Genet. Res., Camb. (1980), **36**, *pp.* 99–109
With 7 figures
Printed in Great Britain

R68.45, a plasmid with chromosome mobilizing ability (Cma) carries a tandem duplication

By G. Rieß,[1] B. W. Holloway[2] and A. Pühler[1]

[1] *Lehrstuhl für Genetik der Universität Bielefeld, Fakultät für Biologie, Postfach 8640, 4800 Bielefeld 1, Federal Republic of Germany*

[2] *Department of Genetics, Monash University, Wellington Road, Clayton, Victoria, 3168 Australia*

(*Received* 24 *April* 1980)

SUMMARY

Plasmids R68 and R68.45 were transferred from *Pseudomonas aeruginosa* to *Escherichia coli* by conjugation. R68.45 was able to mobilize the *E. coli* chromosome from different origins at a frequency of about 10^{-6}/donor cell. With R68, no transfer of chromosomal genes could be detected. Plasmid R68.45 differs from its parent R68 only by an additional DNA segment, 2120 bp long, located close to the kanamycin resistance gene. By restriction enzyme analysis it was shown that the additional DNA segment of R68.45 is a duplication of a pre-existing DNA region of R68. The duplicated region is characterized by the following sequence of restriction sites: A–310 bp–*Sma*I–70 bp–*Pst*I–795 bp–*Pst*I–15 bp–*Kpn*I–540 bp–*Hpa*I–370 bp–B.

The endpoints A and B of the duplicated region were determined by a heteroduplex experiment between *Hin*dIII linearized molecules of R68 and R68.45. It is proposed that this duplication found in R68.45 is responsible for its chromosome mobilizing ability.

1. INTRODUCTION

Plasmid R68.45 is well-known for its chromosome mobilizing ability (Cma) in various gram-negative bacteria including *Pseudomonas aeruginosa* (Haas & Holloway, 1976), *Rhizobium leguminosarum* (Beringer, Hoggan & Johnston, 1978), *Rhizobium meliloti* (Kondorosi *et al.* 1977; Casadesús & Olivares, 1979), *Rhodopseudomonas sphaeroides* (Sistrom, 1977) and *Agrobacterium tumefaciens* (Hamada, Luckey & Farrand, 1979).

R68.45, a derivative of R68, a member of the broad host range incompatibility group IncP-1 (Summers & Jacoby, 1977), was isolated from *P. aeruginosa* crosses between PAO (R68) donors and PAO recipients in which selection for *argB*$^+$ was made (Haas & Holloway, 1976). R68.45 displays Cma in a much wider range of organisms than R68 (Holloway, 1979). Since R68.45 mobilizes bacterial chromosome from a number of different origins, genetic circularity of the bacterial chromosome could be demonstrated in *P. aeruginosa* strain PAT (Watson & Holloway, 1978), *R. leguminosarum* (Beringer *et al.* 1978), and *R. meliloti* (Kondorosi *et al.* 1977). Until now, very little is known about the molecular basis of Cma in R68.45.

0016-6723/80/2828-8100 $01.00

Jacob, Cresswell & Hedges (1977) showed that R68.45 had acquired an additional piece of DNA of the order of 1·4–1·6 Mdal, and van Montagu and Schell (personal communication) had preliminary evidence for an 1800 base pair insertion situated close to the kanamycin resistance marker of R68.45. We have investigated R68 and R68.45 by restriction analysis and heteroduplex experiments and have found that R68.45 differs from R68 by the duplication of a region of DNA already present in R68.

2. MATERIALS AND METHODS

(i) *Media.* The following media were used: PA: Penassay broth 17·5 g per 1000 ml (Difco, Detroit, USA). DM: minimal medium (Davis & Mingioli, 1950). For solid media 18 g agar per 1000 ml were added. Antibiotic containing agar was supplemented with ampicillin (Ap), 100 μg/ml (Hoechst, Frankfurt, Germany); kanamycin (Km), 50 μg/ml (Gruenenthal, Stolberg, Germany); tetracycline (Tc), 10 μg/ml (Hoechst, Frankfurt, Germany); streptomycin (Sm), 200 μg/ml (Serva, Heidelberg, Germany) or nalidixic acid (Nx), 100 μg/ml (Serva, Heidelberg, Germany). Amino acids (Pro: proline; Trp: tryptophan; His: histidine; Leu: leucine) were added at a final concentration of 20 μg/ml and Thi (thiamine) 1 μg/ml.

(ii) *Matings.* 0·1 ml of a mixture of a logarithmic phase donor and a stationary phase recipient were spread on a membrane filter (Sartorius Type SM 11306) put on Penassay agar. Matings were carried out overnight at 37 °C. In order to harvest the mating mixture the filters were vortexed in saline buffer (Na_2HPO_4 7 g, KH_2PO_4 3 g, NaCl 5 g in 1000 ml H_2O). Appropriate dilutions of this mating suspension were plated on selective agar.

(iii) *Plasmid isolation.* Plasmid DNA was isolated by dye-buoyant-density centrifugation of lysed bacterial cells as described by Radloff, Bauer & Vinograd (1967). Cells were lysed by the Sarcosyl method of Bazaral & Helinski (1968) or by a modified cleared method using Triton X100 as detergent (Cannon *et al.* 1974).

(iv) *Buoyant density determination of plasmid DNA.* DNA preparations for buoyant density determinations contained 1–3 μg DNA in 0·4 ml TE (10 mM Tris, 1 mM EDTA, pH 7·5) and CsCl of suprapure grade (Merck, Germany) at a final concentration of 1·71 g/cm^3. The solutions were centrifuged in a Beckman Model E analytical ultracentrifuge at 44000 rev/min and 25 °C. After 24 h the absorption profiles of the resulting gradients at 262 nm were recorded. The buoyant densities were calculated according to the method of Ifft, Volt & Vinograd (1961) using *Micrococcus lysodeikticus* DNA as reference.

(v) *Electron microscopy of DNA.* The methods of Burkardt *et al.* (1978) were used. ColE1 plasmid DNA was used as an internal length standard of $4{\cdot}2 \times 10^6$ D according to Hershfield *et al.* (1974).

(vi) *Restriction endonuclease digestion of DNA.* Restriction endonucleases *Eco*RI, *Pst*I, and *Sma*I were isolated by Dr A. Rösch, Erlangen. *Kpn*I and *Sal*I were gifts from the Institut für Klinische Virologie, Erlangen. *Hin*dIII was supplied by Boehringer, Mannheim and *Hpa*I by New England Biolabs, Beverly, Ma., U.S.A.

All enzymes except *Sma*I were used in 10 mM Tris, 50 mM-NaCl, 10 mM-$MgCl_2$ pH 7·5 restriction buffer. Appropriate amounts of DNA were digested for 1 h at 37 °C with all enzymes except for *Sma*I, which was used for 2 h at 23 °C. Enzymes were heat-inactivated (65 °C for 10 minutes) and the DNA fragments were separated by agarose gel electrophoresis using a tris-acetate-buffer (40 mM Tris, 10 mM sodium acetate, 1 mM EDTA, pH 7·8).

Phage λ DNA digested with *Eco*RI/*Hin*dIII was used as length reference marker. The lengths of *Eco*RI/*Hin*dIII fragments were assumed to be 21805, 5243, 5047, 4214, 3381, 1960, 1911, 1617, 1323, 931, 882, 588 and 98 bp according to Phillipsen & Davis (personal communication, 1978).

3. RESULTS

(i) *R68.45 mobilizes the* E. coli *chromosome*

The plasmids R68 and R68.45 were transferred from *P. aeruginosa* to *E. coli* by conjugation at a frequency of about 5×10^{-1}/donor cell. With respect to antibiotic resistances and transfer functions, both plasmids were found to be absolutely stable in *E. coli*. Within *E. coli* the frequency for plasmid transfer is about

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Genotype or phenotype	Reference or source
Strain		
Pseudomonas aeruginosa		
PAO25	*argF10, leu-10*, FP$^-$	Haas & Holloway (1976)
PAO8	*met-28, ilv-202, str-1*, FP$^-$	Isaac & Holloway (1968)
Escherichia coli		
CSH51	*ara*, Δ(*lac pro*), *strA, thi*	J. F. Miller (1972)
CSH56	*ara*, Δ(*lac pro*), *nalA, thi*	J. F. Miller (1972)
CSH59	*pyrC, trp, strA, thi*	J. F. Miller (1972)
F122	*str-r*	Strain collection, Lehrstuhl Mikrobiologie, Universität Erlangen
F127	*his, nal-r*	Strain collection, Lehrstuhl Mikrobiologie, Universität Erlangen
Plasmids		
R68	Cb Km Tc	Haas & Holloway (1976)

Genetic symbols are those used for *E. coli* (Bachman, Low & Taylor, 1976), except that *str* designates streptomycin resistance. Plasmid phenotype symbols are those proposed by Novick *et al.* (1976). Details of the isolation of the various ECM plasmids used are given in Table 3.

the same for R68 and R68.45. In contrast, the efficiency of chromosome mobilization is at least 100-fold higher with R68.45 than with R68. The mobilization frequency was similar for all markers tested (Table 2).

Table 2. *Selftransmissability of R68.45 and R68 and mobilization of chromosomal genes in* E. coli *by R68.45 and R68*

Donor strain	Recipient strain	R-plasmid transfer (frequency/donor cell)	Chromosomal gene transfer (frequency/donor cell) selected marker	
F122 (R68.45)	F127	$1{\cdot}2\times10^{-1}$	*his*+	$3{\cdot}3\times10^{-6}$
F122 (R68)	F127	$0{\cdot}3\times10^{-1}$	*his*+	$<2\times10^{-8}$
CSH51 (R68.45)	CSH65	$0{\cdot}7\times10^{-1}$	*leu*+	$1{\cdot}5\times10^{-6}$
CSH51 (R68)	CSH65	$1{\cdot}5\times10^{-1}$	*leu*+	$<2\times10^{-8}$
CSH56 (R68.45)	CSH59	$1{\cdot}2\times10^{-1}$	*trp*+	$4{\cdot}0\times10^{-6}$
CSH56 (R68)	CSH59	$1{\cdot}3\times10^{-1}$	*trp*+	$<2\times10^{-8}$

(ii) *R68.45 differs from R68 by an additional DNA segment of 2120 bp*

For molecular studies R68 and R68.45 plasmid DNA was isolated from *E. coli*. By buoyant density centrifugation in the analytical ultracentrifuge, a density of 1·719 g/cm³ was found for R68 as well as for R68.45. Contour length measurements of DNA from R68 and R68.45 in the electron microscope indicated that the DNA of R68 is 19·1 μm ± 0·3 μm in size whereas that for R68.45 is slightly longer (19·7 μm ± 0·4 μm). This difference could also be demonstrated in a heteroduplex experiment. A typical heteroduplex molecule between R68 and R68.45 and its interpretation is shown in Fig. 1. The additional DNA segment of 0·6 μm can be located with reference to the outlooped DNA structure of transposon Tn1, and is situated 9·1 μm or 8·4 μm away from it. The outlooped structure is due to the inverted repeats of Tn1 in each single-strand forming stems, such that after renaturation of the Watson and Crick strand two loops can be distinguished in the heteroduplex molecule at the position where Tn1 is inserted (Burkardt, Rieß & Pühler, 1979*b*).

Plasmid DNA of R68 and R68.45 was digested with the restriction endonucleases *Eco*RI, *Hin*dIII, *Pst*I, and *Sma*I. Like R68, R68.45 carries only one restriction site each for *Eco*RI and *Hin*dIII. In contrast, R68.45 gave an additional fragment of 2120 bp ± 10 bp in the *Sma*I digest and two additional fragments, one 1325 bp ± 10 bp and the other 795 bp ± 10 bp in the *Pst*I digest.

The new 795 bp fragment of R68.45 has the same length as the *Pst*I fragment 5 of R68. *Sma*I and *Pst*I restriction patterns of R68 and R68.45 are shown in Fig. 2. The length determination of 2120 bp by gel electrophoresis for the additional DNA segment of R68.45 is in good agreement with the electron microscope length determinations for R68 and R68.45 molecules. For fragments of this size gel electrophoresis is more precise than electron microscopy so we have taken 2120 bp as the more accurate determination of the length of this fragment.

(iii) *Independently isolated Cma plasmids derived from R68 are identical*

The term Enhanced Chromosome Mobilizing plasmids (ECM) has been proposed for plasmids like R68.45 (Holloway, Krishnapillai & Morgan, 1979*b*) to distinguish

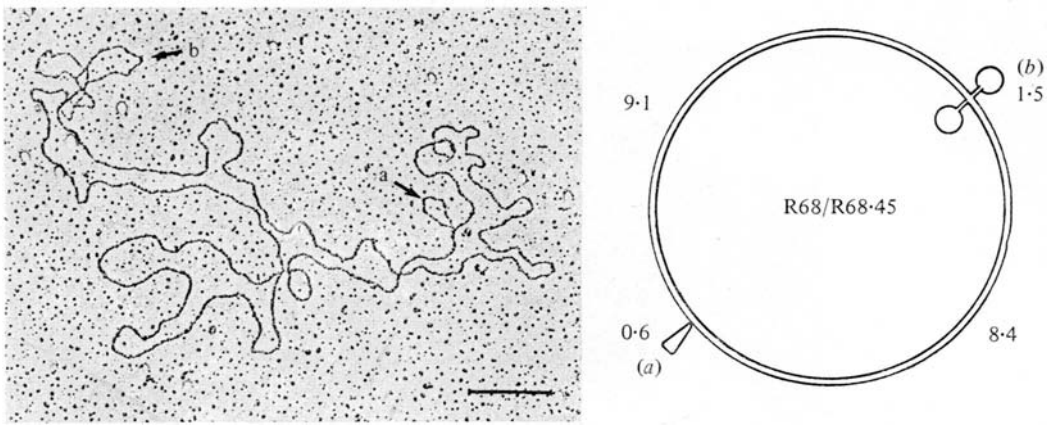

Fig. 1. Heteroduplex molecule between R68 and R68.45 and its interpretation. Lengths are given in μm and were calculated from 5 different molecules. The bar represents 0·5 μm.

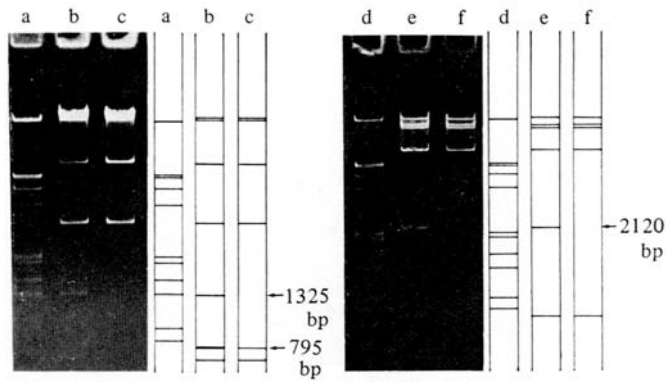

Fig. 2. *Pst*I and *Sma*I fragments of R68 and R68.45 DNA separated on agarose gels. R68 and R68.45 DNA was digested by the restriction endonucleases *Pst*I and *Sma*I. The fragments were separated by agarose gel electrophoresis (1% agarose in tris-acetate-buffer, 100 V, 3 h). Significant differences between R68 and R68.45 are indicated by arrows. The lanes show: (*a*) λ *Eco*RI/*Hin*dIII; (*b*) R68.45 *Pst*I; (*c*) R68 *Pst*I; (*d*) λ *Eco*RI/*Hin*dIII; (*e*) R68.45 *Sma*I; (*f*) R68 *Sma*I.

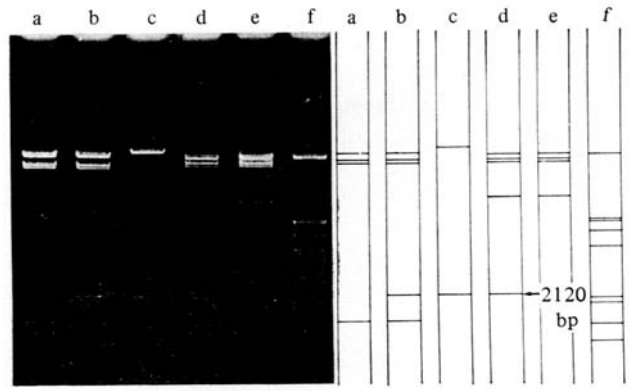

Fig. 3. Restriction endonuclease digestion of R68 and R68.45 DNA with *Kpn*I, *Hpa*I, and *Sma*I. R68 and R68.45 DNA was digested by the restriction endonucleases *Kpn*I, *Hpa*I (R68.45 only), and *Sma*I. The fragments were separated by agarose gel electrophoresis (0·9% agarose in tris-acetate-buffer, 100 V, 2·5 h). The significant difference between R68 and R68.45 is indicated by an arrow. The lanes are: (*a*) R68 *Kpn*I; (*b*) R68.45 *Kpn*I; (*c*) R68.45 *Hpa*I; (*d*) R68.45 *Sma*I; (*e*) R68 *Sma*I; (*f*) λ *Eco*RI/*Hin*dIII.

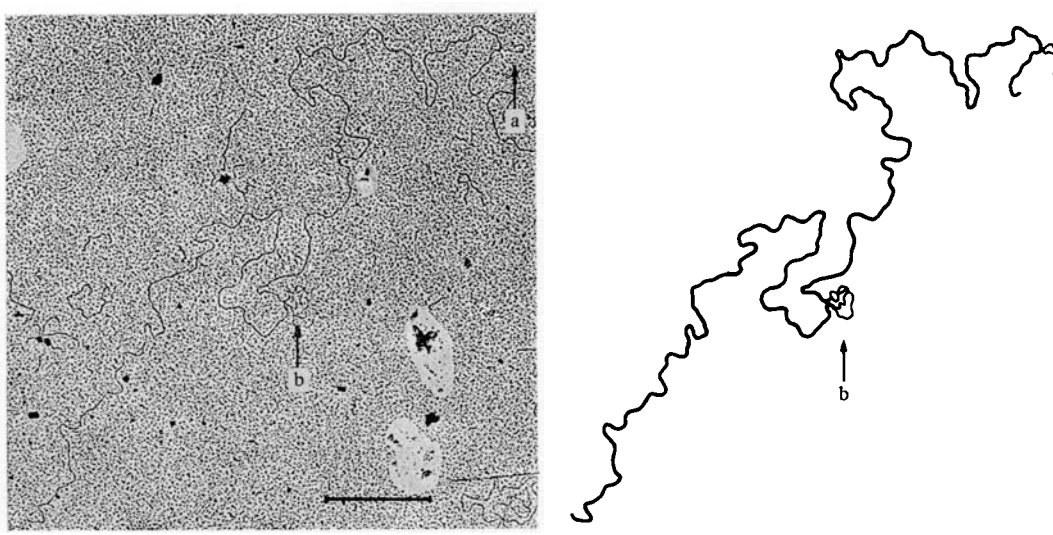

Fig. 5. Heteroduplex molecule between R68 and R68.45 DNA linearized by *Hin*dIII and its interpretation. The bar represents 1 μm.

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them from native Cma plasmids like FP2. A variety of plasmids of this type have now been isolated using selection for different regions of the *P. aeruginosa* PAO chromosome (Holloway, Haas & Morgan, 1979*a*). These were obtained from *P. aeruginosa* crosses using different auxotrophic parental strains and R68 as donor plasmid. Prototrophic recombinants appeared at low frequency, and some of these contained R68 plasmids with Cma. They could be detected by their

Table 3. *Independently isolated ECM plasmids*

Plasmid	Method of isolation
R68.45	From R68, selection for *arg*B$^+$ (Haas & Holloway, 1976)
pMO47	From R18, selection for *hisI V*$^+$ (14′)
pMO60	A native IncP-1 plasmid obtained from a hospital strain of *P. aeruginosa* isolated by H. Matsumoto in Japan and shown by M. Nayudu to have Cma. Its antibiotic resistance phenotype is Cb, Km, Tc
pMO61	From R68, selection for *hisI*$^+$ (13′)
pMO62	From R68, selection for *ilvD*$^+$ (30′)
pMO90	From R68, selection for *cys-5605*$^+$ and *hisI*$^+$ (13′)
pMO91	From R68, selection for *cys-5605*$^+$ and *hisI*$^+$ (13′)
pMO92	From R68, selection for *leu-9001*$^+$ (60′)
pMO93	From R68, selection for *argG*$^+$ (55′)
pMO94	From R68, selection for *ilvD*$^+$ (30′)

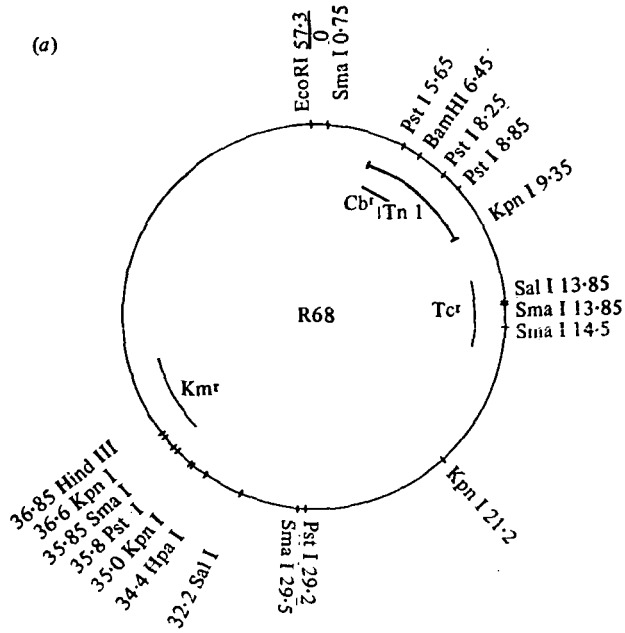
The procedure used for isolating all these above plasmids (except pMO60) was the same as that used in the isolation of R68.45 (Haas & Holloway, 1976). In each case selection was made for an auxotrophic marker in a cross of the type PAO (*aux*$^-$) × PAO (R plasmid), and the recombinants were examined for the presence of an R plasmid with increased Cma in PAO × PAO (R) matings. pMO47 was isolated by M. Nayudu, pMO60, pMO61, pMO62, pMO90, pMO91, pMO92, pMO93 and pMO94 were isolated by C. Crowther.

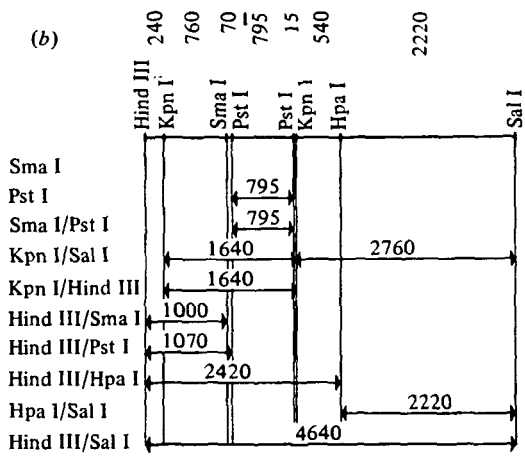
enhanced mobilization activity in further *P. aeruginosa* crosses. Table 3 lists eight such Cma plasmids called pMO47, pMO61, pMO62, pMO90, pMO91, pMO92, pMO93 and pMO94, all isolated in *P. aeruginosa*, and pMO60, a naturally occurring plasmid.

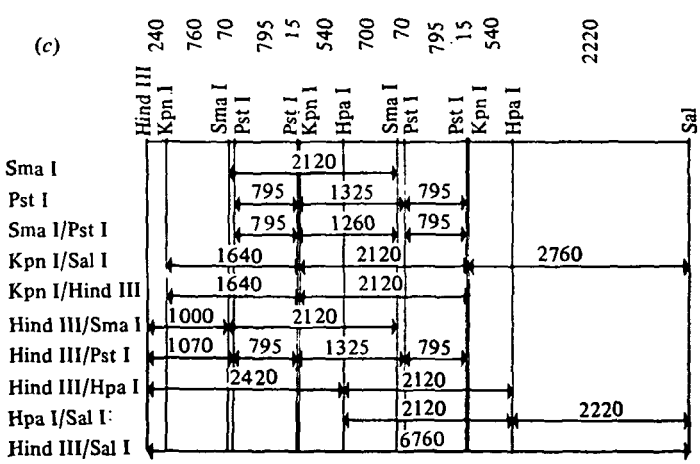
The pMO plasmids were transferred from *P. aeruginosa* to *E. coli* by conjugation and plasmid DNA was isolated from these strains. The *Pst*I and *Sma*I restriction patterns of all pMO plasmids including pMO60, the natural isolate from Japan, were identical to that of R68.45, and gave the same additional DNA segment(s) compared to R68 (data not shown).

(iv) *The additional 2120 bp segment of R68.45 is a duplicated region of R68*

In order to learn more about the additional DNA segment of R68.45, plasmid DNA of R68 and R68.45 was digested with the restriction enzymes *Sal*I, *Hpa*I, and *Kpn*I. In contrast to R68, R68.45 DNA gave an additional fragment of 2120 bp ± 10 bp in the *Kpn*I as well as in the *Hpa*I digest. Fig. 3 compares the restriction patterns of R68 and R68.45 using *Sma*I, *Kpn*I and *Hpa*I. Digestion of







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R68 and R68.45 DNA with restriction endonuclease *Sal*I generated no additional fragment for R68.45, indicating that the duplicated region does not contain a *Sal*I site. However in a *Sal*I/*Hin*dIII double digestion of R68 and R68.45 DNA, the fragment patterns were different: in the R68 pattern there is a 4640 bp ± 20 bp band whereas for R68.45 this is replaced by a 6760 bp ± 20 bp fragment. These two fragments show a difference of 2120 bp in length.

From Fig. 1 it can be seen that R68.45 carries an additional DNA segment not found in R68. This is located on the plasmid chromosome opposite to the location of Tn1 and near the kanamycin resistance gene and the single *Hin*dIII restriction site of RP4 (Barth & Grinter, 1977). When R68 and R68.45 undergo double digestion with *Eco*RI/*Hin*dIII, the results confirm that there is an additional DNA segment integrated into the larger *Eco*RI/*Hin*dIII fragment (Fig. 4*a*).

Utilizing data from double digestions with *Sma*I/*Hin*dIII, *Pst*I/*Hin*dIII, *Pst*I/*Sma*I, *Kpn*I/*Hin*dIII, *Hpa*I/*Hin*dIII, *Sal*I/*Hin*dIII, *Sal*I/*Kpn*I, and *Sal*I/*Hpa*I, a detailed restriction map of the region of the DNA of R68 and R68.45 near the kanamycin resistance gene was constructed. These results are summarized in Figs. 4*b*, *c*. It can be concluded that a DNA segment, pre-existing in R68 and with the following sequence of restriction endonuclease sites appears to be duplicated in R68.45:

*Sma*I–*Pst*I–*Pst*I–*Kpn*I–*Hpa*I.

The duplication hypothesis is confirmed by fragment length measurements: In *Sma*I, *Kpn*I, and *Hpa*I digestions of R68.45 a 2120 bp fragment is always found. The distance between the pairs of *Pst*I sites in the original region and in the duplicated region is also 2120 bp. Likewise the increased size of the *Hin*dIII/*Sal*I fragment of R68.45 compared to R68 is 2120 bp. However the exact location of the duplicated region cannot be determined solely from the restriction data shown in Fig. 4*c*.

(v) *Determination of the precise location of the duplicated region of R68.45 by heteroduplex analysis*

A heteroduplex experiment between R68 and R68.45 plasmid DNA linearized by the restriction endonuclease *Hin*dIII should enable the precise location of the

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FIGURE 4

Fig. 4(*a*–*c*). Restriction maps of plasmids R68 and R68.45.

(*a*) A restriction map of R68. The distances of the different restriction sites to the *Eco*RI site are given in kb. 57·3 kb were taken for the contour length of R68. The *Sal*I and *Kpn*I restriction sites on plasmid R68 were mapped during this work. The precise mapping of the *Hin*dIII/*Sal*I fragment of R68 (32·2 kb–36.85 kb) is shown in Fig. 4*b*. Our R68 map is in good agreement with restriction maps of RP1 (Grinsted, Bennett & Richmond, 1977), of RP4 (DePicker, Van Montagu & Schell, 1979; Priefer *et al.* 1980), and RK2 (Meyer, Figurski & Helinski, 1977).

(*b*) A detailed restriction map of the *Hin*dIII/*Sal*I fragment of R68 (32·2 kb–36·85 kb). The distances are given in bp.

(*c*) A detailed restriction map of the *Hin*dIII/*Sal*I fragment of R68.45 constructed in the same manner as Fig. 4*b*.

duplicated region to be determined. A typical heteroduplex molecule between R68 and R68.45 DNA linearized by *Hin*dIII and its interpretation are shown in Fig. 5. Very close to one end of the molecule there is a single-stranded loop representing the duplicated region of R68.45. In addition, in the middle of the linear molecule a loop structure can be recognized formed by the renaturation behaviour of transposon Tn1.

The distance between the site of insertion of the single-stranded DNA loop and the *Hin*dIII-generated end of the heteroduplex molecule was measured. The sites of loop formation for 28 different heteroduplex molecules were examined and the results are shown in Fig. 6. They occur in an apparently random fashion over

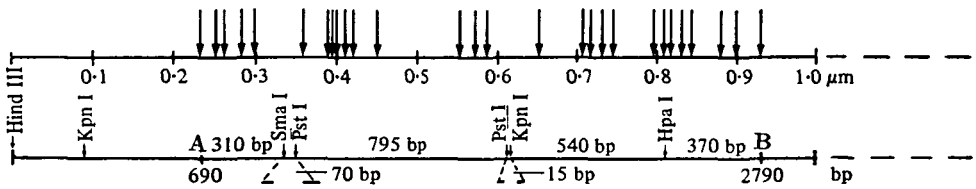

Fig. 6. Distribution of the loop positions of R68/R68·45 heteroduplex molecules in relation to the restriction map of R68. Twenty-eight loop positions of R68/R68.45 heteroduplex molecules were determined by measuring their distances from the *Hin*dIII site of R68. The positions of the different loops are marked by arrows. The R68 restriction map is drawn to scale. The loops are distributed within a region of the R68 map with extremes marked A and B.

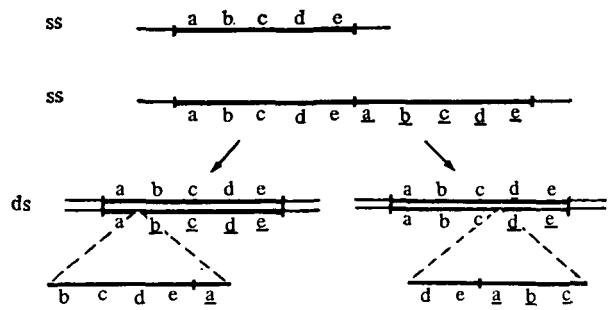

Fig. 7. Heteroduplex formation between two molecules differing in a duplicated region. The two heteroduplex partners are identical except for the tandem duplication of region *a*, *b*, *c*, *d*, *e*. Many different heteroduplex molecules are possible, all of them carrying a loop somewhere within the region *a*, *b*, *c*, *d*, *e*. Two possible heteroduplex molecules are drawn.

a region beginning 0·23 μm from the *Hin*dIII site and extending for 0·7 μm. An explanation as to how the different loop sites arise is shown in Fig. 7. Because the heteroduplex molecule results from the interaction of a duplicated and a non-duplicated region, loops can arise from any point within the region which is duplicated in R68.45. The results enable the most likely location of the duplicated region to be determined: it starts at point A, 690 bp from the *Hin*dIII site and finishes at point B, 2790 bp from the *Hin*dIII site (Fig. 6).

4. DISCUSSION

R68 and R68.45 show no detectable difference in plasmid transfer frequency, stability of plasmid markers in *E. coli* or buoyant density. However, in contrast to R68, the plasmid R68.45 is able to mobilize the bacterial chromosome in *E. coli*. This mobilization occurs from different sites of origin on the chromosome with about the same frequency. These observations are similar to results obtained with R68.45 in chromosome mobilization studies using other bacteria (Haas & Holloway, 1976; Sistrom, 1977; Beringer & Hopwood, 1976; Kondorosi *et al.* 1977; Watson & Holloway, 1978; Casadesús & Olivares, 1979; Hamada *et al.*, 1979). From contour length measurements and heteroduplex experiments we have found that R68.45 differs from its parent R68 by an additional DNA segment of about 0·6 μm. This result is in good agreement with the observation published by Jacob *et al.* that R68.45 is 1·4 Mdal longer than its parent R68.

Restriction analysis showed that this additional DNA segment is 2120 bp long and carries two *Pst*I and one *Sma*I restriction sites. These additional *Pst*I and *Sma*I restriction sites were also found for a range of ECM plasmids constructed in the laboratory and one Cma plasmid isolated from a hospital strain of *P. aeruginosa* (pMO60). This indicates that selection for plasmids with enhanced chromosome mobilization ability is not restricted entirely to laboratory conditions. We conclude that all these different plasmids with Cma are similar in this region and possess the same additional DNA segment.

By restriction endonuclease analysis we have found that the additional DNA segment of R68.45 is a duplication of a region of DNA already present in R68. The existence of this duplication was confirmed by heteroduplex experiments between R68.45 and R68 linearized by *Hin*dIII. Unexpectedly, the loop positions were distributed randomly within a special DNA segment of R68. This observed distribution in position can be satisfactorily explained as an outcome of the tandem duplication of a region of the DNA (Fig. 7). The demonstration of this duplicated segment of DNA in R68.45 now makes very unlikely a previous suggestion that R68.45 arose by means of an insertion-sequence-like element of *P. aeruginosa* integrated into a specific site on the R68 chromosome (Holloway, 1979; Holloway *et al.* 1979*b*; Burkardt *et al.* 1979*a*).

It is interesting to speculate about the origin and function of the duplicated region found in R68.45. We assume that it arises by a mechanism similar to that responsible for intramolecular amplification of antibiotic resistance genes. Yagi & Clewell (1977) reported that the amplification of a Tc^R determinant on plasmid pAMα1 in *Streptococcus faecalis* is dependent on identical sequences in direct repeats flanking the Tc^R determinant. These direct repeats are evidently involved in recombinational amplification. A similar model was published by Schöffl & Pühler (1979) for the amplification of the Tc^R determinant of transposon Tn1771 in *Escherichia coli*. Taking into account these results it is possible that the region of R68 duplicated in R68.45 is also flanked by short direct repeats. It should be noted that we have not entirely excluded the possibility that the duplicated

region of R68.45 arises by transfer of bacterial chromosomal material to R68. It is known that the region of the bacterial chromosome which is selected for the isolation of ECM plasmids is important in that selection for different regions of the chromosome can result in up to 500-fold variation in the frequency of isolation of ECM plasmids (Holloway, Haas & Morgan, 1979*a*). If generation of ECM plasmids is a genetic event solely involving the plasmid DNA, it is difficult to see how selection for different bacterial chromosome regions would have such a marked effect on their generation. Since the additional DNA segment is the only difference between R68 and R68.45 DNA, it is reasonable to assume that this region is responsible for the chromosome mobilizing ability of R68.45. As yet there is little to indicate how such a region can function to mobilize the bacterial chromosome.

This work was supported by a grant of Deutsche Forschungsgemeinschaft (Pu 28/8) and the Australian Research Grants Committee. We would like to thank Carol Crowther and Murali Nayudu for providing various ECM plasmids.

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