Chapter 2

Escherichia coli ColV Plasmid pRK100: Genetic Organisation, Stability and Conjugal Transfer

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ABSTRACT

Uropathogenic *Escherichia coli* strains express chromosomal and plasmid-encoded virulence-associated factors such as specific adhesins, toxins and iron-uptake systems. A ColV plasmid (pRK100) of a uropathogenic strain and its host KS533 were studied. The host strain encodes the K1 capsule, and P and S fimbriae, but neither haemolysin nor the cytotoxic necrotising factor CNF1, indicating that this strain does not harbour a larger pathogenicity island. A restriction map of pRK100 was constructed on the basis of hybridisation experiments and nucleotide sequencing. pRK100 harbours ColV, the conserved replication region RepFIB, the aerobactin-uptake system, a RepFIC replicon and additionally ColIa as well as transposon Tn*5431*. The location of the RepFIC replicon was similar to that in plasmid F. ColV plasmids and F thus share a region spanning more than half the length of plasmid F. Even though their replication and transfer regions are homologous, ColV plasmids are found only in *E. coli* strains. Among the four other species tested, conjugal transfer of pRK100 was demonstrated, with low frequency, only to *Klebsiella pneumoniae*, suggesting that a natural barrier effectively bars transfer. *In vitro* stability of the plasmid with integration into the chromosome to ensure maintenance in the presence of an incompatible plasmid was demonstrated.

Keywords: ColV plasmid, plasmid pRK100, stability, conjugal transfer

1. INTRODUCTION

Pathogenic strains of *Escherichia coli* can cause intestinal and extraintestinal infections as well as new-born meningitis (22). They encode various virulence determinants - adhesins, toxins, capsules, invasins and other virulence factors - on the chromosome, on plasmids, or on the genomes of bacteriophages. Virulence genes can be organised into large chromosomal blocks termed pathogenicity islands (4).

Plasmids are self-replicating and are normally not essential for bacterial growth; however, they often encode antibiotic resistances, colicins and various virulence determinants. Conjugative plasmids, in particular, facilitate the exchange and spread of resistances to antibiotics and chemicals, virulence factors and metabolic properties. The ColV plasmids form a heterogeneous group of large plasmids belonging to the IncFI incompatibility group and encoding the production of colicin V (30). ColV plasmids are harboured primarily by virulent enteric bacteria and encode several virulence-associated properties. Colicin V is a small molecule, but is not SOS inducible and is therefore actually a microcin (7). Recent studies in a chicken embryo model system have shown that an avirulent wild type avian *E. coli* strain transformed with the cloned colicin V genes became virulent, demonstrating that colicin V has a direct role in virulence enhancement (32). Most colicin V plasmids also encode the aerobactin-uptake system and increased serum survival as well as resistance to phagocytosis. They could serve as models for the study of the evolution and molecular biology of other virulence plasmids.

ColV plasmids can harbour more than one replicon. The main replicon is homologous to the RepFIB of plasmid F. This replication region, along with the aerobactin-uptake system, is highly conserved. Some ColV plasmids also carry a replicon homologous to RepFIA of plasmid F and it has been reported that pColV-K30, the prototypic ColV plasmid, also carries an incomplete RepFIC (30) even though its location has not been determined.

Colicin V production was first described more than 70 years ago (14), indicating that ColV plasmids were present in natural *E. coli* populations well before the widespread use of antibiotics. Use of antibiotics has probably selected for carriage of additional virulence and resistance determinants on ColV plasmids.

pRK100 is an approximately 145 kb conjugative plasmid, which was discovered in a uropathogenic *E. coli* strain (34). The plasmid encodes colicins V and Ia, the aerobactin-uptake system and the 16.1 kb transposon Tn*5431* with ampicillin and tetracycline resistance determinants (35, 36). The present work was carried out to study pRK100, to prepare its map, and to gain insight into its evolution, conjugational transfer and stability.

2. MATERIAL AND METHODS

Bacterial strains, plasmids and media

The *E. coli* strains and plasmids used in this study are listed in Table 1. KS533 is a uropathogenic strain, serotype rough:K1:H7 (determined by I. Ørskov), isolated at the Institute of Microbiology, Medical Faculty of Ljubljana. It harbours a large (145 kb) conjugative plasmid, pRK100 (34). *E. coli* HB101 was used as the recipient strain for the conjugative transfer of plasmids pRK100 and RSF2001, a kanamycin-resistant derivative of plasmid F. *E. coli* DH5*a* was used as recipient for recombinant plasmids.

Plasmid pColVK-30, whose map has already been published (29), was used for comparison of probe binding in the hybridisation experiments for IS*1*, RepFIA, RepFIB, RepFIC, the aerobactin-up-

take system and the *tra* region. Plasmids of the Couturier bank of rep probes were used for replicon typing.

The *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Pseudomonas aeruginosa* strains used as recipients in conjugation experiments are clinical isolates. The *Salmonella typhimurium* recipient was LT2.

***Table 1. E. coli* strains and plasmids**

Strain or plasmid	Relevant properties	Source or reference*
Strains		
KS533	Rough:K1:H7 harbouring pRK100	34
HB101	*hsdR hsdM recA13 supE44 leuB6 lacZ proA2*	D. Ehrlich[1]
DH5α	*thi-1 recA hsdR17 lac*	A. Francky[2]
C600	*thi-1 thr-1 leuB6 lacY1* Sm^R	B. Bachmann[3]
AB1133	Sensitive to all colicins	B. Bachmann
KH1038	Sensitive only to ColV	K. G. Hardy[4]
KH1044	Resistant to ColV	K. G. Hardy
Plasmids		
pRK100	ColV plasmid with Tn*5431*	34
pColV-K30	ColV	17
RSF2001	F plasmid with Kn^R	16
pUC19	Ap^R	33
pAlter	Tc^R	Promega
pTC72#24	IS*1*	M. Chandler[5]
pABN1	Aerobactin-uptake system	11
pHK11	Colicin V	13
pED100	*tra* operon	31
pPKL4	Fimbriae type I	23
pRHU845	*pap*/*prs*	23
pANN801-13	*sfa*/*foc*	23
pUBK2404	RepFIB	10
pUBL2440	RepFIC	10
pUBL2422	Rep9	10
pFDH1	pAlter with the 20 kb *Hin*dIII fragment of pRK100	This study
pFDH2	pAlter with the 20 and 12 kb *Hin*dIII fragments of pRK100	This study
pFDS1	pAlter with the 17.5 kb *Sal*I fragment of pRK100	This study
pUX1	pUC19 with 0.9 kb *Eco*RI–*Hin*dIII of pFDH1	This study
pUV55	pUC19 with the 5 kb *Eco*RI fragment of pFDH2	This study
pUX5	pUC19 with the 3.6 kb *Eco*RI fragment of pFDH1	This study
pUX600	pUC19 with 0.6 kb *Pst*I fragment of pUX5	This study

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[3]*E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, CT, USA
[4]Biogen SA, Geneva, Switzerland
[5]Centre de Recherche de Biochimie et Génétique Cellulaire du CNRS, Toulouse, France

Strains were grown in Luria-Bertani (LB) medium. When necessary, LB was supplemented with: ampicillin (Ap, 100 μg/ml); tetracycline (Tc, 10 μg/ml); streptomycin (Sm, 100 μg/ml); chloramphenicol (Cm, 10 μg/ml); kanamycin (Kn, 30 μg/ml). For selection of *S. typhimurium*, *K. pneumoniae*, *Ent. cloacae*, *P. aeruginosa* transconjugants, Simmons citrate media (8) supplemented with the appropriate antibiotics was used.

General DNA manipulation techniques

Prior to DNA manipulation, plasmids pRK100 and pColVK-30 were transferred to *E. coli* HB101. Isolation of plasmid and chromosomal DNA, large-scale isolation of CsCl-purified pRK100, and ligation and transformation experiments were performed as described by Sambrook, *et al.* (27). To visualise shorter restriction fragments pRK100 DNA was concentrated by ultrafiltration through a Centricon concentrator (Amicon) prior to restriction enzyme cleavage. Plasmids pRK100 and pColVK-30 were cleaved with *Eco*RI, *Hin*dIII, *Sal*I, *Xho*I and by double cleavages. Plasmids carrying cloned inserts of pRK100 were additionally cleaved with *Acc*I, *Bam*HI, *Cla*I, *Dra*I, *Pst*I and *Pvu*II. Restriction endonuclease digestions were carried out according to the instructions of the supplier. DNA fragments were purified from agarose gels using the Geneclean II system (Bio101).

Agarose gel electrophoresis and restriction fragment size determination

Restriction enzyme digests of pRK100 were separated by constant field (CFGE) and pulsed-field (PFGE) gel electrophoresis. For separation and size determination of restriction fragments ranging from 0.2 to 7 kb, CFGE with 0.5–2% submarine agarose gels was used. Larger fragments were separated by PFGE (Pharmacia Biotech, Gene Navigator System).

DNA hybridisation for pRK100-encoded determinants

DNA labelling and hybridisation experiments were carried out using the DIG DNA labelling and detection kit (Boehringer). Hybridisation experiments were performed to detect the aerobactin-uptake system, ColV, the *tra* operon, replicons – first to determine, which replication regions were present and subsequently their position (RepFIB, RepFIC, Rep9) – IS*1*sequences and chromosomal P, type I and S-fimbriae. The following labelled probes were used. For the aerobactin-uptake system the 2.7 kb *Sal*I–*Bam*HI fragment of pABN1 (11) with genes *iucA* and *iucB* was used, and for ColV the 0.5 kb *Pvu*II–*Bgl*II fragment of pHK11 with *cvaC* and the overlapping *cvi* genes (13). All *Eco*RI restriction fragments of pRK100 were labelled and used to probe the Couturier bank of rep plasmids (10): for RepFIB the 1.2 kb *Pst*I fragment of pUBL2404; for RepFIC the 0.9 kb *Eco*RI–*Hin*dIII fragment of pUBL2440; for Rep9 the 0.5 kb *Pst*I fragment of pUBL2422 (10). For the *tra* operon, *Eco*RI fragments of pED100 and the 8.3 kb *Eco*RI restriction fragment f6 of pED100 (31) were used, and for detection of IS*1* the 2.9 kb *Pvu*II fragment of pTC72#24 (kindly provided by M. Chandler).

The 0.9 kb *Eco*RI–*Hin*dIII fragment of pFDH1 and the three longest (27, 20 and 17 kb) *Eco*RI fragments of pRK100 were also individually labelled and used to probe *Eco*RI, *Hin*dIII, *Sal*I, *Xho*I and double digestions of pRK100.

Stringent conditions were used for all hybridisation experiments described in this report.

Colicin production

ColV and ColIa production was determined using the overlay method (25) with indicator strains of the Pugsley collection of colicinogenic strains and with strains KH1038 (sensitive only to ColV), KH1044 (resistant to ColV) and AB1133 (sensitive to all colicins).

Resistance to the bactericidal action of serum

E. coli C600 and C600 harbouring pRK100, as well as *E. coli* KS533 with and without pRK100, were tested for serum survival or resistance. Serum survival was tested in the presence of 1%, 2% and 3% human serum (19).

Plasmid stability and curing

Plasmid stability was studied by inoculation of the strain into LB medium without antibiotics and incubating at 37 °C with shaking. The next day the cell suspension was diluted into fresh LB medium and again incubated at 37 °C. After 21 passages the cell suspension was diluted and plated. Colonies were then transferred to grids and tested for antibiotic resistances and colicin production.

Plasmid stability was further tested by introducing pED100, a conjugative derivative of plasmid F also of the IncF1 incompatibility group, into KS533.

Plasmid curing was also performed with acridine orange and SDS treatment (15).

Conjugational transfer experiments

For conjugational transfer of plasmids the donor and recipient strains were overstreaked on an LB plate and incubated overnight at 37 °C. Following the mating period, a portion of the mating mixture was removed from the growth surface with a sterile rod and was streaked on a selection plate. For liquid mating, overnight cultures of donor and recipient strains were diluted 50-fold in LB liquid medium. The donor strain was incubated at 37 °C for 3 h without shaking and the recipient strain for 2 h with shaking. A mating mixture consisting of 0.5 ml of the donor and 4.5 ml of the recipient cultures was prepared and incubated for 20 h at 37 °C. Transconjugants were selected on Simmons citrate agar plates, supplemented with Tc and Ap when testing for transfer of pRK100 and with Kn when testing for transfer of plasmid RSF2001. *E. coli* transconjugants were selected on LB media supplemented with Sm, Ap and Tc for transfer of pRK100 or Sm and Kn for transfer of RSF2001. Negative controls were prepared by plating donor and recipient strains separately on selective media. Transconjugants were screened for plasmid DNA.

Production of type I fimbriae, P fimbriae, S fimbriae, CNF1 and haemolysin

Cleaved KS533 chromosomal DNA was probed for type I fimbriae with the labelled 6 kb *Pst*I fragment of pPKL4, for *pap/prs* with the 4 kb *Hin*dIII–*Eco*RI fragment of pRHU845 and for *sfa*/*foc* with the 6 kb *Cla*I–*Eco*RV fragment of pANN801-13. PCR was carried out with primers specific for the cytotoxic necrotising factor CNF1: CNF1 primer 1 (CTGACTTGCCGTGGTTTAGTCGG) and CNF1 primer 2 (TACACTATTGACATGCTGCCCGGA). PCR was carried out in the following steps: heating at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1.5 min, extension at 72 °C for 2 min, and the final extension for 5 min at 72 °C.

Haemolysin production was tested by plating onto LB plates containing 2% washed sheep erythrocytes.

DNA sequencing

Single- and double-stranded sequencing of cloned pRK100 restriction fragments was performed using the Sequenase version 2.0 Sequencing Kit (USB) and the Silver sequencing kit (Promega). Primers were the commercially available M13/pUC forward and reverse sequencing primers.

3. RESULTS

Location of Tn*5431* and flanking regions

Previously, we determined that pRK100 carries transposon Tn*5431*, encoding resistances to Tc and Ap (35). To determine the position of Tn*5431* on pRK100, three clones carrying the antibiotic resistances of Tn*5431*, pFDH1, pFDH2 and pFDS1, were isolated (Fig. 1). pFDH1 harbours the 20 kb *Hin*dIII fragment, pFDH2 two consecutive *Hin*dIII fragments, 20 kb and 12 kb long (isolated by cloning partially *Hin*dIII-digested pRK100 DNA), while pFDS1 carries the 17.5 kb *Sal*I fragment. Using restriction mapping, hybridisation experiments, and testing with indicator strains for colicin activity it was determined that pFDH1 and pFDS1 harbour an incomplete Tn*5431* and that each plasmid carries one flanking region. pFDH1 carries ColIa, pFDS1 carries ColV, and pFDH2 carries the entire Tn*5431*, ColV and ColIa.

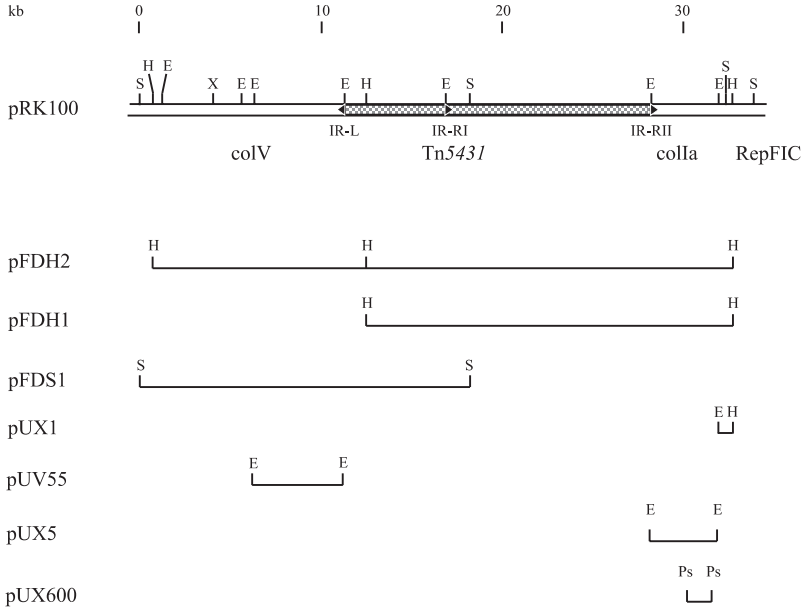

Fig. 1. **Schematic representation of the pRK100 region encoding ColV, Tn*5431*, ColIa, RepFIC and the restriction enzyme maps of clones harbouring restriction fragments of this region**
Restriction sites: E, *Eco*RI; H, *Hin*dIII; Ps, *Pst*I; S, *Sal*I; X, *Xho*I. *Pst*I restriction sites are shown only for pUX600. The hatched area denotes Tn*5431*, with the arrowheads designating inverted repeats (IR).

To confirm the hybridisation and colicin typing results, plasmids pUV55 and pUX5, with 5 kb and 3.6 kb *Eco*RI fragments of pFDH2, respectively were prepared (Figs 1 and 2). Subsequently, the 0.6 kb *Pst*I restriction fragment of pUX5 was subcloned to generate pUX600. Single-stranded sequencing of the pUV55 insert with the reverse sequencing primer and of the pUX5 insert with the forward sequencing primer showed that Tn*5431* was inserted into ColIa at nucleotide position 3424 (determined by alignment with the sequence deposited under EMBL accession number M13819) of the conserved ORF2, which is not involved in ColIa production. Upstream of the IR-L of Tn*5431* are sequences from nucleotide positions 3425 to 3727 of the 3727 bp deposited sequence. Downstream of the Tn*5431* IR-RII are the colicin Ia immunity and structural genes. Single-stranded sequencing

of the 0.6 kb *Pst*I insert of pUX600 with the forward and reverse sequencing primers demonstrated conservation of sequences of the ColIa structural gene, while the ORFI sequences are not conserved. Alignment of the nucleotide sequence obtained by single-stranded sequencing of pUV55 DNA with the forward primer and the ColV sequence deposited under EMBL accession number X57525 showed conservation of nucleotide sequences of the colicin V *cvaC* structural gene and the *cvi* immunity gene. Nucleotide sequencing also showed characteristic 5 bp repeats of target DNA at both Tn*5431* ends, confirming that transposition had occurred.

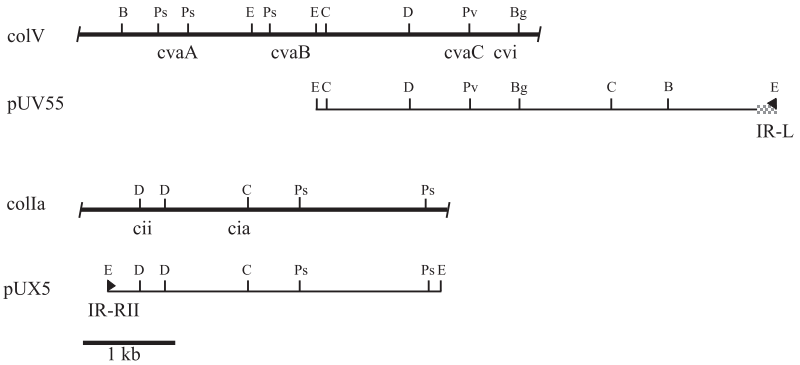

Fig. 2. **Comparison of ColV and ColIa restriction maps**
Top: comparison of the restriction map of ColV, prepared on the basis of sequences deposited under accession numbers X57525 and X57524 and denoted with a heavy line, with the pUV55 restriction map. The shaded line at the right-hand end of the pUV55 map denotes the conserved sequences in the direct vicinity of the ColIa immunity gene from nucleotide positions 3425–3727. The arrowhead denotes the left inverted repeat (IR-L) of Tn*5431*. Bottom: comparison of the restriction map of ColIa, prepared on the basis of the deposited sequence and denoted with a heavy line, with the restriction map of pUX5. The arrowhead denotes the right inverted repeat (IR-RII) of Tn*5431*. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; D, *Dra*I; Pv, *Pvu*II.

Altogether these results demonstrated that Tn*5431* had transposed into a region encoding ColIa and ColV. Tn*5431* is flanked on one side by ColV and on the other by ColIa.

Replication regions, aerobactin system and IS*1*

Waters & Crosa (28) reported conservation of the replication region RepFIB among all ColV plasmids investigated and, further, that most encode the aerobactin iron-uptake system with an upstream and downstream insertion sequence IS*1*. To detect and map these nucleotide sequences we performed hybridisations of specific labelled probes with restriction enzyme digests of pRK100 and, for comparison, with fragments of the prototypic plasmid pColV-K30.

Comparison of the hybridisation signals demonstrated conservation in pRK100 of the replicon RepFIB, a basic replicon found in large plasmids of the IncFI group (12), the aerobactin-uptake system and the two IS*1* sequences (Figs 3, 4 and 5, respectively). Some but not all ColV plasmids also have the RepFIA homologous replicon downstream of the aerobactin-uptake system. Since large plasmids are known to carry more than one replicon, first all *Eco*RI fragments of pRK100 were labelled and used to probe the Couturier bank of rep plasmids. The probe did not hybridise with the 917 bp *Eco*RI fragment of plasmid pUBL2154 harbouring RepFIA, demonstrating that pRK100 does not have this replicon. On the other hand the probe hybridised with fragments corresponding to RepFIC

(the 967 bp *Eco*RI–*Hin*dIII fragment of pUBL2440), Rep9 (the 539 bp *Pst*I fragment of pULB2422), RepFIIA *copA* (the 543 bp *Pst*I fragment of pUBL2401) and RepFIIA *copB* (the 540 bp *Pst*I fragment of pULB2402). All four are known to cross-hybridise. To determine their positions, the labelled Rep-FIC and Rep9 fragments were then used to probe restriction enzyme digests of pRK100. Both probes hybridised to the same two *Eco*RI, *Sal*I and *Hin*dIII fragments, indicating that a replicon(s) of this family is present at two positions of pRK100 (Fig. 6).

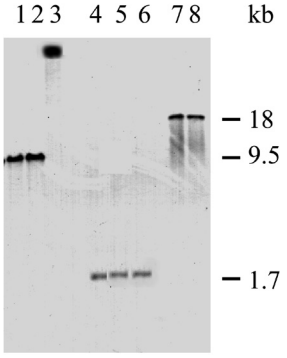

Fig. 3. **Southern blot hybridisation, with the Rep-FIB-specific probe, of pRK100**
pRK100 digested with: lane 1, *Eco*RI; lane 2, *Xho*I/*Eco*RI; lane 3, *Xho*I; lane 4, *Hin*dIII; lane 5, *Xho*I/*Hin*dIII; lane 6, *Eco*RI/*Hin*dIII; lane 7, *Sal*I; lane 8, *Xho*I/*Sal*I.

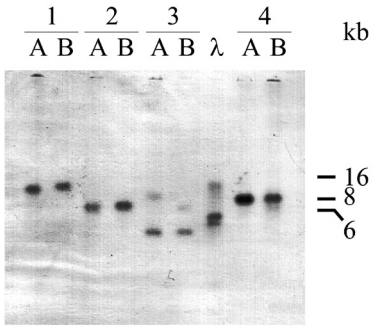

Fig. 4. **Southern blot hybridisation, with the probe specific for the aerobactin-uptake system, of pRK100 and pColV-K30**
pRK100 (lanes A) and pColV-K30 (lanes B) digested with: 1, *Hin*dIII; 2, *Eco*RI/*Hin*dIII; 3, *Bam*HI/*Hin*dIII; 4, *Eco*RI. Lane λ, labelled marker λ DNA digested with *Eco*RI/*Hin*dIII.

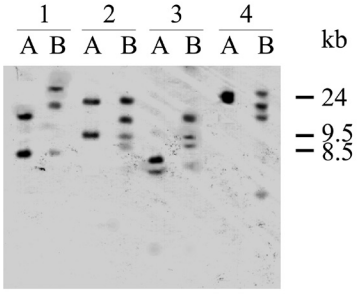

Fig. 5. **Southern blot hybridisation, with the IS*1*-specific probe, of pRK100 and pColV-K30**
pRK100 (lanes A) and pColV-K30 (lanes B) digested with: 1 *Hin*dIII; 2, *Eco*RI; 3, *Eco*RI/*Hin*dIII; 4, *Sal*I.

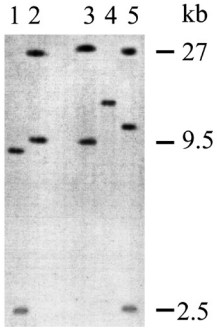

Fig. 6. **Southern blot hybridisation, with the Rep-FIC-specific probe, of pRK100**
pRK100 digested with: lane 1, *Eco*RI/*Sal*I; lane 2, *Eco*RI/*Hin*dIII; lane 3, *Eco*RI; lane 4, *Hin*dIII; lane 5, *Sal*I.

tra region and construction of the pRK100 map

The pRK100 *tra* region was determined by hybridisation of the labelled pED100 *Eco*RI fragments harbouring the *tra* operon of plasmid F and the labelled 8.3 kb *Eco*RI fragment f6, to restriction fragments of pRK100 and pColV-K30 (Figs 7 and 8, respectively).

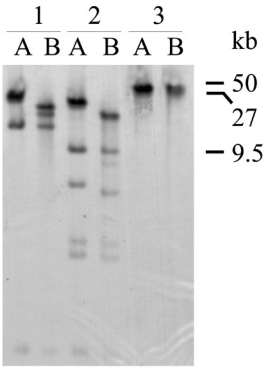

Fig. 7. **Southern blot hybridisation, with *Eco*RI fragments of the F factor *tra* operon, of pRK100 and pColV-K30**
pRK100 (lanes A) and pColV-K30 (lanes B) digested with: 1, *Eco*RI; 2, *Eco*RI/*Sal*I; 3, *Hin*dIII.

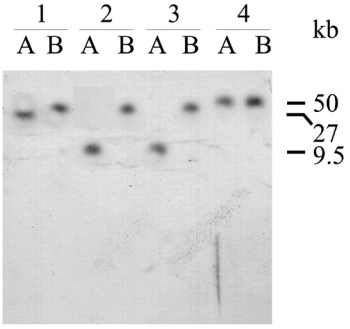

Fig. 8. **Southern blot hybridisation, with fragment f6 of the F factor *tra* operon specific probe, of pRK100 and pColV-K30**
pRK100 (lanes A) and pColV-K30 (lanes B) digested with: 1, *Sal*I; 2, *Eco*RI/HindIII; 3, *Eco*RI; 4, *Hin*dIII.

Comparison of the hybridisation results for the *tra* region with the results obtained with the labelled 0.9 kb *Eco*RI–*Hin*dIII fragment of pFDH1 enabled us to deduce the position of the *tra* operon with regard to ColIa. Further comparison of the above results with the RepFIC and Rep9 hybridisation patterns enabled us to deduce that a RepFIC replicon is downstream of ColIa. This was confirmed by single-stranded nucleotide sequencing with the forward sequencing primer of the pUX1 insert. Subsequently, by analysing the hybridisation results obtained with probes specific for the aerobactin system, IS*1* sequences, RepFIC and with the labelled 0.9 kb *Eco*RI–*Hin*dIII pFDH fragment, we were able to deduce the position of the *tra* operon on pRK100.

*Xho*I cleaves pRK100 at only two sites, resulting in two fragments, of 26 kb and 120 kb. One site was mapped in the ColV region, while the other was deduced from the fact that neither Tn*5431* nor pFDH1 has a *Xho*I site and from fragment patterns of double restrictions.

On the basis of the above-mentioned results together with the fragment patterns of double restrictions, hybridisation experiments using the three longest individually labelled *Eco*RI fragments (27, 20 and 17 kb), and comparison of probe binding to pColV-K30 we were able to complete the restriction map (Fig. 9).

Increased serum survival conferred by pRK100

ColV plasmids have been shown to enhance serum resistance. TraT, the surface exclusion protein of the plasmid transfer system, and the *iss* (increased serum survival) locus, which is linked to the colicin V genes, have been implicated (3). Serum survival was tested for the original clinical strain KS533, strain C600 and C600 harbouring pRK100 in the presence of 1%, 2% and 3% human serum.

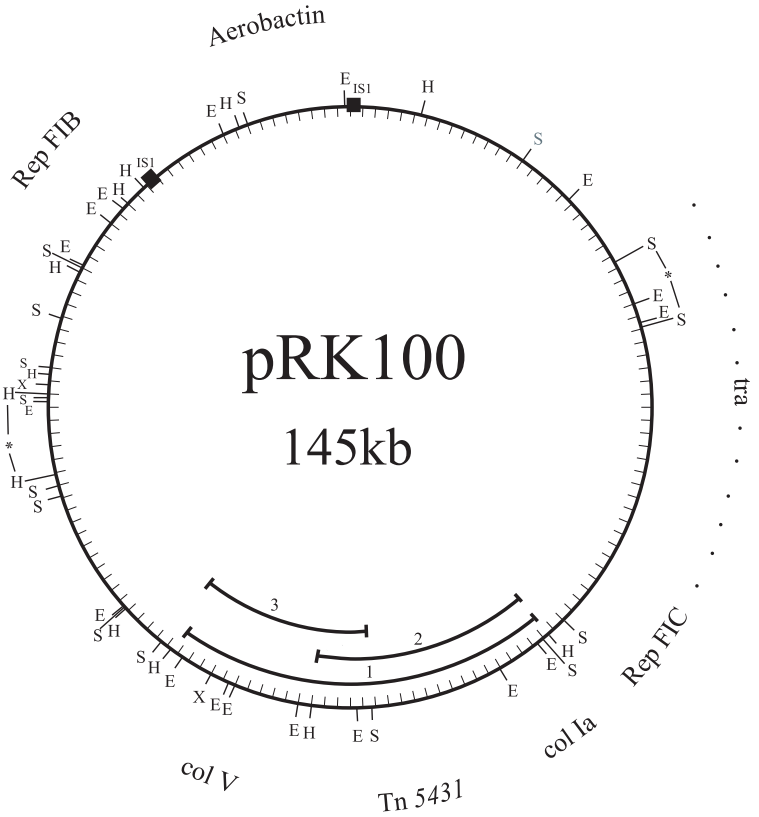

Fig. 9. **Map of pRK100**
On the inside border of the map are the boundaries of clones: 1, pFDH2; 2, pFDH1; 3, pFDHS1. The black boxes denote the two IS*1* insertion sequences. The asterisks in the bracketed area denote a region containing several small *Hin*dIII and *Sal*I fragments.

Table 2. **Survival in human serum of strains with and without pRK100**
Percentage survival is expressed relative to that of bacteria incubated in buffer without serum. The experiment was repeated three times and the results presented are mean values.

Strain	Percentage survival in 1%, 2% and 3% serum:		
	1%	2%	3%
C600	0.4	0.03	0
C600 (pRK100)	1.97	0.13	0
KS533	800	789	679
KS533 (pRK100)	802	800	683

The results (Table 2) show increased survival of C600 harbouring pRK100 in the presence of 1% and 2% serum (five- and fourfold, respectively) while both strains were killed in the presence of 3% serum. In contrast, both the clinical strain KS533 with plasmid pRK100 and strain KS533 without the plasmid exhibited growth at all serum concentrations tested. The K1 capsule thus offers much greater protection than any plasmid-encoded determinant.

Stability and conjugal transfer to other Gram-negative bacteria

Following 21 passages of pRK100 without antibiotic selection 1200 colonies were transferred to grids (LB plates) and tested for antibiotic resistances and colicinogenicity. All colonies retained the characteristics of the initial strain. Stability of pRK100 was also studied by introduction of an incompatible plasmid, pED100, a Cm^R derivative of F. A total of 152 colonies expressing the characteristics of both plasmids, resistances to Ap, Tc, Cm and colicinogenicity, were subsequently further subcultured by replica plating first onto an LB plate and then to an LB plate supplemented with Cm. This procedure was repeated seven times, during which some colonies exhibited gradual loss of the Cm^R character. None of the Cm^R colonies exhibited loss of pRK100. Gel electrophoresis of the isolated plasmid DNA of strains, which stably maintained colicinogenicity and antibiotic resistances of both plasmids (Ap, Tc, Cm), showed the presence of both plasmids or only F plasmid DNA, indicating integration of pRK100 into the chromosome. Only culturing strain KS533 in the presence of SDS yielded two colonies cured of plasmid pRK100.

To determine whether pRK100 can be transferred and maintained in other Gram-negative bacteria, conjugal transfer was attempted to *S. typhimurium*, *K. pneumoniae*, *P. aeruginosa* and *Ent. cloacae*. With plate mating only a small number of *K. pneumoniae* transconjugants harbouring pRK100 were isolated. Liquid matings were also carried out and the transfer frequencies of pRK100 and RSF2001 were compared (Table 3). pRK100 and RSF2001 were both transferred at low frequency to *K. pneumoniae*. On the other hand while the transfer frequency of RSF2001 to *S. typhimurium* was comparable with that to *E. coli*, transfer of pRK100 to *S. typhimurium* was never detected. Transfer of RSF2001 to *Ent. cloacae* was detected only with plate mating.

Table 3. **Plasmid transfer frequency to different species**

Conjugal transfer frequencies are expressed as the proportion of transconjugants to recipients (means of three independent experiments). –, No transconjugants were isolated; ±, a small number of transconjugants were isolated only with plate mating; NT, not tested.

Plasmid	Transfer frequency to				
	E. coli	*S. typhimurium*	*K. pneumoniae*	*Ent. cloacae*	*P. aeruginosa*
pRK100	2.4×10^{-2}	–	1.2×10^{-6}	–	–
RSF2001*	1.1×10^{-2}	3.7×10^{-2}	3.7×10^{-7}	±	NT

* RSF2001 is plasmid F with Kn^R

Chromosomal virulence-associated genes of strain KS533

Hybridisation experiments demonstrated the presence of nucleotide sequences specific for type I, P and S fimbriae. PCR was carried out with primers specific for CNF1 but amplification of the corresponding fragment was not detected. KS533 does not produce haemolysin as determined by growth on blood agar plates.

4. DISCUSSION

Using hybridisation and nucleotide sequencing a map of plasmid pRK100 and its characteristics was constructed. Transposon Tn*5431* has transposed into the direct vicinity of the ColIa immunity and structural genes. Prior to transposition the ColV and ColIa genes were thus linked. The ColV operon has so far been determined only on plasmids of the IncF1 incompatibility group, which share replicon RepFIB, colicin V genes and – in most of the plasmids examined – the aerobactin-uptake

system. On the other hand, examination of ColIa-harbouring plasmids of the *E. coli* Reference Collection (ECOR) showed that ColIa is present on large plasmids with little homology. It was therefore suggested that the ColIa operon or some larger fragment has been transferred between distinct plasmid lineages (26). On pRK100 the conserved ColIa sequences are on one side separated from ColV by approximately 2.6 kb and on the other side, upstream of the conserved RepFIC sequences, by approximately 1.5 kb. Together with the ColIa sequences this is roughly the size of the fragment homologous to different ColIa-carrying plasmids (26). Sequences involved in ColIa transfer could be present in the vicinity of the ColIa and ColV determinants of pRK100.

Using specific probes we demonstrated that pRK100 harbours RepFIB and RepFIC. It is not yet known whether pRK100 carries a complete and functional RepFIC replicon. However, when pRK100 was challenged with an incompatible plasmid, integration of pRK100 into the chromosome or the coexistence of both plasmids was observed, indicating that pRK100 has two functional replication regions. Further, on the basis of binding of probes specific for RepFIC and the *tra* region we demonstrated that in pRK100 the *tra* region is, as in plasmid F, linked to the RepFIC replication region. Other ColV plasmids, including pColV-K30, also harbour RepFIC sequences; however, their locations have not been determined (2).

Colicinogenicity and conjugal transfer contribute to plasmid stability in bacterial populations. Cells, which have lost the plasmid are no longer immune and can be killed by plasmid-harbouring cells. Further, cells, which have lost the plasmid can act as recipients in conjugal transfer. However, our experiments demonstrated pronounced *in vitro* stability of pRK100. Colonies cured of the plasmid were isolated only by SDS treatment. Further, the spontaneous loss of pRK100 upon sub-culturing and storage was never observed. Introduction of an incompatible plasmid could result in integration of pRK100 into the chromosome, probably by recombination between chromosomal and plasmid insertion sequences. Integration of plasmids is known to reduce the expression of plasmid-encoded factors due to differential supercoiling of the integrated plasmid DNA (24). It might also be viewed as a means by which selfish DNA avoids elimination in conditions detrimental to maintenance. The influence of environmental signals in the regulation of integration will be investigated. On plasmids such as pRK100, the maintenance of genes is stable while having, with regard to chromosomal genes, the additional advantage of being able to disseminate through a population by conjugation.

As well as plasmid-encoded determinants, strain KS533 also has chromosomal virulence determinants important for eliciting extraintestinal infections. The K1 capsule is poorly immunogenic and is responsible for immuno-tolerance by the host. Type P fimbriae mediate the initial binding of uropathogenic *E. coli* to its host receptor.

Fifteen per cent of the ECOR strains possess F-related plasmids (5). The *Salmonella* Reference Collection A (SARA) also has approximately the same percentage of F-like plasmids and it has been inferred that F plasmid transfer is an important mechanism of interspecies recombination (6). *Salmonella* IncF1 R plasmids are known to carry the IS*1*-bound aerobactin-uptake system (9) and some *Salmonella* isolates harbour colicin plasmids (1). Besides, some *E. coli* virulence factors might have originated in some other species (20). On the other hand, ColV plasmids have been determined only in *E. coli* even though the replication and transfer regions of plasmid F and ColV plasmids are homologous and exhibit a high degree of sequence conservation (12). It could be that for some reason DNA restriction and modification is more efficient in reducing or eliminating recombinants carrying ColV plasmid than F plasmid recombinants. In our plasmid-transfer experiments only *K. pneumoniae* transconjugants harbouring pRK100 were isolated. By comparing the host range of plasmids RSF2001 and pRK100 we see that the latter cannot be transferred to *S. typhimurium* or *Ent. cloacae* even though both plasmids are transferred to another *E. coli* strain with approximately the same frequencies. The absence of the RepFIA replication region in pRK100 could also be responsible for the plasmid's

limited host range when compared with plasmid F. Plasmid host range is important particularly for transferable plasmids. Conjugal transfer of plasmid-encoded virulence properties to another strain or species could provide quantum leaps toward virulence. The role of RepFIA in plasmid host range will be investigated. As environmental stimuli regulate gene expression and gene transfer (18), experiments will be carried out to determine the *in vivo* stability and transfer of pRK100.

The maps of plasmids pRK100, F and two other known ColV plasmids – pColV-K30 and pColV-B188 – are presented in Fig. 10.

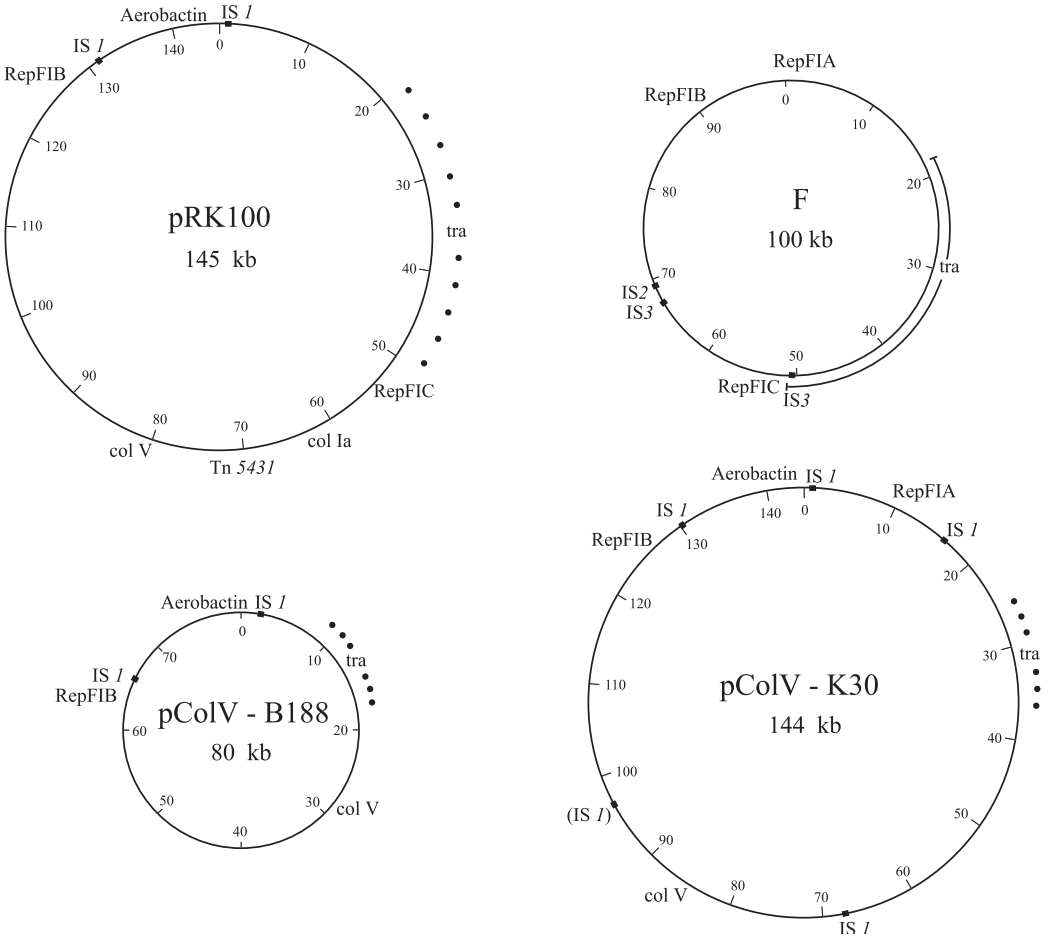

Fig. 10. **Comparison of the map of pRK100 with those of plasmid F (adapted from Neville, *et al.* (21)) and the ColV plasmids pColV-K30 and pColV-B188 (29)**
The location of insertion sequences (IS) is shown by black boxes. The IS*1* copy, which is believed to be partial, is labelled in parentheses. Dotted lines indicate that the location of the *tra* region is approximate, within the limits of available restriction enzyme sites. Numbers within the maps indicate kilobase co-ordinates.

The *tra* regions of the ColV plasmids are approximate, but with regard to the well-studied *tra* region of plasmid F should span about 30 kb. The aerobactin iron-uptake system, which is encoded by most of the ColV plasmids, is found on plasmids and chromosomes among *E. coli*, *Shigella* and

Salmonella species. The ubiquity of the system can be explained by IS*1*-mediated genetic mobility via recombination or probably less frequently by transposition (30). By comparing the maps of the ColV plasmids and plasmid F it can be inferred that acquisition of IS*1* sequences could have been important in the evolution of ColV plasmids. It can be envisaged that the aerobactin system integrated via IS*1*-mediated recombination into the region between the RepFIB and RepFIA replicons of an ancestral F-like plasmid. Recombination between two copies of IS*1* could result in deletion of the RepFIA replication region.

It is evident that pRK100 evolved in several steps. First a segment encoding the ColIa operon was transferred from one plasmid to a ColV plasmid, then transposon Tn*5431* transposed into sequences in the direct vicinity of the ColIa operon. By comparing pRK100 with plasmids F and pColV-K30 (Fig. 10) we see that deletion of the RepFIA replication region could have occurred by IS*1*-mediated recombination. However, other rearrangements must have taken place, as sequences homologous to the RepFIC probe were also detected in the region between RepFIB and the ColV genes. A pronounced clustering of virulence-related properties is also evident in pRK100 when compared with other ColV plasmids. An insertion hotspot could be present in the ColIa region and/or there could be a constraint on insertions in the region between the RepFIB and colicin V genes. The presence of transposable antibiotic resistances on a ColV plasmid is a reflection of the widespread use of antibiotics. The aerobactin iron-uptake system, the *tra* region, the ColV and ColIa genes, and the transposable antibiotic resistance genes together with the replication regions could be considered a stable transferable pathogenicity island.

Plasmid pRK100 and the constructed map will also enable further studies of sequences involved in transfer of the ColIa genes, the role of certain replicons in plasmid host range and gene clustering on large plasmids encoding virulence properties.

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6. REFERENCES

1. **Ayala, F. J., D. E. Krane, and D. L. Hartl.** 1994. Genetic variation in IncI1-ColIb plasmids. *J Mol Evol* **39**: 129–133.
2. **Bergquist, P. L., S. Saadi, and W. K. Maas.** 1986. Distribution of basic replicons having homology with RepFIA, RepFIB, and RepFIC among IncF group plasmids. *Plasmid* **15**:19–34.
3. **Binns, M. M., D. L. Davies, and K. G. Hardy.** 1979. Cloned fragments of the plasmid ColV,I-K94 specifying virulence and serum resistance. *Nature* **279**:778–781.
4. **Blum, G., F. Vincenzo, A. Caprioli, and J. Hacker.** 1995. Gene clusters encoding the cytotoxic necrotizing factor type 1, Prs-fimbriae and *a*-hemolysin form the pathogenicity island II of the uropathogenic *Escherichia coli* strain J96. *FEMS Microbiol Lett* **126**:189–196.
5. **Boyd, F. E., C. W. Hill, S. M. Rich, and D. L. Hartl.** 1996. Mosaic structure of plasmids from natural populations of *Escherichia coli*. *Genetics* **143**:1091–1100.
6. **Boyd, F. E., and D. L. Hartl.** 1997. Recent horizontal transmission of plasmids between natural populations of *Escherichia coli* and *Salmonella enterica*. *J Bacteriol* **179**:1622–1627.

7. **Braun, V., H. Pilsl, and P. Groß.** 1994. Colicins: structures, mode of action, transfer through membranes, and evolution. *Arch Microbiol* **161**:199–206.
8. **Collee, J. G., and R. S. Miles.** 1989. Tests for identification of bacteria, p. 141–160. *In* J. G. Collee, *et al.* (editors), Practical medical microbiology. Churchill Livingstone, Edinburgh.
9. **Colonna, B., M. Nicolette, P. Visca, M. Casalino, P. Valenti, and F. Maimone.** 1985. Composite IS*1* elements encoding hydrozamate-mediated uptake in FI*me* plasmids from epidemic *Salmonella* spp. *J Bacteriol* **162**:307–316.
10. **Couturier, M., F. Bex, P. L. Bergquist, and W. K. Maas.** 1988. Identification and classification of bacterial plasmids. *Microbiol Rev* **52**:375–395.
11. **De Lorenzo, V., A. Bindereif, B. H. Paw, and J. B. Neilands.** 1986. Aerobactin biosynthesis and transport genes of plasmid ColV-K30 in *Escherichia coli* K-12. *J Bacteriol* **165**:570–578.
12. **Gibbs, M. D., A. J. Spiers, and P. L. Bergquist.** 1993. RepFIB: a basic replicon of large plasmids. *Plasmid* **29**: 165–179.
13. **Gilson, L., H. K. Mahatny, and R. Kolter.** 1987. Four plasmid genes are required for colicin V synthesis, export, and immunity. *J Bacteriol* **169**:2466–2470.
14. **Gratia, A.** 1925. Sur un remarquable example d'antagisme entre deux souches de collibacille. *C R Soc Biol* **93**:1041–1042.
15. **Hardy, K. G.** 1987. Plasmids: a practical approach, p. 105–161. IRL Press, Oxford.
16. **Heffron, F., P. Bedinger, J. J. Champoux, and S. Falkow.** 1977. Deletions affecting the transposition of an antibiotic resistance gene. *Proc Natl Acad Sci* **74**:702–706.
17. **Lehrbach, P. R., and P. Broda.** 1984. Molecular comparisons of plasmids isolated from colicinogenic strains of *Escherichia coli*. *J Gen Microbiol* **130**, 401–410.
18. **Mel, S. F., and J. J. Mekalanos.** 1996. Modulation of horizontal gene transfer in pathogenic bacteria by in vivo signals. *Cell* **87**:795–789.
19. **Moll, A., P. A. Manning, and K. N. Timmis.** 1980. Plasmid-determined resistance to serum bactericidal activity: a major outer membrane protein, the *traT* gene product, is responsible for plasmid-specified serum resistance in *Escherichia coli*. *Infect Immun* **28**:359–367.
20. **Mühldorfer, I., and J. Hacker.** 1994. Genetic aspects of *Escherichia coli* virulence. *Microb Pathog* **16**: 171–181.
21. **Neville, F., K. Ippen-Ihler, and R. A. Skurray.** 1996. Structure and function of the F factor and mechanism of conjugation, p. 2377–2401. *In* F. C. Neidhardt, *et al.* (editors), *Escherichia coli* and *Salmonella*, Cellular and molecular biology, 2nd edition. ASM Press, Washington, D. C.
22. **Ørskov, I., and F. Ørskov.** 1992. *Escherichia coli* serotyping and disease in man and animals. *Can J Microbiol* **38**:699–704.
23. **Ott, M., L. Bender, G. Blum, M. Schmittroth, M. Achtman, H. Tschäpe, and J. Hacker.** 1991. Virulence patterns and long-range genetic mapping of extraintestinal *Escherichia coli* K1, K5, and K100 isolates : use of pulse-field gel electrophoresis. *Infect Immun* **59**:2664–2672.
24. **Ott, M.** 1993. Dynamics of the bacterial genome: deletions and integrations as mechanisms of bacterial virulence modulation. *Zentralbl Bakteriol* **278**:457–468.
25. **Pugsley, A. P.** 1985. *Escherichia coli* K12 strains for use in the identification and characterization of colicins. *J Gen Microbiol* **131**:369–376.
26. **Riley, M. A., and D. M. Gordon.** 1992. A survey of Col plasmids in natural isolates of *Escherichia coli* and an investigation into the stability of Col-plasmid lineages. *J Gen Microbiol* **138**:1345–1352.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
28. **Waters, V. L., and J. H. Crosa.** 1986. DNA environment of the aerobactin iron uptake system genes in prototypic ColV plasmids. *J Bacteriol* **167**:647–654.
29. **Waters, V. L., J. F. Perez-Casals, and J. H. Crosa.** 1989. ColV plasmids pColV-B188 and pColV-K30: genetic maps according to restriction enzyme sites and landmark phenotypic characteristics. *Plasmid* **22**:224–248.
30. **Waters, V. L., and J. H. Crosa.** 1991. Colicin V virulence plasmid. *Microbiol Rev* **55**:437–450.
31. **Willets, N., and D. Johnson.** 1981. pED100, a conjugative F plasmid derivative without insertion sequences. *Mol Gen Genet* **182**:520–522.

32. **Wooley, R. E., L. K. Nolan, J. Brown, P. S. Gibbs, and D. I. Bounous.** 1994. Phenotypic expression of recombinant plasmids pKT107 and pHK11 in an avirulent avian *Escherichia coli*. *Avian Dis* **38**:127–134.
33. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–109.
34. **Žgur-Bertok, D., E. Modrič and M. Grabnar.** 1990. Aerobactin uptake system, ColV production and drug resistance encoded by a plasmid from a urinary tract infection *Escherichia coli* strain of human origin. *Can J Microbiol* **36**:297–299.
35. **Žgur-Bertok, D., J. Ambrožič, Z. Podlesek, and M. Grabnar.** 1994. Tn*5431*, a new transposable element composed of Tn*1721*- and Tn*3*-like genes. *Plasmid* **32**:95–99.
36. **Žgur-Bertok, D., J. Ambrožič, and M. Grabnar.** 1996. Tn*5431* arose by transposition of Tn*3* into Tn*1721*. *Can J Microbiol* **42**:1274–1276.