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Maintenance and incompatibility of plasmids carrying the replication origin of the *Escherichia coli* chromosome: evidence for a control region of replication between *oriC* and *asnA*

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ABSTRACT

Plasmids that replicate only by means of the cloned Escherichia coli replication origin (oriC) are called minichromosomes or oriC-plasmids. In this paper it is shown that sequences located between oriC and asnA are involved in maintenance and incompatibility of minichromosomes. These sequences include part of the 16kD and 17kD genes, previously allocated within this region (1,2).

Transcription towards oriC that is initiated at the 16kD promoter, specifically enhances the stability and copy-number of minichromosomes.

Three regions are involved in minichromosome incompatibility. One region, incA, includes the minimal oriC sequence. A second, incB, maps within a 210 base pairs fragment that overlaps the 16kD promoter. The third, incC, encompasses the 17kD gene. Neither one of the regions expresses incompatibility on its own, but the additional presence of one of the others is required.

The data presented indicate that sequences of the 16kD and 17kD genes are part of the replication control system of oriC-plasmids.

INTRODUCTION

Replication of the Escherichia coli chromosome initiates at an unique site, the replication origin (oriC) and proceeds bidirectionally (3). Extensive analysis of this replication origin was facilitated by the isolation of relatively small replicons which contained oriC. oriC has been obtained either as part of a phage or plasmid hybrid (4-6), or as a minichromosome which contains oriC as the single replication origin (7-9). Initiation of replication on minichromosomes resembles that on the E. coli chromosome by its dependency on RNA polymerase mediated transcription, de novo protein synthesis and functional dnaA and dnaC products (9). The nucleotide sequence of the oriC region has been determined (10, 11) and the sequences required for autonomous replication were delimited to a specific region of 245 base pairs (6). The development of an in vitro system for replication of oriC-plasmids renders minichromosomes as a very attractive model system for the study of chromosomal initiation of replication (12). However, there are indications that replication control of oriC-plasmids is not completely identical to that of the chro-

mosome. oriC-plasmids are present in E. coli in numbers that exceed the chromosome three to ten-fold (13). Moreover, these additional copies of oriC in the cell do not seem to interfere with chromosomal initiation of replication (9).

Although the minimal origin sequence of 245 base pairs allows autonomous plasmid replication, adjacent sequences may be present that have more subtle regulatory functions. For example, DNA sequences immediately to the right of oriC are required for efficient bidirectional replication in vivo (14) and they contain a binding site for a membrane protein (15). An incompatibility determinant of oriC-plasmids has recently been localized in this region (16; incB). The copy-number of minichromosomes is not only influenced by neighbouring sequences to the left of oriC (17), but possibly by sequences to the right as well (6). Two genes encoding 16kD and 17kD proteins, that have unknown functions, have been mapped immediately to the right of oriC (1, 2; Fig. 2 and 4). The 16kD protein is well-conserved in a number of gram-negative bacterial species (18) and the leftward transcription from its promoter proceeds past the right end border of the minimal oriC sequence, where it is efficiently terminated (2).

In this paper we have analyzed which sequences in the above mentioned region, between oriC and asnA, can affect minichromosome replication in cis or in trans. For this analysis we used minichromosome pCM959 that consists exclusively of chromosomal DNA and which sequence has been determined (9, 10, 19). Moreover, it has a relatively high copy-number and is inherited rather stable during cell growth when compared to other minichromosomes (this paper). We could show by introducing deletions and insertions into pCM959 that the sequence of the 16kD gene promoter has a positive effect on minichromsome replication (cis-action). Incompatibility experiments performed by cloning fragments of pCM959 into high copy-number vectors showed that three specific regions, including the 16kD promoter, affect minichromosome replication in trans. However, in none of these experiments an interference with host chromosomal replication was observed. The implications of these results will be discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids

All strains are E. coli K12 derivatives. CM987 F^- asnA asnB thi λ^s recA1 is a derivative of strain ER (10). M152 F^- galK rpsL λ^- recA3 (20) and N23-53 F^- thr galT trp phe tyr rpsL λ^- recA41 (21) were obtained from B. Bachmann.

Minichromosomes used are pCM959 and pCM961 (9). oriC-plasmid pEM120 was made by insertion of an Ava II fragment conferring kanamycin resistance (Tn903), into the Eco RI site of pCM961. Plasmids pEM35, pEM123, pEM124, pEM127, pEM134 and pEM190 are insertion or deletion derivatives of minichromosome pCM959 and are described in the Results section.

For the construction of oriC-vector hybrids, pCM959 or its derivatives served as the source of DNA fragments inserted in pBR322 or pAT153. The oriC-vector hybrids are listed in Table I.

Enzymes

Restriction enzymes, calf intestinal phosphatase, T_4 DNA ligase, polynucleotide kinase and nuclease BAL-31 were from New England Biolabs, Boehringer Mannheim or Amersham. Nuclease S1 was from Sigma. These enzymes were used according to the manufacturer's recommendations.

DNA Sequencing.

The chemical method of Maxam and Gilbert (24) was used to determine the extent of the deletions generated by BAL-31 in pEM123 and pEM124. In plasmid pEM123 the deletion endpoints are at positions 385 and 497 and in plasmid pEM124 at positions 352 and 1184. The method was also employed to analyze the sequence at the junction of the deletions generated in plasmids obtained after a blunt-end ligation procedure that followed nuclease S1 treatment. The deletion endpoints have been determined for the following plasmids: pEM127 (positions 289 and 727), pEM193 (position 781), pEM197 (position 418 and 781), pEM198 (position 418 and 835).

Bacterial Transformation.

E. coli cells were made competent and were transformed with plasmids essentially according to Cohen et al. (25) or Maniatis et al. (26).

Plasmid Isolation and Copy-number Determination.

Plasmid DNA was isolated by a scaled-up procedure of the alkaline lysis method of Birnboim and Doly (27). If necessary the DNA was further purified by ethidiumbromide-CsCl isopycnic centrifugation or Bio-Gel A50m chromatography. Extraction of plasmid DNA from small cultures was by the method of Holmes and Quigley (28). Copy-numbers were determined in total lysates from cultures subjected to propidium iodide-CsCl equilibrium density centrifugation (8).

Assay for Plasmid Maintenance and Incompatibility.

The segregation kinetics of minichromosomes in E. coli recA strains were determined as follows. A fresh colony from a transformation plate was inoculated in medium and grown under selective conditions. For pCM959 and some of its derivatives in strain CM987 this is enriched minimal medium with selection

Table I. List of oriC-vector hybrid plasmids[a]

Plasmid	Relevant Properties[b]	Source, reference or construction[c]
pEM90	Tc^r oriC$^+$ asnA$^+$	pCM959 at PstI (position 2566):: PstI of pBR322. Orientation II
pEM174	Tc^r oriC$^+$	ΔMluI fragment (position 777 to 1793) of pEM90
pEM73	Tc^r oriC$^+$ asnA$^+$	pEM124 at PstI (position 2566):: PstI of pBR322. Orientation I
pEM132	Tc^r oriC$^+$ asnA$^+$	pEM127 at PstI (position 2566):: PstI of pBR322. Orientation II
pEM88	Tc^r oriC$^+$ asnA$^+$	pCM959 at PstI (position 488):: PstI of pBR322. Orientation I
pEM89	Tc^r oriC$^+$ asnA$^+$	Same as pEM88. Orientation II
pEM199	Tc^r oriC$^+$	PvuI fragment (position-241 to 934) pEM127:: PvuI of pBR322. Orientation I
pOC67	Tc^r oriC$^+$	HindII fragment (position-178 to 1495) of pCM959:: Hind II of pBR322 (position 3906*). Orientation I. From W. Messer
pEM193	Tc^r	Δ PstI-MluI fragment (position 3608* to 777) of pOC67
pEM197	Tc^r oriC$^+$	Δ XhoI-MluI fragment (position 417 to 777) of pOC67
pEM198	Tc^r oriC$^+$	Same as pEM 197 but deletion to position 835 by the nuclease S1 treatment
pEM179	Tc^r	ΔPstI fragment (position 3608* to 488) of pOC67
pEM204	Tc^r	PstI-HindIII fragment (position 3608* to 29*) of pEM179 cloned in same sites of pAT153
pEM171	Tc^r asnA$^+$	PstI fragment (position 488 to 2566) of pCM959:: PstI of pBR322. Orientation I
pEM108	Tc^r asnA$^+$	Same as pEM171. Orientation II
pEM172	Tc^r	ΔPvuI fragment (position 934 to 3734*) of pEM171
pEM208	Tc^r	PstI-HindIII fragment (position 3608* to 29*) of pEM172 cloned in same sites of pAT153
pOC62	Tc^r oriC$^+$	PvuI fragment (position-241 to 934) of pCM959:: PvuI of pBR322. Orientation I. From W. Messer
pEM175	Tc^r oriC$^+$	Δ XhoI-MluI fragment (position 417 to 777) of pOC62
pOC42	Tc^r oriC$^+$	PstI fragment (position 2566 to 488) of pCM959:: PstI of pBR322. Orientation I (22)
pEM203	Tc^r oriC$^+$	Same as pOC42 but in pAT153

[a] The term oriC-vector hybrid plasmids indicates the cloning of a fragment of pCM959 into pBR322 or pAT153. It does not refer to the actual presence of the functional oriC sequence.

[b] oriC$^+$ indicates that the minimal DNA fragment required for autonomous replication is present. Tc^r is resistance to tetracycline

[c] Orientation I and II indicate the orientation of the cloned sequences against the vector DNA. In orientation I the direction of transcription of the Ap gene of pBR322 is from right to left with respect to the inserted chromosomal sequences (Fig. 4A). Symbols Δ and :: mean a deletion and insertion, respectively. Map positions are shown in Fig. 4A. Symbol * indicates a position in the pBR322 sequence (23).

for asn$^+$ (9). Strains with minichromosomes having an antibiotic resistance marker were grown in TY broth (8) supplemented with ampicillin (50 µg/ml) or kanamycin (50 µg/ml). At a cell density of about 1 x 10^8/ml the culture was diluted in fresh medium without selection for the oriC-plasmid and was kept in exponential phase. At intervals aliquots were spread on TY agar plates. After incubation at 37°C at least 100 single colonies were tested for the presence of the asnA$^+$ or antibiotic resistance marker.

The rate of exclusion of oriC-plasmid pEM120 from strain CM987 originally harbouring both pEM120 and one of the oriC-vector hybrids was determined in a similar way. In this case, initial growth in TY broth with selection for both plasmids is subsequently followed by growth in broth with selection for the oriC-vector hybrid plasmid only (tetracyclin; 10 µg/ml). Samples are spread on TY agar plates containing tetracyclin and single colonies were tested for resistance to kanamycin.

RESULTS

Comparison of the stability of minichromosomes.

In the absence of selective pressure minichromosomes are rapidly lost from a bacterial population (7-9). This poor stability has hampered a study on incompatibility of oriC-plasmids. Recently, Yamaguchi et al. (16) claimed that oriC-plasmids are better maintained in certain recA3 or recA41 strains. However, we observed that the minichromosomes pOC15 and pOC24 (29), which are comparable to the plasmids used by Yamaguchi et al. (16), are as unstable in strains M152 recA3 (Fig. 1A) and N23-53 recA41 as in other recA strains (not shown). The discrepancy between these results is not clear to us.

Furthermore, the minichromosomes pEM120 and pCM959 are maintained to a better extent, irrespective of the recA strain used (Fig. 1 and 3). More than 60% of the cells still contain the plasmids after 15 generations of growth in the absence of selective conditions. The increased stability of pEM120 and pCM959 compared to pOC15 and pOC24 most likely reflects a higher plasmid copy-number. Whereas the copy-number of pCM959 is about 9 (Table II; 13), that of pOC15 and pOC24 is about 4 (13, 30). The relatively high stability of pEM120 and pCM959 allowed us to analyze not only incompatibility properties of oriC-plasmids but also to perform more detailed studies on the maintenance of minichromosomes.

Maintenance of pCM959 derivatives.

In minichromosome pEM35 the 16kD gene was destroyed by inserting an AvaII fragment, conferring kanamycin resistance, into the single ClaI site of pCM959 (Fig. 2). Compared with pCM959, pEM35 is a very unstable oriC-plasmid (Fig. 1B). The reduction in stability might be caused by inactivating the 16kD protein or some specific sequence in this region. These two possibilities can be ruled out by plasmids pEM123 and pEM127. pEM123 is a 112 base pairs BAL-31 generated deletion at the XhoI site of pCM959 (Fig. 2). pEM127 is a AccI-EcoRV deletion of pCM959 that covers almost the complete coding region for the 16kD protein without affecting the promoter sequence of the gene (Fig. 2). Both

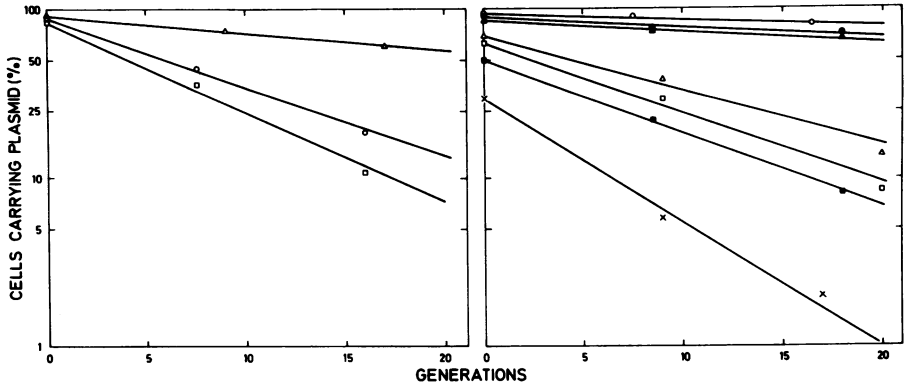

Fig. 1. A. Rate of loss of minichromosomes from growing M152 recA3 cells. The first point on each plot represents the percentage of plasmid containing cells at the time when cells grown in selective medium are diluted into non-selective medium. Plasmids tested are pEM120 (Δ), pOC15 (o) and pOC24 (□). B. Rate of loss of minichromosomes from growing CM987 recA1 cells. Plasmids tested are pCM959 (●), pEM127 (o), pEM123 (▲), pEM190 (Δ), pEM35 (□), pEM124 (■) and pEM134 (x). The assay for maintenance of pEM134 was performed at 30°C.

plasmids are maintained as stable as pCM959 (Fig. 1B). By removing 832 base pairs with BAL-31 from XhoI cut pCM959 DNA, a plasmid was obtained (pEM124) with an impaired stability (Fig. 1B, 2). In this plasmid the deletion has proceeded beyond the 16kD promoter region into the 17kD gene. Sequence analysis

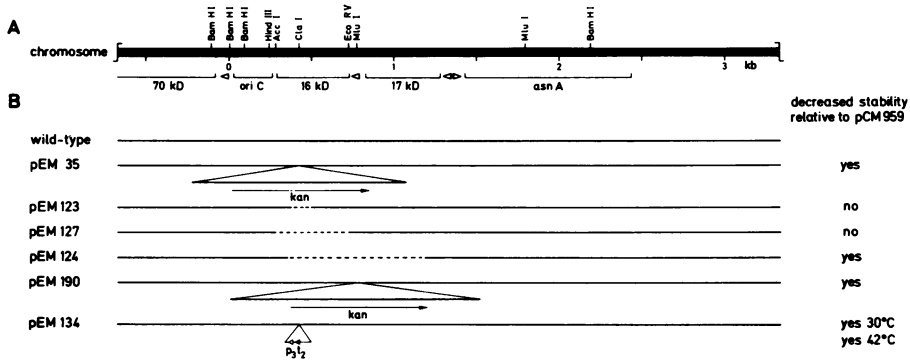

Fig. 2 Construction of pCM959 derivatives.
A. Linear map of pCM959 opened at the point of circularization (19) of the E. coli chromosome. Arrows (▷) indicate promoter regions and direction of transcription.
B. Maps of pCM959 insertion and deletion derivatives. Dotted lines represent the size and position of deletions. (—►) indicates the direction of transcription of the kanamycin resistance gene. (◁) and (◄) indicate the position and orientation of the promoter of the preprimer RNA and of the temperature sensitive terminator of gene H, respectively (32).

defined the endpoints of the deletion at position 352 and 1184 of the oriC map (10, 11). The right end boundary of the region required for autonomous replication (position 267; 6) is thus unaffected in pEM124.

Previous studies demonstrated that transcription initiated at the 17kD gene promoter is relatively weak compared with transcription initiated at the 16kD gene promoter (2; our unpublished observations). Therefore, it seems likely that the reduced stability of pEM124 is caused by a diminished transcription entering oriC. The importance of the 16kD promoter mediated transcription is supported by the significant difference in stability between oriC-plasmids pEM190 and pEM205. In these plasmids an MluI fragment conferring kanamycin resistance, derived from Tn903 (pKT071; 31) was inserted in either one of the two MluI restriction sites on pCM959 (19; Fig. 2). In plasmid pEM190 this insertion is at the MluI site at position 777 and thereby destroys the 16kD gene promoter. In plasmid pEM205 the insertion is in the asnA gene and has no effect on the stability (not shown).

To determine whether an increased level of transcription entering oriC may also interfere with the stability of minichromosomes plasmid pEM134 was constructed. In this oriC-plasmid a 160 base pairs TaqI-Hpa II fragment derived from Clo DF13 cop 1 ts that contains the rho-independent temperature sensitive transcription terminator t_2(ts) and the primer RNA promoter region P3 (32; A. v. Putten personal communication) has been inserted into the ClaI site of pCM959. The orientation of the cloned fragment in pEM134 is such that transcription from the primer promoter is in the direction of oriC (Fig. 2). At 30°C only transcription from the primer promoter should reach oriC. At 42°C, when the transcription terminator is not functioning, additional transcripts from the 16kD gene promoter will run into oriC. As shown in Fig. 1B a very drastic reduction in stability of pEM134 is observed at 30°C. This instability is more prominent at 42°C, since even under selective conditions, the amount of plasmid containing cells never exceeded 5% of the population at this temperature.

From the observations mentioned above we conclude that the 16kD gene promoter cannot simply be replaced by other promoter sequences. However, we have not directly compared the transcription frequency in these derivatives of minichromosome pCM959. Therefore, at present it is not clear to what extent counterclockwise transcription into oriC may deviate without significantly disturbing the stability of minichromosomes.

Plasmid instability is an indication for either a defect in replication control or an impaired partitioning mechanism of plasmid molecules at cell

division. Copy-number determinations showed a small but reproducable decrease in copy-number from about 9 for pCM959 to about 5 for pEM124 (Table II). This finding favours the hypothesis that transcription from the 16kD gene promoter, although not essential for oriC functioning, has a positive effect on minichromosome replication. In the next section we will present more evidence that supports the involvement of the 16kD gene promoter region in the replication control system of oriC on minichromosomes.

Mapping of regions on pCM959 involved in incompatibility of oriC-plasmids

Two related plasmids that are not coinherited stably during growth in the same bacterial cell are called incompatible. Plasmid incompatibility may result from competition for one or more steps of replication or partitioning control. In a number of cases a characterization of incompatibility properties of related plasmids was a succesful approach for understanding replication control mechanisms (33). Recently it was shown that minichromosomes express incompatibility (16). Two regions conferred incompatibility towards oriC plasmids. One region, designated incA, includes the minimal region required for autonomous replication. The second region, incB, is immediately adjacent to oriC between positions 280 and 1050 (Fig. 4A). The 16kD gene promoter is within this latter region and we have determined whether it contributes to expression of incompatibility. This was studied by constructing plasmids consisting of pBR322 and various DNA segments from the oriC region (Table 1, Fig. 4). These oriC-vector hybrids that use the replication system of pBR322 are present at high copy-numbers and are stably maintained, even during growth in non-selective medium. The incompatibility conferred by the cloned fragments was determined by their ability and the rate at which they cause exclusion of the relatively stable minichromosomes pCM959 (not shown) or pEM120. It should be mentioned that under these experimental conditions the growth rate and cell morphology of the E. coli strain CM987 do not seem to be affected by the presence of the pBR322-oriC hybrids and minichromosomes used in this study (data not shown). Some representative segregation curves of the oriC-plasmid pEM120 are shown in Fig. 3. In Fig. 4B the incompatibility evoked by the relevant oriC-vector hybrid plasmids is expressed as the percentage of cells still carrying pEM120 after 15 generations of growth in non-selective medium.

A drastic difference in expression of incompatibility is observed when minichromosomes pCM959 and some of its deletion derivatives are inserted at the PstI site at position 2566 into pBR322 (Fig. 4B, lines 1-4). The incompatibility conferred by oriC-vector hybrid plasmids pEM90 and pEM132 is completely lost in pEM73 and pEM174 (Fig. 3, 4). The results suggest that

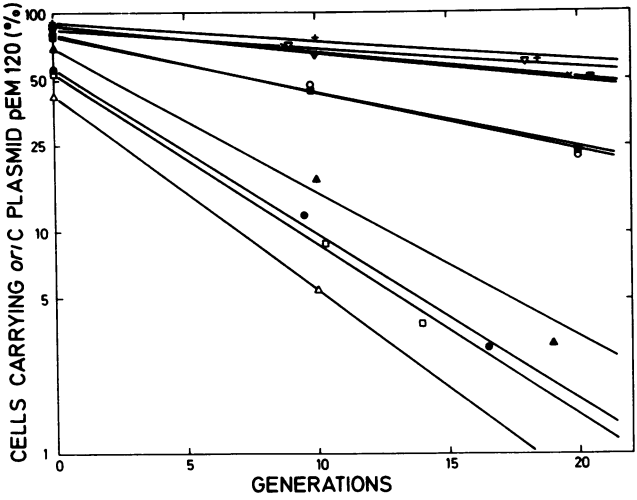

Fig. 3 Rate of loss of oriC-plasmid pEM120 from growing CM987 recA1 cells containing various oriC-pBR322 hybrid plasmids. Plasmids tested are pBR322 (+), pEM174 (▽), pEM73 (▼). pEM193 (x), pEM132 (□) pEM90 (△), pEM197 (■), pEM179 (○), pEM199 (▲) and pEM88 (●).

incompatibility determinants towards oriC-plasmids are only present within a DNA segment that covers the sequences from the MluI site (position 777) to the sequence at position 1184. At the left hand side this segment is bounded by the EcoRV site (position 723). We also may conclude that, in contradiction to a previous report (16), the minimal oriC sequence cannot cause a significantly enhanced exclusion of minichromosomes by itself (see also pOC42, pEM198, pEM175; Fig. 4). A direct confirmation for the presence of the above-mentioned incompatibility region was obtained by the construction of oriC-vector hybrids such as pEM179 (Fig. 4; line 5). However, the rate of exclusion of the oriC-plasmid pEM120 from cells carrying pEM179 is reduced to an intermediate level when compared to its exclusion in the presence of pEM90 or pEM132 (Fig. 3, 4). Obviously, genetic information outside the region contained in pEM179 is necessary to allow full expression of incompatibility. As we will argue below, these sequences are within or close to the region required for autonomous replication.

In an attempt to localize the incompatibility region between positions 723 and 1184 more precisely, hybrid plasmids pEM172 and pEM193 were constructed (Fig. 4B, lines 6 and 7). Neither plasmid caused a significant expression of incompatibility despite an extensive overlap between the cloned chromosomal sequences. Surprisingly, we found that if these same (or even smaller) DNA

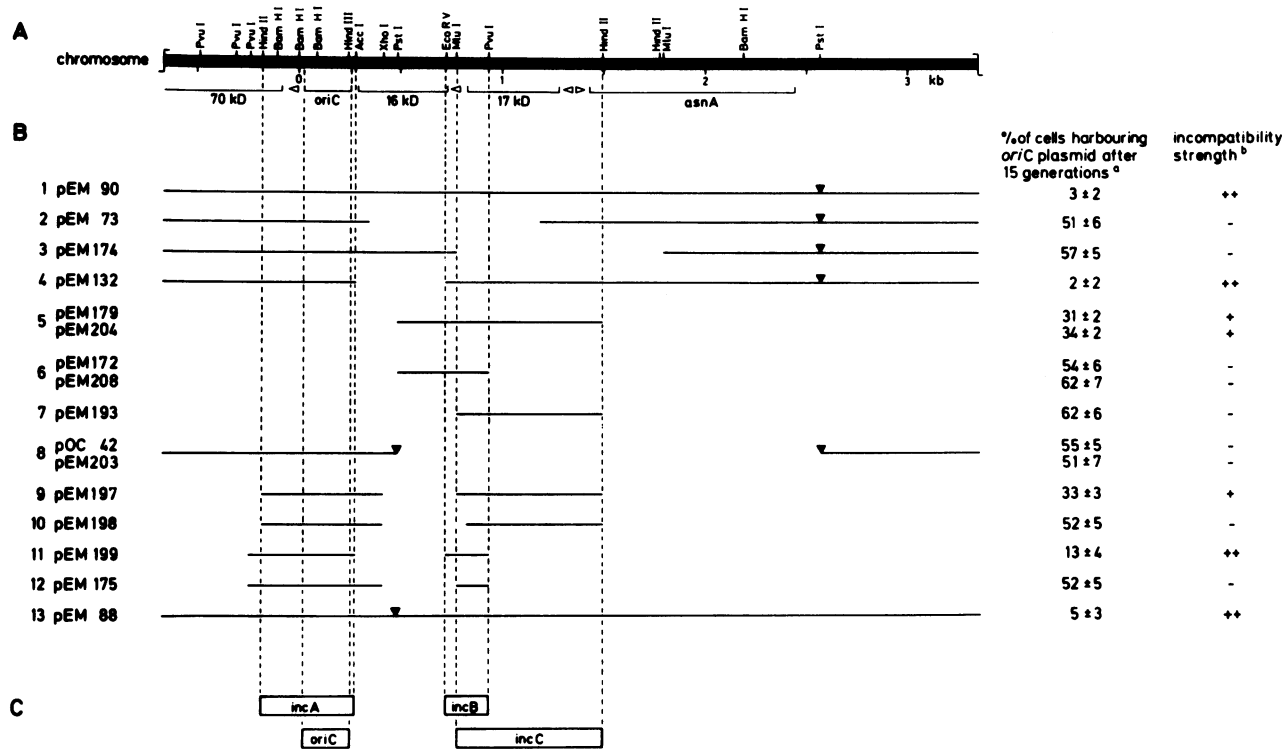

Fig. 4 Incompatibility expressed by DNA fragments inserted into pBR322 and pAT153. (▼) indicates the PstI site at which ligation to the vector occurred. [a] The numbers given are mean values of at least three independent experiments with their standard deviations. [b] ++ indicates a strong incompatibility: less than 15 percent of the cells carry test plasmid after 15 generations of growth; + indicates a weak incompatibility: between 25 and 40% percent of the cells still carry the test plasmid; - indicates no incompatibility: more than 50 percent of the cells have retained the test plasmid after 15 generations of growth.

segments are present on hybrid plasmids together with a DNA segment encompassing the minimal oriC sequence, the expression of incompatibility is restored (compare pEM172 with pEM199 and pEM193 with pEM197 in Fig. 4B). However, the effect of pEM199 on the segregation of the oriC-plasmid is much more prominent than that of pEM197. Whereas the expression of incompatibility by pEM199 is of a strength comparable to that of pEM90 or pEM132, the expression by pEM197 is at the intermediate level of pEM179. Therefore, we conclude that two incompatibility determinants, designated incB and incC, are located in the region between position 723 and 1495 (Fig. 4C). Neither incB nor incC can evoke plasmid incompatibility on its own. Either the presence of both inc-regions on the same plasmid or the separate cloning of incB or incC on a plasmid containing the sequences between position -178 and 285 (Fig. 4C) results in incompatibility. This latter region contains the sequences required for autonomous replication and is designated incA (16; Fig. 4C).

Further mapping of incB and incC was enabled by the construction of pEM198 and pEM175, since both plasmids do not express incompatibility (Fig. 4B, lines 10 and 12). The left end of incB is thus located between the EcoRV and MluI sites (positions 723 and 777, respectively) and its right end is between the MluI and PvuI site (positions 777 and 934, respectively). For incC the boundaries are between the MluI site (position 777) and position 835 at the left and between the PvuI and the HindII site (positions 934 and 1495, respectively) at the right. At this point it should be noted that the MluI site (position 777) is located within the 16kD gene promoter region between its Pribnow box and -35 region. The Eco RV site (position 723) is located downstream the Shine-Dalgarno sequence of the 16kD gene, in the very proximal part of its protein coding region (19, 34). The end point of the deletion present in plasmid pEM198 is at position 835. This is in the very distal part of the protein coding region of the 17kD gene which is terminated before the MluI site at position 777 (19). From the observations it is tempting to postulate that the 16kD promoter sequence is responsible for the expression of incompatibility by incB. The most likely candidate for the expression of incC seems to be the transcript or the protein made by the complete 17kD gene.

Interaction between incA and incB.

In the first section we presented evidence that the transcription that initiates at the 16kD gene promoter and that enters oriC has a positive effect on minichromosome replication. We have investigated whether this transcription activity contributes to the strong incompatibility expressed by incA plus incB (e.g. pEM90, pEM132 and pEM199). For this purpose we have

Table II Plasmid copy-numbers

Plasmid	Copy-number[a]
pCM959	9[b]
pEM124	5[b]
pBR322	15
pAT153	28
pEM90	29
pOC42	26
pEM203	60
pEM208	40

a The copy-number is given as the average number of plasmid molecules per chromosome equivalent
b Correction was made for plasmid-free cells

constructed plasmid pEM88 (Fig. 4B, line 13). In this hybrid plasmid minichromosome pCM959 is cloned in such a way that incA and incB are separated by the complete pBR322 sequence. From Fig. 3, showing the strong incompatibility conferred by pEM88, we conclude that the mere presence of incA and sequences of the 16kD gene promoter (incB) are sufficient to express strong incompatibility.

In agreement with an earlier report (2) we observed that the copy-number of oriC-vector hybrids is increased in case the intact oriC sequence is present on the plasmid (Table II; compare pBR322 with pEM90 and pOC42). This raises the question whether the cooperative action of incA plus incB and incA plus incC is simply the result of an increased dosage of incB or incC incompatibility determinants. In that case, the mere presence of incB or incC in about 30 copies or more per cell should be able to promote exclusion of oriC-plasmids. This was tested by making use of the pBR322 copy-number mutant pAT153 that has a 1.5-3 times higher copy-number than pBR322 itself (35, Table II). Hybrid plasmids pEM203, pEM204 and pEM208 were constructed by recloning the oriC sequences of pOC42, pEM179 and pEM172 in an identical way into pAT153 (Fig. 4B, lines 5, 6 and 8). From the data shown in Table II and Fig. 4 we conclude that the mere presence of incB or incC in about 40 copies per cell (pEM204, pEM208) is not sufficient to stimulate incompatibility. Furthermore, these experiments demonstrate that: (i) the DNA sequences in or close to the minimal oriC region comprise an actual incompatibility determinant, incA (ii) even at very high copy-numbers hybrid plasmids that do contain only incA (pEM203) do not express incompatibility on their own (iii) the maximal rate of

exclusion of oriC-plasmids that is possible by incB in conjunction with incC seems to be reached with the pBR322 hybrid (compare pEM204 with pEM179).

Orientation dependency of expression of incompatibility.

The oriC-vector hybrids shown in Fig. 4 lines 5 to 13 all have the inserted DNA present in orientation I (Table I). We observed that both a strong (e.g. pEM88 vs. pEM89; Table I) and weak expression of incompatibility (e.g. pEM171 vs. pEM108; Table I) are totally absent if the cloned fragment in pBR322 is inserted in orientation II (results not shown). This orientation dependency was not observed in case pCM959 or its derivatives (Fig. 4, lines 1-4) were linearized at the PstI site at position 2566 and inserted at the PstI site of pBR322. These results indicate that rightward transcription of incB and incC sequences, initiated at the pBR322 β-lactamase promoter, interferes with the expression of incompatibility. An identical orientation dependency was observed with recombinants obtained by insertion of fragments in the Tet gene of pBR322 (not shown).

DISCUSSION

It has been shown that minichromosomes are rapidly lost from growing cells in absence of selective pressure (8, 9). However, we observed that some minichromosomes (e.g. pCM959 and pEM120) are inherited rather stably. Still the rate of loss per cell generation is much faster than would be expected for a random partitioning of plasmids at a copy-number of 9. This makes it very unlikely that a specific partitioning system is present on these oriC-plasmids. Since the poor maintenance observed with some of the deletion and insertion mutants of pCM959 is accompanied with a decrease in plasmid copy-number we infer that this reduced stability reflects at least a defect in replication control.

We have shown that inactivation of the 16kD gene promoter (pEM190) or replacement of this promoter by other promoter sequences (pEM124, 134) caused a drastic reduction in stability. On the other hand, a deletion of almost the complete coding region of the 16kD protein itself did not affect minichromosome stability at all (pEM127). From the analysis of these mutants we propose that transcription of oriC sequences, initiated at the 16kD gene promoter, positively affects the copy-number of minichromosomes. Yet, whether this effect is exerted at the level of initiation or termination of transcription remains unclear.

The high stability of pCM959 and pEM120 allowed us to study whether sequences within or nearby oriC can also affect minichromosome stability in

trans. This type of incompatibility experiments may provide a better insight in the replication control of minichromosomes. For this purpose various portions of the region near oriC were cloned into the high copy-number vector pBR322. Analysis of the rate of exclusion of plasmid pEM120 from cells that contain one of these pBR322-oriC hybrids revealed the existence of three incompatibility determinants. One region was mapped between positions -178 and 285 and overlaps the region that was previously designated incA (16). This region contains the minimal sequences required for autonomous replication (6). A second region, previously allocated between positions 280 and 1050 and designated incB (16), was delimited to a fragment between positions 723 and 934. This trans-acting 210 base pairs fragment covers the 16kD gene promoter region. Since we have shown that the 16kD gene promoter is also involved in the maintenance of minichromosomes (a cis-action), we suggest that a regulatory sequence contained within this promoter region is responsible for incB incompatibility. We have also identified a third incompatibility region, incC, that is located between positions 777 and 1495. As shown in Fig. 4 the incB and incC regions seem to overlap to some extent. However, we conclude that they are distinct incompatibility determinants since they cause exclusion of pEM120 at clearly different rates.
From its location we suggest that the transcript of the 17kD gene or the 17kD protein itself expresses some regulatory function that is responsible for incC incompatibility.

We could not demonstrate that the inc-regions can express incompatibility on their own. Incompatibility towards oriC-plasmids was only observed when at least two inc-regions were present on the same hybrid plasmid. On the contrary, Yamaguchi et al. (16) claim that both incA and incB determine incompatibility independently. This discrepancy could neither be solved by cloning incA and incB into pAT153, a copy-number mutant of pBR322. The data obtained with these hybrids (pEM203, incA; pEM208, incB Fig. 4) demonstrate that a high dosage of incompatibility determinants, incA and incB do not express incompatibility independently. These results do not exclude that at a still higher dosage incompatibility can be expressed independently (see also below).

To determine whether the transcription of the incA-region, promoted by the incB-region is responsible for the cooperative action of incA plus incB, plasmid pEM88 was constructed. In this hybrid plasmid incA and incB are separated by the complete pBR322 sequence. Since pEM88 still expresses strong incompatibility it seems plausible that a regulatory sequence contained within the promotor region of the 16kD gene is responsible for incB incompatibility

rather than the transcription event itself.

While the effect of insertions or deletions on minichromosome stability suggests that transcription initiated at the 16kD promoter has a positive effect on minichromosome replication, its regulatory function is still unclear from these studies. Yet, the trans effect of the 16kD gene promoter, observed in the incompatibility experiments, suggests that a diffusable gene product is involved. Based upon these results it seems plausible that incB incompatibility results from titrating a control element involved in the transcription process. However, the observations that incompatibility is only evoked towards oriC on minichromosomes and that incB cannot exert incompatibility on its own (at a dosage of about 40, pEM208) argues against this hypothesis. In view of these apparently conflicting data it is of importance to mention some recent experiments. It was observed that certain dnaA^{ts} strains cannot be transformed by pBR322-oriC hybrids containing incA plus incB (i.e. pEM90, pEM199) at the permissive temperature. Furthermore, transformants are obtained at high frequency with hybrid plasmids containing incA, incB, incA plus incC and incB plus incC (Meijer and Stuitje, manuscript in preparation). These experiments demonstrate that incA plus incB can also act in trans towards oriC on the chromosome in E. coli strains with partial defects in the dnaA replication initiation protein. Moreover, these results and the data presented in this paper substantiate the suggestion of Hansen et al. (36) that the dnaA protein binds not only to a specific sequence present in the promoter region of the dnaA operon and its 4 homologous counterparts in the oriC sequence (incA), but also to the same sequence present in the 16kD promoter region (incB). We suggest that incA and incB incompatibility results from the titration of dnaA protein, whereas incC probably enhances the affinity of the dnaA protein towards its recognition sequence. Since it has been proposed that the dnaA gene product is autoregulated (36) only extensive titration will result in a decreased level of this protein. This may explain why incompatibility is only observed when at least two inc-regions are present on the hybrid plasmid. This hypothesis also predicts that by a further increase in dosage incA and andB eventually should be able to exert incompatibility on their own.

To account for the difference in incompatibility exerted towards oriC on minichromosomes and oriC on the host chromosome we suggest that initiation of replication on minichromosomes is limited by the concentration of dnaA protein, whereas in E. coli strains wild type for the dnaA protein chromosomal initiation is limited by some other initation factor or structural component. The observation that minichromosomes are present in E. coli in copy-numbers

that exceed that of the chromosome 3 to 10-fold (13) may reflect this difference in initation of replication.

Sofar no data has been obtained about the actual function of the 16kD gene promoter, the dnaA protein and the 17kD gene product in the initiation of DNA replication. Therefore further studies are focussed on resolving the molecular basis of the genetic data presented in this paper.

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