JOURNAL OF BACTERIOLOGY, Sept. 1983, p. 1185–1191
0021-9193/83/091185-07$02.00/0

Vol. 155, No. 3

Nucleotide Sequence of an Incompatibility Region of Mini-Rts1 That Contains Five Direct Repeats

YOSHIYUKI KAMIO AND YOSHIRO TERAWAKI*

Department of Bacteriology, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto, 390 Japan

Received 24 March 1983/Accepted 14 June 1983

The plasmid mini-Rts1, consisting of an *Eco*RI/*Hin*dIII fragment of about 1.8 kilobases (kb), contains an incompatibility determinant in its *Eco*RI/*Acc*I region (0.5 kb). The nucleotide sequence of this incompatibility fragment was determined. A most striking feature of the sequence is the presence of five 24-base pair direct repeats. Four out of the five repeating units, which are contained in a 0.2-kb *Eco*RI/*Hin*cII fragment, were cloned en bloc in pACYC184 and found to express Rts1-specific incompatibility. In addition, the copy number of the mini-Rts1 plasmid appeared to be increased threefold upon removal of the 0.2-kb incompatibility region (*inc*I) from the plasmid. This deletion derivative of mini-Rts1, as well as mini-Rts1, was maintained stably at 37°C, but was cured at a high frequency at 42°C. A possible role of the repeated nucleotide sequence was discussed. By subcloning the mini-Rts1 DNA, a second *inc* determinant (*inc*II) was found on the *Acc*I fragment, which is contiguous to the 0.5-kb *Eco*RI/*Acc*I fragment.

Studies on plasmid replication have concentrated on their control mechanisms, and significant progress has been made for many plasmids in relation to the structure and function of incompatibility. In ColE1, an RNA of 108 nucleotides inhibits primer formation for the initiation of DNA replication (26). In IncFII plasmids, such as R1, R6-5, and NR1 (or R100), the incompatibility determinant also expresses its function mainly through a small RNA molecule (7, 12, 19, 22, 25). In λ*dv*, R6K, and F plasmids, the presence of a repeated nucleotide sequence has been demonstrated at the replication origin region, and the repeats are found to interact with a positively operating initiator protein (9, 16, 21, 27).

Recently, the replication region from the large, stringent-type plasmid Rts1 (24) that belongs to the IncT group (5) was isolated. The basic replicon, mini-Rts1, is approximately 1.8 kilobases (kb) in length, and an *inc* determinant is located in a portion close to its *Eco*RI end (11, 23). In the present study, we determined the nucleotide sequence of the incompatibility region consisting of an *Eco*RI/*Acc*I fragment of 496 base pairs (bp) and found five 24-bp direct repeats to cluster within a 194-bp area. Four out of the five repeating units were cloned, and their functions were investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* K-12 strains JC1569 (*recA gal leu his arg met str*) (3) and JG112 (*polA lacY thy str*) (14) were used as host cells of plasmids. Plasmids used are listed in Table 1.

Media. Penassay broth (Difco Laboratories, Detroit, Mich.) was used for cultivation of bacteria throughout this study except that L broth was used for transformation.

Enzymes. All the restriction endonucleases used in this study, T4 DNA ligase, and T4 polynucleotide kinase were purchased from Takara Shuzo (Kyoto, Japan). S1 nuclease was obtained from Bethesda Research Laboratories, Gaithersburg, Md. Treatment of the DNA with these enzymes was done as recommended by the suppliers.

Preparation of plasmid DNA and isolation of restricted fragments. Plasmid DNA was prepared mainly according to the method of Clewell and Helinski (4). JC1569 harboring plasmid was grown in Penassay broth. For preparing pBR322- or pACYC184-chimeric plasmid DNA, 100 μg of chloramphenicol per ml was added to the culture when it reached an optical density of 0.5, and growth was allowed to continue overnight. A Brij cleared lysate was concentrated by adding 10% polyethylene glycol 6000 and 0.1 volume of 5 M NaCl (10). The cleared lysate was centrifuged to equilibrium in an ethidium bromide-cesium chloride solution at 35,000 rpm for 13 h at 20°C, using a Beckman rotor VTi65. The purified plasmid DNA was cleaved with restriction endonuclease and electrophoresed in a 6 or 8% polyacrylamide gel. The DNA fragments were extracted from the gel according to the method of Murotsu and Matsubara (15).

Nucleotide sequence determination. Nucleotide sequence was determined by the method of Maxam and Gilbert (13). The 5′ ends of restricted fragments were labeled with [γ-^{32}P]ATP (>7,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) by T4 polynucle-

TABLE 1. Plasmids[a]

Plasmid	Cloned fragment of Rts1	Vector or drug resistance fragment	Drug resistance	Reference
NR1			Sp/Sm Cp Tc Sa	17
R401			Ap Sm	5
pBR322			Ap Tc	1
pACYC184			Tc Cp	2
pTW601	mini-Rts1[b]	Sp fragment of NR1	Sp/Sm	11
pTW602	mini-Rts1	pBR322	Ap	11
pTW603	mini-Rts1ΔE/HII[c]	pBR322	Ap	This study
pTW604	mini-Rts1ΔE/HII	Sp fragment of NR1	Sp/Sm	This study
pTW701	E/HII fragment[d]	pACYC184	Tc	This study
pTW801	*Acc*I fragment[e]	pBR322ΔS[f]	Ap	This study

[a] Drug abbreviations: Sp, spectinomycin; Sm, streptomycin; Cp, chloramphenicol; Tc, tetracycline; Sa, sulfonamide; Ap, ampicillin.
[b] The 1.8-kb *Eco*RI/*Hin*dIII fragment of Rts1 capable of autonomous replication.
[c] Mini-Rts1 with its *Eco*RI/*Hin*cII fragment of 216 bp deleted.
[d] *Eco*RI/*Hin*cII 216-bp fragment containing four repeating units.
[e] The 1.1-kb *Acc*I fragment of mini-Rts1.
[f] pBR322 deleted for its *Sal*I(*Acc*I) site.

otide kinase. The labeled fragments were separated by a secondary cleavage with different restriction endonucleases. Dimethyl sulfate was used for the guanine reaction, piperidinum formate for adenine-guanine, hydrazine for cytosine-thymidine, and hydrazine in the presence of 5 M NaCl for the cytosine reactions. The products were electrophoresed on polyacrylamide gels (0.05 by 60 cm; 16 and 8%) containing 8.3 M urea and were autoradiographed on RX film (Fuji Film, Tokyo, Japan).

Incompatibility test. Incompatibility between two plasmids was examined by transforming one plasmid into the *recA* host JC1569 harboring the other plasmid. Transformation was carried out essentially according to Cohen et al. (6). After a heat pulse at 42°C for 5 min, a mixture of the donor DNA and the competent cells was incubated in L broth at 37°C for 90 min before plating onto a selective plate. The transformant was selected either for a donor marker only or for a combination of donor and recipient markers. When transformants were obtained on the plate containing a donor marker drug, 100 colonies were picked from the plate and examined for resident marker resistance. The transformants, which produced colonies on the plate containing both drugs, were picked separately and grown in broth without drugs. After 6 h of incubation at 37°C, the culture was streaked onto a Penassay broth agar plate, and the resulting colonies were examined for their resistance to both drugs by replica plating.

RESULTS

Nucleotide sequence of *Eco*RI/*Acc*I fragment. An incompatibility determinant of mini-Rts1 is located within its *Eco*RI/*Acc*I fragment, which is about 0.5 kb in length (11). We determined the nucleotide sequence of this fragment isolated from pTW602 (mini-Rts1 plus pBR322). The sequencing strategy and the nucleotide sequence are shown in Fig. 1 and 2.

The Inc fragment was composed of 496 bp. A most striking feature of the sequence was the presence of 24-bp direct repeats which appeared five times at positions between nucleotides 48 and 241 (in Fig. 2, shown with underlines). Among the five repeating units (numbered I, II,

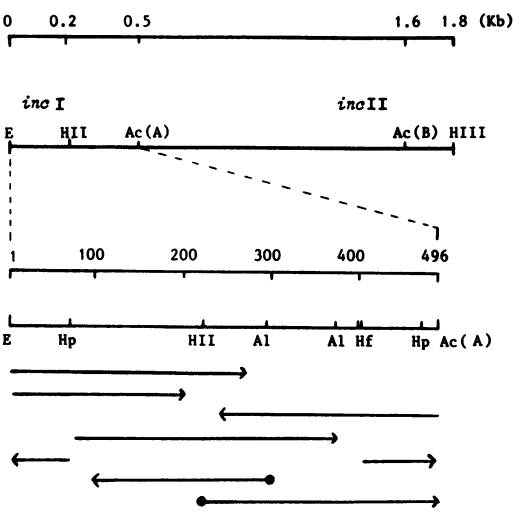

FIG. 1. Physical map of mini-Rts1 consisting of the 1.8-kb *Eco*RI/*Hin*dIII fragment of Rts1. The second *inc* gene (*inc*II), located within the 1.1-kb *Acc*I fragment, has not been precisely mapped yet. The lower section shows the restriction cleavage map and sequencing strategy of the 0.5-kb *Eco*RI/*Acc*I fragment encoding *inc*I. Numbers show bp length starting at the *Eco*RI recognition sequence. The arrows indicate the direction and extent of the nucleotide sequence determined and are aligned in the 5′ to 3′ direction. Abbreviations: E, *Eco*RI; HIII, *Hin*dIII; Ac, *Acc*I; Hp, *Hap*II; HII, *Hin*cII; Al, *Alu*I; Hf, *Hin*fI. The *Eco*RI linker (●—) was ligated to the *Alu*I or *Hin*cII blunt end to label the 5′ end.

FIG. 2. Nucleotide sequence of the *Eco*RI/*Acc*I Inc fragment. The sequence is arranged such that 1 is the first nucleotide in the recognition sequence for *Eco*RI and 496 is the last nucleotide for the *Acc*I recognition sequence. The upper strand is shown in the 5′ to 3′ direction. The underlined sequences indicate the 24-bp direct repeats. The dashed line shows a half-sized repeating unit. The bold lines are for the two pairs of inverted repeats, a and b.

III, IV, and V from the *Eco*RI site), II, III, and IV were found to consist of a 24-bp identical sequence except for a 1-bp difference in the IInd unit (Fig. 3). The Vth unit, being mostly distorted in its sequence, was separated by a *Hin*cII restriction site at position 216 from the remaining four repeating units. In addition, the 11 bases at positions 20 to 30 were identical to the internal sequences of the IIIrd and IVth units except for a 1-bp difference. Two pairs of inverted repeats consisting of 10 and 11 nucleotides are shown in Fig. 2 with bold underlines.

Four reading frames which could encode a polypeptide of larger than 5,000 daltons were found. However, neither an apparent RNA polymerase binding sequence (18) nor a ribosome binding sequence (20) was detected in front of the respective start codon ATG or GTG.

Incompatibility of the *Eco*RI/*Hin*cII fragment. To investigate the function of the 0.2-kb *Eco*RI/*Hin*cII fragment that contains the four repeating units, this fragment was cloned into the *Eco*RI site of pACYC184 after ligating an *Eco*RI linker (Genex Corp., Gaithersburg, Md.) to the *Hin*cII end of the fragment (Fig. 4A and B). This chimeric plasmid, pTW701, was examined for its incompatibility toward pTW601 (mini-Rts1 plus spectinomycin fragment) and toward the IncT plasmid R401.

After selection with a combination of donor and resident markers, pTW701 could not be transformed into JC1569 harboring pTW601 (Table 2). Although doubly infected cells were obtained by selecting transformants for only the donor marker, their growth on the plate containing 30 μg of spectinomycin per ml was poor in comparison with that of JC1569 with only pTW601. Furthermore, the resident plasmid pTW601 was lost from the doubly infected cells during 6 h of subsequent cultivation in broth without selective drugs. In contrast, pTW701 was introduced successfully into the R401 harboring cells, and both plasmids were inherited stably in the absence of drug. These results indicate that the incompatibility encoded on the 0.2-kb region is specific for Rts1. At the same time, the findings suggest that another *inc* determinant(s), common to the IncT group plasmids, should exist on the mini-Rts1 genome, since pTW601 and R401 are incompatible (Table 2, line 3). This led us to examine the incompatibility of the *Acc*I fragment which is contiguous to the 0.5-kb *Eco*RI/*Acc*I fragment.

Incompatibility of the *Acc*I fragment. Because pBR322 has two *Acc*I restriction sites, one of which is also cleaved with *Sal*I and *Hin*cII, the *Sal*I/*Acc*I site was deleted by digesting pBR322 with *Sal*I and S1 nuclease. Unexpectedly, a derivative, named pBR322ΔS, has a deletion of about 0.3 kb surrounding the *Sal*I/*Acc*I site. The 1.1-kb *Acc*I fragment isolated from the *Acc*I-digested pTW602 DNA was ligated to the unique *Acc*I site of pBR322ΔS (Fig. 5). The recombinant obtained, pTW801, was tested for its incompatibility toward pTW601. No transformants were isolated on the plate containing both drugs (Table 2, line 4), and all the transformants obtained by selecting only for a donor marker were found to have lost the resident pTW601. Thus, the second *inc* determinant (*inc*II) of mini-Rts1 is located on the *Acc*I fragment. Whether this fragment is incompatible with R401 could

I) TTCCCCCTGACACACACTTTACCG

II) TTCCCCCTGACGCACACCTTGCCA

III) TTCCCCCTGACACACACCTTGCCA

IV) TTCCCCCTGACACACACCTTGCCA

V) ACCCCCCTGACACACACTTTTCAT

FIG. 3. Nucleotide sequence of five repeated sequences. The sequence is arranged in the 5′ to 3′ direction, and the Roman numerals represent the repeating units in the order of the *Eco*RI to *Acc*I site direction. The underlined residues indicate nonconsensus bases.

not be tested, since pTW801 and R401 are both ampicillin resistant.

It should be mentioned that pTW801 was not capable of replication in the JG112 (*polA*) host (data not shown), indicating that the 0.5-kb *Eco*RI/*Acc*I region (or a part of it) is essential for the autonomous replication of mini-Rts1.

Effect on plasmid replication of removing the 0.2-kb *Eco*RI/*Hin*cII fragment from mini-Rts1. As described, the 0.2-kb region contained four repeating units and expressed Rts1-specific incompatibility. To remove this region from mini-Rts1, pTW602 was doubly digested with *Hin*dIII and *Hin*cII, and an *Eco*RI linker was ligated to the *Hin*cII end of the isolated *Hin*dIII/*Hin*cII fragment of about 1.6 kb. The obtained deletion derivative of mini-Rts1 (named in short mini-Rts1ΔE/HII) has a *Hin*dIII and an *Eco*RI end, which was ligated to either pBR322 or the spectinomycin resistance fragment derived from NR1 (Fig. 4A and C). The chimeric plasmids constructed were termed pTW603 and pTW604, respectively. Both plasmids transformed JG112 and JC1569 at high frequency, meaning that mini-Rts1 is capable of autonomous replication without the 0.2-kb region.

To estimate the copy number of mini-Rts1ΔE/HII, the level of ampicillin resistance of pTW603 in JG112 was compared with that of pTW602, since plasmid-specified ampicillin resistance is known to be proportional to the gene dosage (28). The resistance conferred by pTW603 was at least three times higher than that conferred by pTW602 (Table 3). It should be noted that, in both chimeric plasmids, mini-Rts1 and mini-Rts1ΔE/HII are inserted into the *Eco*RI-*Hin*dIII region of pBR322. Accordingly, it may be rational to compare the ampicillin resistance of the plasmid in the JG112 host as a function of the copy number controlled by the Rts1 replicon. Similarly, the spectinomycin resistance specified by pTW604 increased significantly in comparison with that of pTW601 (Table 3). As previous work suggests that the copy number of mini-Rts1 is about 3 per host chromosome (11), that of mini-Rts1ΔE/HII was estimated to be

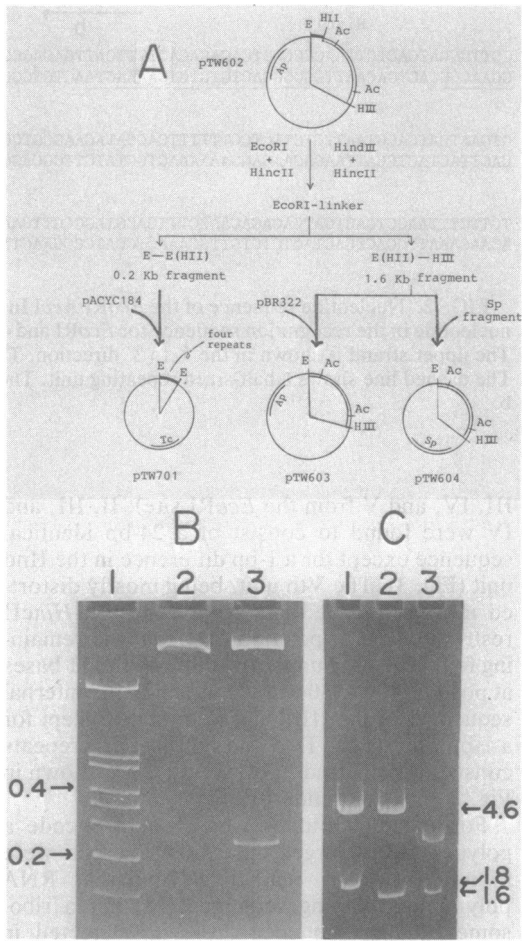

FIG. 4. Cloning or deletion of mini-Rts1 region containing four repeating units. (A) To clone the repeat region, shown with a bold line, or to remove it from mini-Rts1, pTW602 (mini-Rts1 plus pBR322) DNA was digested with either *Eco*RI plus *Hin*cII or *Hin*dIII plus *Hin*cII. In both cases, an *Eco*RI linker was ligated to the *Hin*cII blunt end. The resulting *Eco*RI fragment of 0.2 kb was ligated to pACYC184, and the *Eco*RI/*Hin*dIII fragment of 1.6 kb was ligated to pBR322 or to the spectinomycin fragment of NR1. (B) Restriction cleavage patterns of plasmid DNA. DNA digested with restriction endonuclease was electrophoresed in a 6% polyacrylamide gel. Lane 1, pBR322 digested with *Hin*fI was used as a molecular size standard; lane 2, pACYC184/*Eco*RI; lane 3, pTW701/*Eco*RI. (C) Restriction cleavage patterns of chimeric plasmid DNA. DNA doubly digested with *Eco*RI and *Hin*dIII was electrophoresed in a 0.8% agarose gel. Lane 1, pTW602; lane 2, pTW603; lane 3, pTW604. The numbers on the sides are molecular size in kb.

TABLE 2. Incompatibility of cloned mini-Rts1 components[a]

Plasmid		No. of transformant selected by:		% of resident in transformant[b]
Donor	Resident	Both markers	Donor marker	
pTW701	pTW601	$<1 \times 10$	2.7×10^4	48
pTW701	R401	2.4×10^4	2.1×10^4	100
pTW601	R401	$<1 \times 10$	$<1 \times 10$	
pTW801	pTW601	$<1 \times 10$	9.8×10^3	0
pTW603	pTW601	$<1 \times 10$	1.5×10^4	0

[a] JC1569 harboring pTW601 was grown in the presence of 30 μg of spectinomycin per ml to avoid generation of plasmid-cured cells. Approximately 0.5 μg of donor DNA was used to transform JC1569 harboring a resident plasmid. Drugs used for selection were 10 μg of tetracycline (for pTW701), 30 μg of ampicillin (for pTW801, pTW603, and R401), and 30 μg of spectinomycin (for pTW601) per ml.

[b] Each of 100 colonies that developed on plates selected for the donor marker was examined for the resident marker by scoring resistance.

about 10 per host chromosome. Thus, the 0.2-kb *inc* region (*incI*) containing four direct repeats appears to control the copy number of mini-Rts1. As expected, mini-Rts1ΔE/HII is incompatible with pTW601, since pTW603 could not be transformed into JC1569 harboring pTW601 (Table 2, line 5).

Stability of mini-Rts1ΔE/HII. The mini-Rts1 plasmid is maintained stably at 37°C, but it is cured at a high frequency at 42°C (11). To determine the stability of mini-Rts1ΔE/HII, pTW604 was used for the study, since this recombinant plasmid has no replication unit other than the Rts1 replicon. By repeated dilution, a culture of JC1569 harboring pTW604 or pTW601 was maintained in exponential growth for more than 100 generations at 37°C, during which curing of the plasmids was examined periodically. pTW604 as well as pTW601 were maintained stably at the permissive temperature (Fig. 6). In contrast, at 42°C, pTW604 began to be lost at 4 h (corresponding to about eight generations) after the temperature shift, and the fraction of plasmid-containing cells decreased exponentially, showing a rate of curing like that of pTW601 (Fig. 6). The only difference observed between the two plasmids was that the start of curing of pTW604 was delayed by about two generations, which should be due to a larger copy pool of pTW604 than that of pTW601.

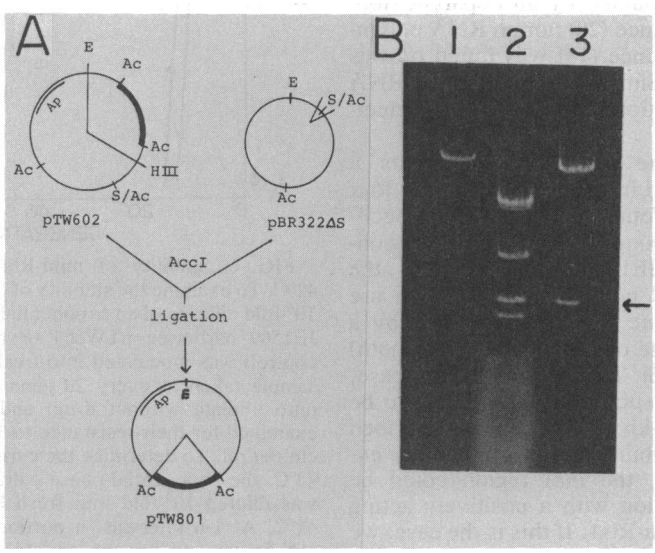

FIG. 5. Cloning of the 1.1-kb *Acc*I fragment of mini-Rts1. (A) An *Acc*I fragment of 1.1 kb (shown with a bold line) was isolated from pTW602 DNA digested with *Acc*I endonuclease and cloned to the unique *Acc*I site of a derivative of pBR322, in which the *Sal*I/*Acc*I cleavage site of pBR322 was deleted by treating with *Sal*I and S1 nuclease. (B) Restriction cleavage patterns of plasmid DNA. Restricted DNA was electrophoresed in a 1.0% agarose gel. Lane 1, pBR322/*Eco*RI; lane 2, pTW602/*Acc*I; lane 3, pTW801/*Acc*I. Note that the 1.1-kb *Acc*I fragment indicated by an arrow is present in both lanes 2 and 3.

TABLE 3. Level of drug resistance[a]

Plasmid	Ampicillin resistance (μg/ml) in JG112 (*polA*)	Spectinomycin resistance (μg/ml) in JC1569 (*recA*)
pTW602	400	NT[b]
pTW603	1,500	NT
pTW601	NT	100
pTW604	NT	300

[a] A 0.1-ml volume of exponentially growing cells (4×10^3 to 6×10^3 per ml) was spread onto a Penassay broth agar plate containing different concentrations of ampicillin or spectinomycin. The level of resistance was the maximum concentration of the drug that allowed development of isolated, but not small, colonies on the plate after 18 h of incubation at 37°C.

[b] NT, Not tested.

We conclude from the curing kinetics that mini-Rts1ΔE/HII is stably inherited at 37°C, and its replication is still temperature sensitive as is that of its parent plasmids mini-Rts1 and Rts1. This suggests in turn that the *inc*I region is not involved in either maintenance or temperature sensitivity of mini-Rts1.

DISCUSSION

An incompatibility determinant of Rts1 is located in a 0.5-kb *Eco*RI/*Acc*I fragment of mini-Rts1 (11). In the present study, we determined the nucleotide sequence of the 0.5-kb Inc fragment and demonstrated the presence of five 24-bp direct repeats. Neither a coding frame for a polypeptide accompanied by an apparent ribosome binding sequence (20) nor an RNA polymerase binding sequence (18) was found on this 0.5-kb fragment. Similarly, no distinct RNA polymerase recognition sequence (8) was detected.

By subcloning the mini-Rts1 components, a 0.2-kb *Eco*RI/*Hin*cII fragment that contains four direct repeats was found to express Rts1-specific incompatibility along with apparent copy control function. In ColE1 and IncFII plasmids, the incompatibility and copy control function are expressed by the *inc* gene product, i.e., by a small RNA molecule or a polypeptide (or both) (7, 12, 19, 22, 25, 26). This may not be our case, however, since no such product is likely to be made from the 0.2-kb *inc*I region as described (although the possibility is not completely excluded). Therefore, the *inc*I region could be needed for interaction with a positively acting gene product of mini-Rts1. If this is the case, we can imagine a mechanism for incompatibility/copy control exerted by this region as follows. When pTW601 and pTW701 coexist, a hypothetical positive factor from the former would be titrated by the excess units of the repeats in pTW701, resulting in curing of pTW601. Conversely, if some of the repeats were deleted from mini-Rts1, the surplus positive factor could induce frequent initiation of replication leading to a higher copy number for pTW603 and pTW604. The 35,000-dalton polypeptide that is found in vivo as a gene product of mini-Rts1 (11) might be a candidate for the positively operating substance.

As demonstrated in pTW801, mini-Rts1 has another *inc* determinant (*inc*II) within the 1.1-kb *Acc*I fragment. The 0.2-kb *inc*I fragment expresses an incompatibility function only toward Rts1, whereas the whole mini-Rts1 is incompatible with both Rts1 and R401. Therefore, the

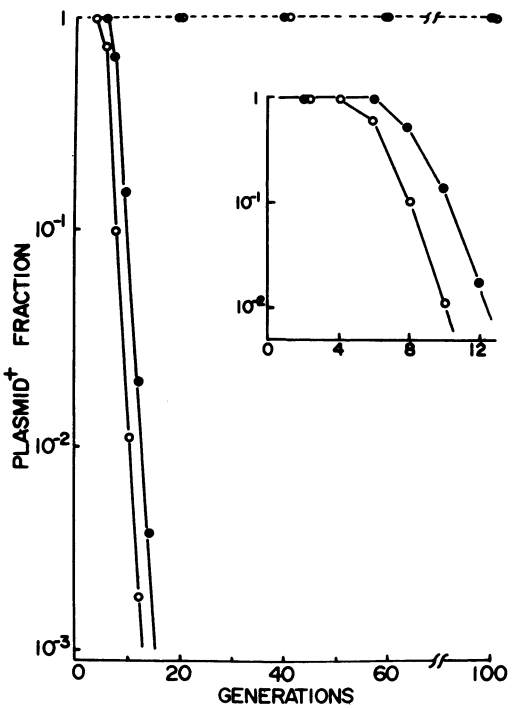

FIG. 6. Stability of mini-Rts1ΔE/HII at 37 and 42°C. To examine the stability of the plasmid at 37°C, a 10^6-fold dilution of an exponentially growing culture of JC1569 harboring pTW604 or pTW601 (used as a control) was inoculated into fresh Penassay broth. A sample taken at every 20 generations was streaked onto a plate without drug, and 100 colonies were examined for their resistance to 30 μg of spectinomycin per ml. To determine the curing of the plasmids at 42°C, the exponential-phase culture prepared at 37°C was diluted 10^4-fold into fresh broth and shifted to 42°C. At 1-h intervals, a portion of the culture was withdrawn and an appropriate dilution was spread onto a plate with or without 30 μg of spectinomycin per ml, and the number of colonies developing on the plates was compared. The inset shows the initial phase of the plasmid curing at 42°C with expanded scale in the abscissa. Symbols: ——, 42°C; ----, 37°C; ●, pTW604; ○, pTW601.

incII region might determine IncT incompatibility, although this has not been verified yet. Sequencing analysis of the *Acc*I fragment would aid in clarifying the nature of the incompatibility encoded on this region.

It is already known that λ*dv* and R6K have four 18-bp and seven 22-bp direct repeats in the replication origin region, respectively (9, 21), and *O* and π proteins that are positively acting molecules bind to the repeating sequences (21, 27). Also, mini-F contains two clusters of 19-bp repeats separated by the region encoding a hypothetical 29,000-dalton initiator protein (16). Furthermore, each fragment containing the four and five units of the 19-bp direct repeats has been cloned separately and expresses F-specific incompatibility (H. Tsutsui, personal communication).

An interesting characteristic of mini-Rts1 is that it is still capable of efficient replication even after four out of the five repeats are deleted. To determine the effect on the plasmid replication of removing the remaining repeat from mini-Rts1ΔE/HII, and to examine whether the mini-Rts1 genome contains another cluster of direct repeats like mini-F are our immediate research subjects. In addition, the location of the replication origin and of the coding frame for the 35,000-dalton protein should be defined.

ACKNOWLEDGMENTS

We thank Asao Fujiyama for instruction with the nucleotide sequence determination technique and computer analysis of the sequence and Kenichi Matsubara for critical reading of the manuscript.

This work was supported in part by grants from the Ministry of Education, Science and Culture, Japan, The Yakult Foundation, and from N. Aikō's Foundation.

LITERATURE CITED

1. **Bolivar, F., R. L. Rodriguez, P. J. Green, M. C. Betlach, H. L. Heynecker, H. W. Boyer, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multiple cloning system. Gene **2**:95–113.
2. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmids. J. Bacteriol. **134**:1141–1156.
3. **Clark, A. J., M. Chamberlin, and R. P. Boyce.** 1966. Abnormal metabolic response to UV light of a recombinant deficient mutant of *E. coli* K 12. J. Mol. Biol. **19**:442–453.
4. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. **62**:1159–1166.
5. **Coetzee, J. N., N. Datta, and W. Hedges.** 1972. R factors from *Proteus rettgeri*. J. Gen. Microbiol. **72**:543–552.
6. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69**:2110–2114.
7. **Easton, A. M., and R. H. Rownd.** 1982. The incompatibility product of IncFII R plasmid NR1 controls gene expression in the plasmid replication region. J. Bacteriol. **152**:829–839.
8. **Gilbert, W.** 1976. Starting and stopping sequences for RNA polymerase, p. 193–205. *In* R. Losick and M. Chamberlin (ed.), RNA polymerase. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Hobom, G., R. Grosschedl, M. Lusky, G. Sherer, E. Schwarz, and H. Kössel.** 1979. Functional analysis of the replicator structure of lambdoid bacteriophage DNAs. Cold Spring Harbor Symp. Quant. Biol. **43**:165–178.
10. **Humphreys, G. O., G. A. Willshaw, and E. S. Anderson.** 1975. A simple method for the preparation of large quantities of pure plasmid DNA. Biochim. Biophys. Acta **383**:457–463.
11. **Itoh, Y., Y. Kamio, Y. Furuta, and Y. Terawaki.** 1982. Cloning of the replication and incompatibility regions of a plasmid derived from Rts1. Plasmid **8**:232–243.
12. **Light, J., and S. Molin.** 1982. The sites of action of the two copy number control functions of plasmid R1. Mol. Gen. Genet. **187**:486–493.
13. **Maxam, A., and W. Gilbert.** 1977. A new method for DNA sequence analysis. Proc. Natl. Acad. Sci. U.S.A. **74**:560–564.
14. **Miller, J., J. Manis, B. Kline, and A. Bishop.** 1978. Nonintegrated plasmid-folded chromosome complexes. Plasmid **1**:273–283.
15. **Murotsu, T., and K. Matsubara.** 1980. Role of an autorepression system in the control of λ*dv* plasmid copy number and incompatibility. Mol. Gen. Genet. **179**:509–519.
16. **Murotsu, T., K. Matsubara, H. Sugisaki, and M. Takanami.** 1981. Nine unique repeating sequences in a region essential for replication and incompatibility of the mini-F plasmid. Gene **15**:257–271.
17. **Nakaya, R., A. Nakamura, and Y. Murata.** 1960. Resistance transfer agents in Shigella. Biochem. Biophys. Res. Commun. **3**:654–659.
18. **Pribnow, D.** 1975. Nucleotide sequence of an RNA polymerase binding site at early T7 promoter. Proc. Natl. Acad. Sci. U.S.A. **72**:784–788.
19. **Rosen, J., T. Ryder, H. Ohtsubo, and E. Ohtsubo.** 1981. Role of RNA transcripts in replication incompatibility and copy number control in antibiotic resistance plasmid derivatives. Nature (London) **290**:794–797.
20. **Shine, J., and K. Dalgarno.** 1974. The 3′ terminal sequence of *E. coli* 16S rRNA: complementary to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. U.S.A. **71**:1342–1346.
21. **Stalker, D. M., R. Kolter, and D. R. Helinski.** 1979. Nucleotide sequence of the region of an origin of replication of the antibiotic resistance plasmid R6K. Proc. Natl. Acad. Sci. U.S.A. **76**:1150–1154.
22. **Stougaad, P., S. Molin, and K. Nordström.** 1981. RNA molecules involved in copy number control and incompatibility of plasmid R1. Proc. Natl. Acad. Sci. U.S.A. **78**:6008–6012.
23. **Terawaki, Y., Y. Kobayashi, H. Matsumoto, and Y. Kamio.** 1981. Molecular cloning and mapping of a deletion derivative of the plasmid Rts1. Plasmid **6**:222–234.
24. **Terawaki, Y., H. Takayasu, and T. Akiba.** 1967. Thermosensitive replication of a kanamycin resistance factor. J. Bacteriol. **94**:687–690.
25. **Timmis, K. N., H. Danbara, G. Brady, and R. Lurz.** 1981. Inheritance functions of group IncFII transmissible antibiotic resistance plasmids. Plasmid **5**:53–75.
26. **Tomizawa, J., T. Itoh, G. Selzer, and T. Som.** 1981. Inhibition of ColE1 primer formation by a plasmid-specified small RNA. Proc. Natl. Acad. Sci. U.S.A. **78**:1421–1425.
27. **Tsurimoto, T., and K. Matsubara.** 1981. Purification of bacteriophage λ*O* protein that specifically binds to the origin of replication. Mol. Gen. Genet. **181**:325–331.
28. **Uhlin, B. E., and K. Nordström.** 1977. R plasmid gene dosage effects in *Escherichia coli* K-12: copy mutants of the R plasmid R1*drd-19*. Plasmid **1**:1–7.