The Region Controlling the Thermosensitive Effect of Plasmid Rts1 on Host Growth Is Separate from the Rts1 Replication Region

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Rts1 is a high-molecular-weight (126 × 10^6) plasmid encoding resistance to kanamycin. It expresses unusual temperature-sensitive phenotypes, which affect plasmid maintenance and replication, as well as host cell growth. We have cloned the essential replication region of Rts1 from pAK8, a smaller derivative which is phenotypically similar to Rts1. Restriction endonuclease digests of isolated pAK8 deoxyribonucleic acid were allowed to "self-ligate" (ligation without an additional cloning vector) and subsequently were used to transform Escherichia coli strain 20SO to kanamycin resistance. Screening of these strains for the phenotypes of thermosensitive host growth and temperature-dependent plasmid elimination demonstrated that these two properties were expressed independently. Furthermore, it was shown that the Rts1 replication locus per se is not necessarily responsible for altered host growth at the nonpermissive temperature. The kanamycin resistance fragment of pAK8 was also cloned into pBR322. Electrophoretic analysis of BamHI restriction enzyme digests of this plasmid and similar digests of an Rts1 miniplasmid has allowed the identification of an 18.6-megadalton fragment carrying the replication locus and a 14.1-megadalton fragment carrying the kanamycin resistance gene.

Rts1 is a high-molecular-weight R factor of the T-incompatibility group (4) which confers upon its host resistance to kanamycin. When examined in Escherichia coli 20SO, the Rts1 plasmid copy number is stringently regulated (10), and the molecular size is approximately 126 megadaltons (Mdal) (10, 11). The unique feature of Rts1 is the thermosensitivity of strains harboring this plasmid during incubation at 42.5°C. At 42.5°C, the efficiency of conjugal transfer is markedly diminished (30), and the Rts1-mediated resistance to T-even bacteriophages is not expressed (12, 17, 36). In addition, there is a detrimental effect on the growth of cells carrying Rts1 (27, 35), and significant membrane lesions have been observed (12). Furthermore, an alteration occurs in the control of autonomous maintenance, causing the subsequent appearance of R− segregants (11). The ability of Rts1 DNA to replicate as a closed covalent circle (CCC) is impaired at 42.5°C (11, 33), which may correlate with the effect of Rts1 on host growth at the nonpermissive temperature.

Recently, two groups have selected strains carrying the Rts1 genome in a chromosomally integrated state by integrative suppression (37) and directed transposition (28). Comparison of the patterns of thermosensitivity expressed by Rts1 in an integrated state to that of autonomously replicating Rts1 derivatives revealed that reduced conjugal transfer and T-even phage restriction are under a control separate from that of thermosensitive host growth (tsg) and inhibition of Rts1 CCC formation (28, 29, 37). These findings are consistent with the idea that the tsg phenotype is closely linked to the control of Rts1 replication. Recently, a deletion map of Rts1 has been prepared by examination of an integrated Rts1 plasmid (25), and tsg was localized at or near the anticipated Rts1 replication region, adjacent to the gene(s) coding for IncT.

We have cloned Rts1 genes by using the cloning vehicle pBR322 (2), as well as by "self-ligation," which avoids the use of additional cloning vehicles or exogenously added drug resistance markers (7, 31). In this study, we report the characterization of a series of Rts1 miniplasmids generated by this self-ligation method with respect to the phenotypes of thermosensitive host growth (tsg) and stability of plasmid maintenance.
MATERIALS AND METHODS

Bacterial strains, media, and biochemicals. Media used for bacterial plating was MacConkey Agar (Difco) or L-agar (20). Liquid media included the following: heart infusion broth (Difco), Trypticase soy broth (BBL Microbiology Systems), L-broth, and M9-glucose minimal medium (9). Kanamycin sulfate (20 \(\mu\)g/ml), ampicillin (50 \(\mu\)g/ml), and tetracycline (20 \(\mu\)g/ml) were from Sigma. Endonuclease BamHI was from Boehringer Mannheim and Bethesda Research Labs, EcoRI was from Boehringer Mannheim, Sall was from Miles, and T4 DNA ligase was from Miles Labs and Bethesda Research Labs. Bacterial strains used have been described elsewhere (11, 34).

Alkaline sucrose gradient analysis. The conditions used for alkaline sucrose gradient analysis have been described in detail previously (11, 34).

Isolation of plasmid DNA. Plasmids pBR322, pFY603, and pFK004 were isolated by ethidium bromide-cesium chloride centrifugation from overnight cell cultures as previously described (34), except that a solution of 0.4% Triton X-100 in 62.5 mM EDTA-50 mM Tris-hydrochloride (pH 8.0) was substituted for Brij detergent to accomplish lysis of cells containing pFY603 and pFK004. Ethidium bromide was removed from the collected plasmid band by extracting twice with CsCl-saturated isopropanol, and solutions were finally dialyzed extensively against a buffer containing 0.1 mM EDTA-10 mM Tris-hydrochloride (pH 7.5).

Substantial quantities of Rts1 and pAK8 were prepared by a scaled-up modification of the procedure of Hansen and Olsen (15). Samples (500 ml) were grown to 1.0 \(\times\) 10\(^8\) cells per ml in M9 media, chilled quickly, and pelleted in a Beckman JA14 rotor at 0°C. Cells were washed with 50 ml of 10 mM sodium phosphate, pH 7.0, and centrifuged again. Pellets were then resuspended in 23.4 ml of 25% sucrose-0.05 M Tris-hydrochloride, pH 8.0, 1.8 ml of lysozyme (10 mg/ml in the same buffer) was added, and cells were incubated at 0°C for 5 min. After the addition of 9.4 ml of 0.25 M EDTA (pH 8.0) and another 5 min on ice, lysis was accomplished with 9.4 ml of sodium dodecyl sulfate (20% in 0.05 M Tris-hydrochloride-0.02 M EDTA, pH 8.0). Samples were alternately mixed and heated as described elsewhere (15). Samples were denatured with 9.4 ml of 3 M NaOH, mixed for 3 min, and then renatured after the addition of 18.8 ml of 2 M Tris-hydrochloride, pH 7.0. Chromosomal DNA was precipitated with 12.5 ml of the sodium dodecyl sulfate solution and 23.5 ml of 5 M NaCl; after chilling overnight at 4°C, samples were centrifuged at 12,500 rpm for 35 min at 0°C in a Beckman JA 14 rotor. The supernatant fluids were decanted and combined with 0.313 volumes of 42% polyethylene glycol (in 0.01 M sodium phosphate, pH 7.0), and kept for 6 h at 4°C. DNA was pelleted at 6,000 rpm, 4°C, for 6 min in a JA 14 rotor and purified in ethidium bromide-cesium chloride gradients as described above.

The DNA concentration was measured by the fluorescence method (16).

Restriction endonuclease digestion. Conditions for restriction enzyme reactions (50 \(\mu\)l) were as specified by the companies from which the enzymes were obtained. Sall digests were dialyzed extensively against 10 mM Tris-hydrochloride (pH 7.5) before use in cloning experiments.

Ligation. DNA digests (50 \(\mu\)l) obtained by EcoRI, BamHI, and Sall digestion were mixed with an equal volume of salts mixture to obtain a final concentration of 10 mM MgCl\(_2\), 25 mM NaCl, 0.05 mM Na\(_2\)ATP, 10 mM dithiothreitol, and 50 mM Tris-hydrochloride (pH 7.5). T4 DNA ligase (2 U, Miles Laboratories) was added, and samples were incubated at 12°C for 13 h.

Transformation. Competent cell cultures were prepared and transformed as described by Cohen et al. (5). Transformation mixtures were diluted with L-broth and incubated at 32°C for 4 to 6 h. Transformants were selected on MacConkey agar containing kanamycin or ampicillin.

Screening of Rts1 miniplasmid strains for thermosensitive growth. Colonies were picked from MacConkey-kanamycin plates and inoculated into 2 ml of M9-glucose medium. After overnight growth at 32°C, duplicate samples (approximately 4 \(\times\) 10\(^7\) cells/ml) of M9-glucose (2 ml) were incubated at 32 and 42.5°C for 12 h.

Screening for elimination of Rts1 miniplasmids. Overnight cultures inoculated from a MacConkey-kanamycin plate were grown at 32°C in 2 ml of M9-glucose medium. Cultures were diluted into duplicate samples of 2 ml of tryptic soy broth to give a final concentration of 10\(^6\) to 10\(^7\) cells/ml. Samples were incubated at 32 and 42.5°C for 20 to 24 h, and then streaked onto MacConkey plates. After overnight incubation, these “masters” were replica plated onto MacConkey and MacConkey-kanamycin plates and incubated at 32°C.

Kinetics of growth and elimination of plasmid-containing strains. Log-phase cultures at 32°C in M9-glucose media were diluted to a density of 0.01 to 0.03 optical density units at 540 nm and incubated at 32 and 42.5°C for 0, 1.5, 3, 5.5, and 9 h. Optical density measurements were taken, and serial dilutions of the cultures were plated onto MacConkey agar. Replica plating onto MacConkey-kanamycin was done to determine the extent of curing of the plasmid.

Agarose gel electrophoresis. DNA samples were subjected to electrophoresis through 0.7% agarose-89 mM Tris-2.5 mM EDTA-89 mM boric acid at 4.5 V/cm for 3 h as described by Meyers et al. (22).

Neutral CsCl gradient analysis. Cells were grown and lysed (24) and samples were subjected to centrifugation in CsCl as described previously (34).

RESULTS

Properties of the Rts1 derivative pAK8. Plasmid pAK8 is a spontaneous Rts1 derivative reported previously (32) which has a smaller molecular weight than Rts1. As shown in Fig. 1, pAK8 sediments more slowly than Rts1 through an alkaline sucrose gradient. The distance sedimented by pAK8 relative to Rts1 (\(D_{pAK8}/D_{Rts1} = 0.81\)) gives an approximate molecular mass for pAK8 of 82 Mdal (10, 32). The proportion of plasmid DNA to chromosomal DNA was 0.022 and 0.033 in strains carrying
pAK8 and Rts1, respectively (Rts1 data not shown).

The plasmid copy number (CCC molecules/chromosomal genome) was calculated to be 1.2 and 1.3 for pAK8 and Rts1, respectively, taking into consideration the loss of CCC plasmid DNA in alkaline sucrose analysis as previously described (11). To measure the buoyant density of pAK8 and Rts1, they were placed in Enterobacter cloacae and subjected to neutral CsCl density gradient centrifugation. The density of pAK8 thus measured was 1.706 g/cm³, identical to the value for Rts1 (14). In this bacterium also, 1.3 copies of pAK8 and 1.4 copies of Rts1 per chromosome exist, assuming that the molecular weight of the chromosome for the host bacterium is similar to that of E. coli, 2.5 × 10⁹ (6).

A comparison of Rts1 and pAK8 (Table 1) shows that they are identical with respect to various properties. Digestion of Rts1 and pAK8 with restriction endonuclease BamHI demonstrated substantial homology of the electrophoretic profiles of the digests (Fig. 2). Eight bands corresponding to molecular masses of approximately 22.9, 18.6, 14.1, 8.0, 6.0, 4.3, 3.7, and 1.1 Mdal were present in BamHI digests of pAK8. With the exception of the 3.7-Mdal fragment, which may represent a "fusion band," the remaining seven fragments were present in digests of Rts1 as well. Rts1 also contained additional

### TABLE 1. Comparison of the genetic characteristics of the thermosensitive R plasmids Rts1 and pAK8

<table>
<thead>
<tr>
<th>Property tested</th>
<th>Rts1</th>
<th>pAK8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incompatibility groupa</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>Entry exclusion against R401b</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>TS conjugal transferc</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>TS CCC DNA formationd</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Host growth inhibition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(42°C)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Kanamycin resistance level/ (µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>1,000</td>
<td>1,000</td>
</tr>
<tr>
<td>T-even phage restrictione</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Copy number (CCC molecules/host genome)</td>
<td>1.35</td>
<td>1.25</td>
</tr>
</tbody>
</table>

a Rts1 was transferred from E. coli 20SO to E. coli CSH2 Nal' (nalidixic acid-resistant [34]) carrying a T-group Ap' plasmid, R401 (4). It was observed that R401 was eliminated from 19 of 20 clones tested; the remaining one clone carried a recombinant between Rts1 and R401. With pAK8, R401 was eliminated from 16 of 20 clones tested, with four recombinants. The presence of recombinant plasmids was confirmed by cotransferability of the drug-resistance markers of both plasmids. Late log-phase cultures grown at 30°C in L broth were used, and bacterial mating was performed at 30°C for 90 min.

b R401 was transferred from R⁺ E. coli 20SO to E. coli CSH2 Nal', E. coli CSH2 Nal' (Rts1), and E. coli CSH2 Nal' (pAK8) at a frequency of 5.3 × 10⁻⁵, 3.3 × 10⁻⁶, and 4.6 × 10⁻⁶, respectively. Late log-phase cultures grown at 30°C in L broth were used, and bacterial mating was performed at 30°C for 90 min.

c,d,e Experimental conditions employed were those described in references 36, 33 and 34, and 9 and 34, respectively. TS, Temperature sensitive.

d Results were obtained by replica plating at 37°C.

f Experimental conditions were those described in reference 36. Relative efficiency of plating of T4 phage on E. coli 20SO with or without R plasmids was as follows (plaque counts on E. coli 20SO grown at 30°C were taken as 1.00); 1.10 for R⁺ cells grown at 43°C, <0.006 for Rts1 cells grown at 30°C, 0.10 for Rts1 cells grown at 43°C, 1.04 for pAK8 cells grown at 30°C, and 0.0 for pAK8 cells grown at 43°C. The partial "restriction" observed in cells harboring pAK8 grown at 43°C may be due to alterations of the cell envelope (12, 25).
bands at positions of roughly 1.0, 1.25, 2.3, 3.0, and 9.3 Mdal, and at least one extra band greater than the 22.9-Mdal fragment of pAK8. The degree of homology observed is consistent with the hypothesis that pAK8 was generated by a simple deletion event from Rts1. Similar results have been obtained with other restriction enzymes (data not shown).

Insertion of Rts1 and pAK8 fragments into the cloning vehicle pBR322. We tested various restriction enzymes for their ability to clone the kanamycin resistance determinant by insertion into the cloning vehicle pBR322 (2). The results of experiments inserting BamHI and Sall digests of Rts1 and pAK8 DNA into pBR322 are shown in Table 2. With Rts1 DNA, a substantial number of kanamycin sensitive inserts (pBR322 derivatives with inserts other than the kanamycin gene) could be obtained. Although no kanamycin-resistant (Km') transformants were isolated with the enzyme BamHI, a large number could be obtained with Sall. In contrast, a small number of Km' transformants could be obtained with BamHI with pAK8 DNA. This difference between Rts1 and pAK8 may possibly be due to the larger size of Rts1; after digestion, certain size classes of DNA fragments presumably have a lower frequency of ligation and subsequent transformation (13). Attempts to screen pBR322 containing Rts1 or pAK8 inserts for the capacity to mediate T4 phage restriction have so far been unsuccessful. The results summarized in Table 2 encouraged us to use pAK8 DNA for the cloning of the Rts1 replication region by self ligation.

Formation of Rts1 miniplasmids. In the experiment described in Table 3, pAK8 DNA was digested completely with restriction enzymes, self-ligated with T4 ligase, and subsequently used to transform E. coli 20SO. By using this method, a substantial number of Km' colonies were isolated. The plasmids carried by strains transformed with self-ligated pAK8 DNA were called Rts1 miniplasmids. The difficulty in obtaining Sall miniplasmids is possibly due to the large fragment size obtained after digestion by this enzyme. Agarose electrophoresis analysis (data not shown) indicated that two fragments of the Sall digest have a combined mass of 71 Mdal. Preliminary analysis suggested that the single Sall miniplasmid obtained may have been the result of a large deletion from one of these fragments (manuscript in preparation).

Experiments with randomly selected clones

![Figure 2](http://jb.asm.org/)

**Figure 2. Similarity of restriction enzyme BamHI fragments of Rts1 to those of pAK8 DNA.** Plasmid DNA was digested with BamHI and subjected to agarose gel electrophoresis as described in the text. Lane 1, 1.5 μg of Rts1 DNA; lane 2, 1.2 μg of pAK8 DNA. Molecular masses shown (Mdal) were obtained by computing relative migration compared to λ HindIII digests used as standards.

<table>
<thead>
<tr>
<th>DNA employed (μg)</th>
<th>Restriction enzyme used</th>
<th>Transformants phenotype</th>
<th>Transformants/μg of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rts1</td>
<td>pAK8</td>
<td>pBR322</td>
<td>BamHI</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.5</td>
<td>BamHI</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.5</td>
<td>BamHI</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>BamHI</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>0.5</td>
<td>BamHI</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.9</td>
<td>Sall</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.9</td>
<td>Sall</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.9</td>
<td>Sall</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.9</td>
<td>Sall</td>
</tr>
</tbody>
</table>

* Ligation mixtures containing DNA as shown were used to transform competent E. coli 20SO.

* Among 300 colonies examined, none was resistant to T4.
indicated that transformants have lost the capacity for conjugal transfer of drug resistance, thus ruling out the possibility that a small background of intact pAK8 DNA caused the acquisition of resistance to kanamycin. Colonies tested thus far have expressed the IncT phenotype (data not shown), suggesting that these transformants are indeed derived from the parent plasmid pAK8.

Characterization of the replication properties of the Rts1 miniplasmids. Transformants were screened for two thermosensitive Rts1 phenotypes: the inhibition of host growth at 42.5°C (tag), and the elimination of the plasmid, detected by the concomitant loss of kanamycin resistance. It was found that, with respect to stability, miniplasmids could be grouped into three main categories (Table 4); some plasmids are stable at both 32 and 42.5°C, others are stable at 32°C but eliminated at 42.5°C, and the remainder are only partially stable at 32°C and completely eliminated at 42.5°C. Interestingly, only 5 of 63 strains tested expressed the tag− phenotype; these belonged to various classes with respect to plasmid elimination (stability). In other words, stable plasmids exist which are either tag− or tag+

Kinetics of cell growth and plasmid elimination in E. coli pFY603. pFY603 is an Rts1 miniplasmid constructed with endonuclease BamHI. It is unstable during prolonged incubation at nonpermissive temperature and does not express the tag phenotype (Class 11, Table 4). Cell growth and maintenance of kanamycin resistance were monitored over a period of 9 h in strains harboring Rts1 and pFY603, and compared with a thermosensitive (tag+) miniplasmid, pFY505 (Fig. 3). At 42.5°C cultures carrying Rts1 show a markedly diminished turbidity after 9 h of growth, compared with strains carrying pFY603 which grow well at 42.5°C. Although a minor degree of growth inhibition was observed with pFY603, another plasmid belonging to this class (pFY601) had no appreciable effect on the host growth (data not shown). Even after 9 h of growth at 42.5°C, 89% of the 20SO pFY603 culture retained resistance to kanamycin (Fig. 3B). Therefore, the lack of tag expression was not due to the rapid emergence and growth of R− segregants. It should be mentioned that at about 10^5 cells per ml, segregation of Rts1 can occur at 42°C (11). In the experiment shown in Fig. 3, the initial cell concentration was about 10^9/ml, which generally does not lead to loss of Rts1. As expected from the tag+ phenotype of pFY505, kinetic studies revealed that, under the conditions for growth assessment, this plasmid strongly inhibited host growth at 42.5°C (Fig. 3C).

Identification of restriction fragments carrying the replication and kanamycin regions. Rts1 miniplasmids should contain a minimum of two restriction fragments; the fragment carrying the replication region and the fragment carrying Km+. (If these loci were closely linked, a single-fragment miniplasmid might be gener-

### Table 3. Isolation of small plasmid derivatives by restriction endonuclease digestion and self-ligation of pAK8 DNA

<table>
<thead>
<tr>
<th>Restriction endonuclease used</th>
<th>pAK8 DNA (μg)</th>
<th>Kanamycin-resistant transformants obtained</th>
<th>Kanamycin resistant transformants/μg of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>5</td>
<td>267</td>
<td>53</td>
</tr>
<tr>
<td>BamHI</td>
<td>15</td>
<td>77</td>
<td>5.1</td>
</tr>
<tr>
<td>EcoRI</td>
<td>5</td>
<td>29</td>
<td>5.8</td>
</tr>
<tr>
<td>EcoRI</td>
<td>15</td>
<td>65</td>
<td>4.3</td>
</tr>
<tr>
<td>Sali</td>
<td>5</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Sali</td>
<td>15</td>
<td>0</td>
<td>&lt;0.07</td>
</tr>
</tbody>
</table>

*Plasmid (pAK8) DNA was completely digested with restriction endonucleases, and T4 DNA ligase was added to mediate ligation of the DNA fragments. The ligation mixtures were then mixed with competent E. coli 20SO cells, and kanamycin-resistant transformants were selected on plates containing kanamycin.

### Table 4. Maintenance of Rts1 miniplasmids and inhibition of host cell growth

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Total tested</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>tag−</td>
<td>tag+</td>
<td>tag−</td>
</tr>
<tr>
<td>BamHI</td>
<td>35</td>
<td>0</td>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td>EcoRI</td>
<td>27</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sali</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*Class I, less than 10% loss of plasmid at either 32 or 42.5°C; class II, less than 10% loss at 32°C and more than 10% loss at 42.5°C; class III, more than 10% loss at 32°C and more than 90% loss at 42.5°C. Curing was tested by replica plating onto plates containing kanamycin after 20 to 24 h of growth at 32 and 42.5°C in liquid media as described in the text. tag+ indicates that strains express the phenotype of inhibited growth at 42.5°C; tag− indicates that strains are not thermosensitive for growth. Strains were classified as tag+ if the ratio of the turbidity at 42.5°C to that at 32°C was less than 0.3 after 12.5 h of growth in M9 medium.
plasmid elimination during short-term incubation at 42.5°C. (A) 20SO Rts1; (B) 20SO pFY603 (class II tag*); (C) 20SO pFY505 (class II tag*). Duplicate cultures were inoculated into M9-glucose media supplemented with 2 μg of thiamine per ml and incubated at 32 and 42.5°C. Cell growth was monitored, and the percentage of R' segregants was determined. Symbols: ○, optical density at 540 nm, 32°C; ●, optical density at 540 nm, 42.5°C; ▼, percent Km', 32°C; ■, percent Km', 42.5°C.

Fig. 3. The kinetics of cell growth and lack of plasmid elimination during short-term incubation at 42.5°C. (A) 20SO Rts1; (B) 20SO pFY603 (class II tag*); (C) 20SO pFY505 (class II tag*). Duplicate cultures were inoculated into M9-glucose media supplemented with 2 μg of thiamine per ml and incubated at 32 and 42.5°C. Cell growth was monitored, and the percentage of R' segregants was determined. Symbols: ○, optical density at 540 nm, 32°C; ●, optical density at 540 nm, 42.5°C; ▼, percent Km', 32°C; ■, percent Km', 42.5°C.

Fig. 4. Identification of the BamHI fragments carrying the kanamycin resistance and pAK8 replication regions. Plasmid DNA was digested with BamHI and subjected to agarose gel electrophoresis as described. Lane 1, 1.2 μg of pAK8; lane 2, 0.3 μg of pFK004; lane 3, 0.3 μg of pFY603; lane 4, 0.25 μg of λ HindIII digest (New England Biolabs).

DISCUSSION

Several laboratories have applied the technique of molecular cloning to select for the replication regions of both relaxed (7) and stringently controlled (23, 31) plasmids. These studies revealed that, although replication functions may be clustered in a small region of the plasmid genome (1, 7, 23), the origin of replication is not identical to the incompatibility and copy control loci (7, 26). In addition, multiple origins have been detected which are associated with inverted repeat structures (1, 7).

In spite of the numerous studies carried out on plasmids, surprisingly little is known about host-plasmid relationships at a molecular level. Rts1 expresses multiple temperature dependent effects on the host bacteria and, along with several other plasmids (19), represents a class of extrachromosomal genetic elements which are ideal for studies of host-plasmid interactions. Since the Km resistance of Rts1 appears to be due to the enzyme aminoglycoside 3'-phosphotransferase (8) whose gene is not susceptible to BamHI, Sall, and EcoRI (3), we have con-
structed kanamycin-resistant Rts1 miniplasmids by using these restriction enzymes. Our initial characterization of strains harboring Rts1 miniplasmids has led us to make several significant conclusions about the relationship among the various temperature-dependent phenotypes of Rts1.

Although the phenotypes of Rts1 include resistance to copper ions (18), production of a DNase (21), temperature-dependent inhibition of CCC plasmid DNA transfer (11), thermosensitive conjugal transfer (30), and restriction of T4 phage growth (12, 17, 36), we have limited our consideration to four properties of Rts1: stable replication and maintenance, kanamycin resistance, temperature-sensitive host growth (tsg), and temperature-dependent plasmid elimination. First, it appears that the replication region of these Rts1 miniplasmids is distinct from the region influencing host growth. This conclusion is based on the observation that, among the transformants harboring miniplasmids, both tsg- and tsg+ transformant strains exist. Of 63 transformants tested, only 8% showed a significant decrease in host growth at the nonpermissive temperature. One Rts1 miniplasmid, pFY603, which is tsg+, consisted of two fragments: one encoding kanamycin resistance (14.1 Mdal), and the other (18.6 Mdal) responsible for replication. (The possibility exists that part of the replication region may extend into the kanamycin resistance fragment.) In contrast, pFY505, which is tsg-, contained four extra bands after digestion with BamHI in addition to those present in pFY603 (manuscript in preparation). These observations are consistent with the notion that the extra bands of pFY505 which do not carry the replication locus may contain the thermosensitive region(s) influencing the growth of strains harboring Rts1. This last point is important because recent genetic evidence has suggested that the replication region of Rts1 may be linked to, or identical with, the tsg locus (25), although other genetic studies were against this conclusion (29). The use of molecular cloning techniques has enabled us to separate these phenotypes in Rts1 miniplasmids, despite seemingly close linkage by genetic analysis. In considering the temperature-dependent growth effects, one should not forget the possibility that an Rts1 derivative may have the characteristic of integrating into the host chromosome at 42°C, although it remains autonomous at 32°C. In such a case, one would not expect tsg expression because this phenotype may be suppressed when the plasmid is integrated into the host chromosome (37). Although this situation is rare, we have observed such a case with AB1157/Rts1 (unpublished observation).

A second conclusion which can be drawn is that the kanamycin resistance locus may not play any role in expression of the tsg phenotype or elimination of the plasmid. Thus, the kanamycin-resistant miniplasmids may be either tsg+ or tsg-, and vary greatly with regard to stability. We have tentatively classified them into three major groups: those which were easily eliminated, those which were eliminated at 42.5°C only, and those which were not eliminated at all. The varying degrees of stability shown by Rts1 miniplasmids led to our third important conclusion, that this property is affected by more than one site on the plasmid. In other words, the temperature-dependent elimination of Rts1 may be under the control of multiple genes. Alternatively, one can postulate that a single gene controls temperature-dependent elimination, but the size of the plasmid or the presence of other specific genes may influence the degree of expression of this locus. This hypothesis was strengthened by a separate observation that many of the pBR322 inserts described in Table 2 are readily eliminated from the host bacteria under nonselective conditions (unpublished observation). We have already reported that Rts1 does not influence the replication of a coexisting plasmid, indicating that there is no trans effect (11). Experiments are currently in progress to isolate the gene(s) responsible for thermosensitive host growth and plasmid elimination.

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