isl-1*, *thr-2*, *ura-1*, *leu-1* and *pro-2*, were closely linked. Analysis of genotypes of the recombinants (Table 3) suggests the order *isl-1*, *thr-2*, *ura-1*, *leu-1*, *pro-2*. The arrangement of loci near *thr-2* is probably the same as that in *K. pneumoniae*, in which a group of closely linked loci are known to exist near *thr* with the order *thr*, *ura*, *ara*, *leu*, *pan*, *pro* (Matsumoto & Tazaki, 1970, 1971). The results given in Tables 4 and 5 suggest that *isl-1*, *arg-5*, *arg-52*, *met-2*, *met-12*, *thr-1*, *leu-1* and *pro-1* are also closely linked.

Growth of both R 122 (carrying *arg-5*) and R 123 (carrying *arg-52*) was supported only by arginine and not by its precursors. In *K. pneumoniae* two genetically different groups of arginineless auxotrophs that responded only to arginine have been obtained. The mutation site of one of them (*arg-9*) was mapped near *ilv*, while the other was located in the *lys–ser* region of the linkage map of *K. pneumoniae* (Matsumoto & Tazaki, 1970). In addition, in intraspecies crosses of *K. pneumoniae* the mutation site of *isl* mutants which could grow in the presence of isoleucine was mapped very near the *ilv* locus (H. Matsumoto & T. Tazaki, unpublished results).

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Table 3. *Genotypes of recombinants for the* isl-1, thr-2, ura-1, leu-1 *and* pro-2 *loci*

Selected loci	Genetic markers tested					No. of recombinants obtained
	isl-1	*thr-2*	*ura-1*	*leu-1*	*pro-2*	
K. pneumoniae	+	+	+	+	+	.
E. aerogenes R 114	−	−	−	−	−	.
isl-1	(+)	+	+	+	+	18
	(+)	+	+	+	−	6
	(+)	+	+	−	−	2
	(+)	+	−	−	−	3
	(+)	−	−	−	−	71
thr-2	+	(+)	+	+	+	21
	−	(+)	+	+	+	37
	+	(+)	+	+	−	12
	−	(+)	+	+	−	15
	+	(+)	+	−	−	1
	−	(+)	+	−	−	6
	−	(+)	−	−	−	10
ura-1	+	+	(+)	+	+	16
	−	+	(+)	+	+	29
	+	+	(+)	+	−	13
	−	+	(+)	+	−	35
	+	+	(+)	−	−	2
	−	+	(+)	−	−	6
	−	−	(+)	+	−	1
leu-1	+	+	+	(+)	+	9
	−	+	+	(+)	+	12
	+	+	+	(+)	−	10
	−	+	+	(+)	−	30
	−	−	+	(+)	+	5
	−	−	+	(+)	−	5
	−	−	−	(+)	−	4
pro-2	+	+	+	+	(+)	25
	−	+	+	+	(+)	31
	−	−	+	+	(+)	3
	−	−	−	−	(+)	34

(+) indicates that the recombinants were selected for this marker.

It is therefore presumed that *arg-5* and *arg-52* are mutations in the gene corresponding to *arg-9* (Matsumoto & Tazaki, 1970).

Both R 122 and R 123 required methionine but their growth responses to its precursors were different (Table 1), indicating that *met-2* and *met-12* are mutations in different genes. These genetic and biochemical results suggest that the order of the five loci near *isl-1* is *isl-1*, *met-2* (*arg-5*, *arg-52*), *met-12*. The other three loci, *met-1*, *his-1* and *lys-3*, were not linked to any of the loci described above.

All the recombinant strains obtained in crosses between *K. pneumoniae* and *E. aerogenes* R 114 were also tested for their production of ornithine decarboxylase and urease, for their motility in semisolid agar and for their somatic antigens,

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Table 4. *Genotypes of recombinants for the* isl-1, met-2, arg-5, thr-1, leu-1 *and* pro-1 *loci*

Selected loci	Genetic markers tested: *isl-1*	*met-2*	*arg-5*	*thr-1*	*leu-1*	*pro-1*	No. of recombinants obtained
K. pneumoniae	+	+	+	+	+	+	.
E. aerogenes R122	–	–	–	–	–	–	.
isl-1	(+)	+	+	+	+	+	7
	(+)	+	+	+	+	–	5
	(+)	+	+	+	–	–	1
	(+)	+	+	–	–	–	82
	(+)	+	–	–	–	–	4
	(+)	–	–	–	–	–	3
met-2	+	(+)	+	+	+	+	10
	+	(+)	+	+	+	–	8
	+	(+)	+	+	–	–	3
	+	(+)	+	–	–	–	72
	+	(+)	–	–	–	–	8
	–	(+)	+	–	–	–	1
arg-5	+	+	(+)	+	+	+	3
	+	+	(+)	+	+	–	5
	+	+	(+)	+	–	–	2
	+	+	(+)	–	–	–	82
	–	+	(+)	–	–	–	4
	–	–	(+)	–	–	–	4

(+) indicates that the recombinants were selected for this marker.

since these characteristics differed in the parental strains. The results of these tests are shown in Table 6. An interesting observation was that in some cases non-motility, a genetic characteristic of the donor, was transferred to the motile recipient. Ninety-seven per cent of the recombinants which had received the chromosomal fragment covering the region between *isl-1* and *thr-2* were found to be non-motile or very sluggishly motile in semisolid agar. The recombinants which did not receive at least a part of this chromosomal fragment were still motile. These results suggest that a gene or genes responsible for the motility of *E. aerogenes* lies between *isl-1* and *thr-2*.

(ii) *Analysis of genetic linkage map of* E. aerogenes

A part of the genetic linkage map of *E. aerogenes* constructed from the results shown in Tables 3–5 is drawn in Figure 1. This part is similar to that of *K. pneumoniae*.

(iii) *Genetic stability of hybrids between* K. pneumoniae *and* E. aerogenes

Hybrids obtained in intergeneric crosses have often proved to be genetically unstable, giving segregants with high frequency. This instability has been attributed to partial heterodiploidy, the transferred genetic element remaining unintegrated and being lost on subsequent cell division (Baron *et al.* 1968; Baron,

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Table 5. *Genotypes of recombinants for the* isl-1, arg-52, met-12, thr-1, leu-1 *and* pro-1 *loci*

Selected loci	Genetic markers tested						No. of recombinants obtained
	isl-1	*arg-52*	*met-12*	*thr-1*	*leu-1*	*pro-1*	
K. pneumoniae	+	+	+	+	+	+	.
E. aerogenes R 123	−	−	−	−	−	−	.
isl-1	(+)	+	+	+	+	+	9
	(+)	+	+	+	+	−	13
	(+)	+	+	+	−	−	1
	(+)	+	+	−	−	−	69
	(+)	+	−	−	−	−	2
	(+)	−	−	−	−	−	8
arg-52	+	(+)	+	+	+	+	14
	+	(+)	+	+	+	−	4
	+	(+)	+	+	−	−	2
	+	(+)	+	−	−	−	70
	+	(+)	−	−	−	−	3
	−	(+)	+	−	−	−	4
	−	(+)	−	−	−	−	3
met-12	+	+	(+)	+	+	+	15
	+	+	(+)	+	+	−	8
	+	+	(+)	+	−	−	4
	+	+	(+)	−	−	−	63
	−	+	(+)	−	−	−	1
	−	−	(+)	−	−	−	4

(+) indicates that the recombinants were selected for this marker.

Table 6. *Biochemical, cultural and serological characteristics of recombinants between* K. pneumoniae *and* E. aerogenes *R114*

	Characteristics tested				No. of recombinants obtained
	Ornithine decarboxylase	Urease	Motility*	Somatic antigen	
K. pneumoniae	−	+	−	Kl-117†	.
E. aerogenes	+	−	+	Ea-144†	.
Recombinants	+	−	+	Ea-144	251
	+	−	− or ±	Ea-144	221‡

* ± indicates very sluggish motility.

† These designations are tentative.

‡ Ninety-seven per cent of these belonged to the genotypes to which all or a part of the chromosomal fragment between *isl-1* and *thr-2* has been transferred (see text).

Spilman & Carey, 1960; Falkow, Rownd & Baron, 1962). Therefore, a hybrid strain between *K. pneumoniae* and *E. aerogenes* was tested for its genetic stability. *K. pneumoniae* was mated to *E. aerogenes* R 114, selection was made for the *isl-1*$^{+}$ marker, and the hybrid obtained, RC 6, was studied in detail. RC 6 grew in minimal medium supplemented with methionine and histidine, indicating that the chromosomal fragment covering at least the region between *isl-1* and *pro-2* had been

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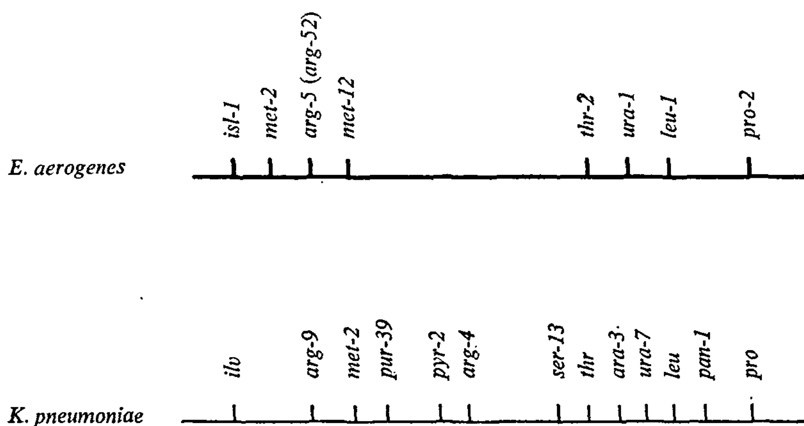

Fig. 1. Genetic linkage maps of *E. aerogenes* and *K. pneumoniae*. The map has been drawn approximately according to the frequency of crossing-over between the markers and the frequency of transfer of unselected markers. The location of *arg-52* may be the same as that of *arg-5* (see text). A segment of the linkage map of *K. pneumoniae* is presented for comparison.

transferred. If part or all of the chromosomal fragment transferred formed a duplication of the corresponding chromosomal region of the recipient, then RC6 should give segregants unable to grow on minimal medium plus methionine and histidine. RC6 was therefore subcultured successively 50 times on nutrient agar slants, and about 3000 colonies were tested for spontaneous segregants by replica plating after every five transfers. No spontaneous segregants were found amongst 34000 colonies tested. In addition, cultures of RC6 inoculated from the 31st agar slant subculture were irradiated with u.v. or grown in the presence of acriflavin and survivors tested for segregants by the same method. Six u.v.-induced and one acriflavin-induced mutants, but no segregants, were detected from the 8600 and the 15400 colonies tested, respectively.

4. DISCUSSION

The occurrence of intergeneric recombination between strains of *K. pneumoniae* and *E. aerogenes* has been observed. The donor in the crosses was a male strain of *K. pneumoniae* previously employed in intraspecies matings, and the recipient was *E. aerogenes*. So far as the author is aware nothing has been published previously on genetic recombination between chromosomal genes of these two enterobacterial species. The size of the chromosomal fragment transferred varied with the recombinant. Often, recombinants received a large fragment covering the chromosomal region between *isl-1* and *pro-2*. The one hybrid of *K. pneumoniae* and *E. aerogenes* tested appeared to be genetically stable. This indicates that in this hybrid the transferred fragment had been integrated, implying close homology between the DNA of these species. However, further experiments will be necessary to decide whether the chromosomal fragments transferred are always integrated

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or are integrated only in a particular region where there is a high level of homology. In view of their close genetic relationship, as shown above, and their phenotypic similarities, it seems reasonable to place *K. pneumoniae* and *E. aerogenes* very close together taxonomically.

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