Characterization and Incompatibility Properties of ROM\textsuperscript{-} Derivatives of pBR322-based Plasmids

By MARILYN E. NUGENT,* TERENCE J. SMITH† AND WILLIAM C. A. TACON

Searle Research and Development, PO Box 53, Lane End Road, High Wycombe HP12 4HL, UK

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We have characterized a copy number mutant of the pBR322-based plasmid pWT111. A single nucleotide transversion in loop II' of RNAI results in an eightfold increase in plasmid copy number. Removal of the rom coding region from pWT111cop results in a further sixfold increase in copy number. We present evidence that ROM is involved in the strong incompatibility effect seen between pMB1 and ColE1 type plasmids.

INTRODUCTION

Two negatively acting components are involved in controlling the copy number of ColE1- and pMB1-type plasmids. One is a 108 bp RNA molecule, RNAI, which interacts with the primer of replication RNAII and inhibits its processing by RNAase H (Tomizawa et al., 1981). The other is a 63 amino acid protein ROM (RNAI inhibition modulator; Tomizawa & Som, 1984). The reduction of plasmid copy number caused by rom can be explained by enhancement of the binding of RNAI to RNAII by the ROM protein (Tomizawa & Som, 1984). Control of plasmid copy number is related to plasmid incompatibility, a mechanism preventing stable co-existence of two similar plasmids in the same cell. Although two ColE1- or pMB1-derived plasmids can co-replicate in the same cell for many generations there is minimal co-replication of a ColE1-derived and a pMB1-derived plasmid, the latter type rapidly excluding the former (Hashimoto-Gotoh & Timmis, 1981). A comparison of the RNAI sequence from ColE1 and pMB1 indicates a single base pair difference in loop I' (Tomizawa & Itoh, 1981) and it has been proposed that the strong incompatibility is due to a differential sensitivity to RNAI.

We have characterized a copy number mutant of the pBR322-based plasmid pWT111 (Tacon et al., 1980). A single base pair change in the loop II' region of pWT111cop RNAI leads to an eightfold increase in copy number. To increase copy number further we have made a ROM\textsuperscript{-} derivative of pWT111cop. We have compared the copy number of this derivative with the ROM\textsuperscript{-} derivatives of pWT111 and pBR322. From a study of the incompatibility properties of these plasmids we present evidence that ROM is involved in the strong incompatibility effect seen between pMB1- and ColE1-derived plasmids.

METHODS

Bacterial strain and plasmids. The Escherichia coli K12 strain WT217, a RecA\textsuperscript{-} derivative of MC1061 (Casabadan et al., 1980), was used. Plasmids are listed in Table 1.

DNA preparation and gel electrophoresis. This was as described by Tacon et al. (1980).

DNA sequencing analysis. This was done by the method of Maxam & Gilbert (1977). See Results for further details.

Copy number determinations. Cell pellets from an equivalent of 1 ml of cells with an OD\textsubscript{600} of 2.0 were used for plasmid DNA extraction using the acid phenol method of Kado & Liu (1981). Cells were sedimented by centrifugation for 2 min in an Eppendorf centrifuge, washed in 500 μl saline and then resuspended in 100 μl lysis
buffer. After lysing for 30 min at 65 °C an equal volume of acid phenol was added, and the phases were mixed gently by inverting the tube four times and then separated by centrifugation for 6 min. A 50 μl sample of the aqueous phase was taken off and added to 10 μl Ficoll running buffer; 20 μl of this mix was run on 1% (w/v) agarose Tris/borate/EDTA gels as described by Tacon et al. (1980). After staining with ethidium bromide, gels were photographed using Polaroid 667 film and photographs scanned with a Joyce-Loebl Chromoscan 3. For high-copy-number plasmids it was necessary to dilute plasmid DNA preparations 2-, 5- and 10-fold to determine a DNA concentration that would not be beyond the response of the film used. Standard plasmid preparations of WT217 (pBR322) and WT217 (pAT153) were run on each gel and the integral of the Chromoscan trace of these was compared to the test plasmids.

**Results**

**Construction of ROM- derivatives of pWT111 and pWT111cop**

pWT111 is a pBR322-based vector containing the trp promoter and part of the trpE coding sequence (Fig. 1; Tacon et al., 1980). We previously isolated a spontaneously arising high-copy-number derivative of pWT111 which was designated pWT111cop (Tacon et al., 1980). This derivative has a copy number six to eight times higher than pWT111 (Table 2).

To determine whether the mutation(s) responsible for this increased copy number was due to an altered RNAI we sequenced the entire RNAI region of pWT111cop from the XhoII site, 29 bp upstream of the start of RNAII transcription, towards the PvuII site located in the rom gene. A single base pair change was found 56 bp downstream of the RNAII transcription startpoint. The mutation, a G:C to T:A transversion, results in an RNAI with an altered loop 11' (Fig. 2a). Similar changes in loop 11' have been shown by others to be the cause of increased copy number (for a review see Davison, 1984). To confirm that this mutation was unique to pWT111cop, the loop 11' regions of pWT111 and pBR322 were also sequenced. No changes were found from the published sequence of Sutcliffe (1979).

In case other mutations causing copy number increase might be present in the rom region we sequenced the rom promoter and coding region of pWT111 and pWT111cop. This was accomplished by sequencing from the AvaII site, 156 bp from the start of the gene, towards the RsaI site 178 bp downstream of the coding region. Although the coding region was identical to that published by Sutcliffe (1979) for pBR322 we noted two base differences in the 5' untranslated portion of the rom gene. These differences have also been found by us on sequencing pBR322 in this region (Fig. 2b). These two bases are missing from the rom promoter-proximal region of ColEl (Cesareni et al., 1982). Having established that the high copy number of pWT111cop was not due to any changes in the rom coding region we made higher copy number derivatives of pWT111 and pWT111cop by deleting a portion of the rom region. pWT111 and pWT111cop were digested with PvuII and the 2383 bp origin-containing fragment was religated (Fig. 1). The resulting plasmids, pWT1118 and pWT1115, respectively, had copy numbers approximately six times those of their parent plasmids (Table 2, Fig. 3). The copy number of the ROM- derivative of pBR322, pAT153, is two to three times that of the parent plasmid. The larger increase in copy number seen with pWT1115 and pWT1118 may be due to their smaller size, i.e. the overall amount of plasmid DNA is similar in cells containing pAT153 and in those containing pWT1118.

**Table 1. Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Comments</th>
<th>Reference</th>
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<tr>
<td>pBR322</td>
<td>pMB1 RNAI, ROM+, Ap&lt;sup&gt;R&lt;/sup&gt; Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Bolivar et al. (1977)</td>
</tr>
<tr>
<td>pWT111</td>
<td>pMB1 RNAI, ROM+, Ap&lt;sup&gt;R&lt;/sup&gt; Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Tacon et al. (1980)</td>
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<tr>
<td>pWT111cop</td>
<td>Mutant RNAI, Ap&lt;sup&gt;R&lt;/sup&gt; Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This paper</td>
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<tr>
<td>pWT1115</td>
<td>ROM- derivative of pWT111cop</td>
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</tr>
<tr>
<td>pWT1118</td>
<td>ROM- derivative of pWT111</td>
<td>This paper</td>
</tr>
<tr>
<td>pAT153</td>
<td>ROM- derivative of pBR322</td>
<td>Twigg &amp; Sherratt (1980)</td>
</tr>
<tr>
<td>pML21</td>
<td>Mini-ColEl, ROM-, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Hershfield et al. (1976)</td>
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**ROM^- derivatives of pBR322**

EcoRI site and upstream HindIII site deleted in the construction of pWT111

Insertion of 500 bp HindIII trp promoter fragment

**Fig. 1.** Map of pBR322 showing the regions implicated in copy number control and plasmid incompatibility. The steps involved in the construction of the trpE fusion vector pWT111 are also illustrated. HaeII fragments B and H were deleted from pBR322 to generate the ROM^- derivative pAT153 (Twigg & Sherratt, 1980).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size (bp)</th>
<th>Copy number relative to pBR322</th>
<th>Copy number per chromosome</th>
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<tr>
<td>pWT111</td>
<td>4823</td>
<td>1</td>
<td>50*</td>
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<tr>
<td>pWT111cop</td>
<td>4823</td>
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<td>2383</td>
<td>40–48</td>
<td>2000–2400</td>
</tr>
<tr>
<td>pML21</td>
<td>10230</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>pAT153</td>
<td>3658</td>
<td>2–3</td>
<td>100–150</td>
</tr>
</tbody>
</table>

* This assumes a copy number for pBR322 of 50 copies per chromosome (Hashimoto-Gotoh & Timmis, 1981).

**Incompatibility properties of pWT111cop, pBR322, pWT1118, pWT1115 and pAT153**

Our sequencing analysis of pWT111cop showed that this plasmid had an altered RNAI, and because previous authors have indicated that this would lead to altered incompatibility properties (reviewed by Davison, 1984), we investigated this by co-transforming an *E. coli* K12 strain with our test plasmids and the ColE1 derivative pML21. pML21 is a mini-ColE1 derivative and our restriction enzyme analysis indicates that it does not contain the *rom* coding region.
In a preliminary study designed to look at strong incompatibility effects we co-transformed our test plasmids pWT11cop, pBR322, pWT1115, pWT1118 and pAT153 with pML21 into the E. coli K12 strain WT217 selecting for the presence of both plasmids by using kanamycin and carbenicillin. Double transformants were restreaked again selecting for both plasmids. Double transformants carrying pML21 with pWT11cop, pWT1115, pWT1118 and pAT153 grew well on double selection and the presence of both plasmids in these double transformants was confirmed by the acid phenol plasmid preparation technique. Double transformants carrying pBR322 and pML21 grew very poorly on the initial transformation plates and failed to grow on re-streaking. This strong incompatibility effect seen with pBR322 and pML21 has been reported previously, pBR322 rapidly excluding pML21. We expected to see altered incompatibility properties with pWT11cop and pWT1115 because of their altered RNAI. The incompatibility results with pWT1118 and pAT153, both of which are ROM- but have the pBR322 RNAI region, indicate that the absence of rom from these plasmids leads to a loss of the strong incompatibility effect seen with pBR322 and pML21.

The altered incompatibility properties of pAT153 as compared with pBR322 were also tested by growth of double transformants in broth without selection over a longer period. pML21 and pBR322 (or pAT153) were co-transformed into competent WT217 cells and the transformation mixtures added to 10 ml volumes of L broth. These were incubated at 37°C for 30 min, then samples were removed and plated onto different selection plates (zero time, Fig. 4). The cultures were kept shaking at 37°C and samples were removed at the times indicated in Fig. 4. Exponential growth was ensured by continued subculture. Double transformants carrying pML21 and pBR322 were not seen after 7 h from the start of the transformation (Fig. 4a), but
Fig. 3. A 1% agarose gel of acid phenol preparations from E. coli WT217 containing (a) pWT1118, (b) pWT1115, (c) pML21, (d) pAT153, (e) pBR322, (f) pWT111cop and (g) pWT111.

Fig. 4. Incompatibility properties of pBR322 (a) and pAT153 (b) with pML21. Cells were plated on nutrient agar (WT217 cells) (●), nutrient agar supplemented with 25 μg kanamycin ml⁻¹ (▲), nutrient agar supplemented with 200 μg carbenicillin ml⁻¹ (▽), and nutrient agar supplemented with both antibiotics (■). Results are from a typical experiment from amongst three independent repeats.

double transformants carrying pML21 and pAT153 were still present after 50 h from the start of the transformation (Fig. 4b). Double transformants carrying pML21 with pWT111cop, pWT1115 and pWT1118 were also tested by this method. Double transformants were still present 28 h from the start of the transformation in all three transformations and the growth profiles were similar to those of double transformants carrying pML21 and pAT153.
DISCUSSION

Our results indicate that ROM is involved in the strong incompatibility effect seen between the ColE1-based plasmid pML21 and pBR322. The ROM− derivative of pBR322, pAT153, does not exhibit strong incompatibility towards pML21. From an analysis of the effect of different RNAI mutations on plasmid incompatibility, Davison (1984) has proposed that the loop II′ region of RNAI regulates replication by base pairing with complementary nucleotides in loop I of RNAII. The nucleotides in loop I/I′ and loop II/II′ of ColE1 are identical whereas pBR322 differs from ColE1 in this region by a single nucleotide change in loop I/I′ (Fig. 2a). The strong incompatibility seen between pBR322 and pML21 may be due to the loop II′ of pBR322 RNAI having greater complementarity to the loop I region of pML21 than to its own loop I.

The deletion of rom from pBR322 has a dramatic effect on incompatibility. Recent work by Tomizawa & Som (1984) also indicates that the incompatibility exerted by RNAI is enhanced by the presence of ROM. ROM enhances the binding of RNAI to RNAII, which from the model proposed by Davison (1984) is thought to involve loop II′ of RNAI and loop I of RNAII. The absence of ROM may also increase the interaction of loop II′ RNAI with its complementary loop II in RNAII. This may not be as inhibitory to replication as the loop II′/I interaction.

The point mutation that we have characterized in loop II′ of pWT111cop RNAI is a G to U transversion that has not been previously isolated by others selecting for pBR322 copy mutants. pWT111, the ROM− derivative of pWT111cop, has an extremely high copy number (approximately 2000 copies per chromosome). We have attempted to make a similar high-copy-number derivative which expresses the recombinant protein urogastrone, but this plasmid was unstable, presumably due to the high level of expression of urogastrone being detrimental to the cell. It could only be isolated in a strain with a chromosomal mutation that resulted in a 20-fold lowering of plasmid copy number (W. C. A. T., unpublished results). These high-copy-number vectors may be of use if the cellular level of ROM can be manipulated to give conditional high copy number vectors (M. E. N., unpublished results).

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REFERENCES


