NOTES

Second-Site Revertants of the P1 copN22 Copy Mutant

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Received 11 September 1989/Accepted 29 January 1990

Miniplasmids with the P1 copN22 mutation have a copy number about seven times that of the wild type. Selection for reduced copy number from this plasmid led to the isolation of second-site pseudorevertants, called poc mutants. DNA sequence analysis showed that all six independent poc mutants have a single base change in the same codon of the repA gene. This implicates the amino acid at this location, either directly or indirectly, in interactions important for copy number control.

The P1 prophage is normally maintained as a plasmid at a copy number of one per Escherichia coli chromosome (17, 20). The minimal P1 plasmid replicon consists of a 245-base-pair region containing the replication origin and a gene (repA) which codes for a protein required for replication (3, 4). A 19-base-pair inexact repeated sequence that occurs five times within the origin region is responsible for the binding of RepA (1, 3). Binding of RepA to this repeated region, called incC, appears to be responsible for the positive role of RepA in replication and for the negative auto-regulation of the repA gene (9).

Directly downstream of the repA gene, nine additional inexact copies of the RepA-binding repeat are found (3). This region, called incA, is dispensable for replication (16). However, incA affects the copy number of the P1 replicon, presumably by binding RepA and making it unavailable for binding at the origin of replication (7, 8, 16).

P1 plasmid prophage mutants have been isolated which are maintained at a copy number five to eight times that of P1 wild type (20). DNA sequence analysis of these P1 cop mutants showed that each mutation resulted in a single amino acid change in RepA (5).

To identify other potential genes involved in regulating P1 copy number, we selected for reversion of the high copy number of P1 copN22 miniplasmids. Plasmid pEU3.0 (Fig. 1) (B. J. Froehlich and J. R. Scott, submitted for publication) consists of a 6.7-kilobase (kb) P1 copN22 minireplicon plus an 8.7-kb fragment of pIF11, which contains the kanamycin/neomycin resistance gene aphA-1 from Tn903 (14). This was used for the isolation of the first of the poc mutants. To preclude the possible problem of instability in low-copy-number revertants, the complete P1 partition region from pALa284, which contains the same P1 fragment as pALa270 (2), was inserted at the BamHI site of pEU3.0 to make pEU4.0 (Fig. 1). Plasmid pEU4.0 was used for the isolation of the five other poc mutants.

Nitrous acid mutagenesis of E. coli K-12 strain N99 (13) containing either pEU3.0 or pEU4.0 was performed as described by Miller (15). Independent overnight cultures of the mutagenized cells were plated on tryptone plates (19) containing 20 µg of neomycin per ml, and the plates were incubated overnight at 37°C. Colonies were screened for plasmids with reduced copy number by replica plating onto tryptone plates containing 5,000 µg of neomycin per ml. This drug concentration distinguishes the parental strain with the P1 copN22 miniplasmid, which grows with 100% efficiency, from a strain with P1 cop* . To confirm the presence of a reversion of the cop phenotype, the efficiency of plating of candidate strains was determined at both drug concentrations. Six independent mutants (called poc) that grew with 100% efficiency on plates containing the drug at 20 µg/ml but not on plates containing the drug at 5,000 µg/ml were isolated.

To determine whether this reduced level of neomycin resistance was plasmid encoded, plasmid DNA was isolated (6) from each mutant strain and transformed into E. coli K-12 strain N99, made competent by the method of Cohen et al. (10). Two transformants containing the plasmid DNA from each poc mutant were tested for efficiency of plating on tryptone plates with 20 and 5,000 µg of neomycin per ml. All six retained the poc phenotype, indicating that the mutation is located on the P1 miniplasmid. Thus, no host mutations were isolated by this procedure.

Because the reduced level of drug resistance may have resulted from a mutation in the neomycin resistance gene, and not from an alteration in plasmid copy number, the copy number of the poc mutant plasmids was measured directly. Log-phase cultures of the plasmid-containing cells were

<table>
<thead>
<tr>
<th>poc mutant plasmid*</th>
<th>Genotype</th>
<th>Relative copy no.*</th>
<th>% Error</th>
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<tbody>
<tr>
<td>pEU3.01 poc-1</td>
<td>poc-1</td>
<td>0.15</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>par</td>
<td>0.16</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.19</td>
<td>19</td>
</tr>
<tr>
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<td>poc-6</td>
<td>0.23</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.27</td>
<td>10</td>
</tr>
<tr>
<td>pEU4.08 poc-8</td>
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<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.27</td>
<td>7</td>
</tr>
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</table>

* pEU3.01 is the poc-1 derivative of plasmid pEU3.0 (Fig. 1). pEU4.06 and pEU4.08 are poc-6 and poc-8 derivatives, respectively, of plasmid pEU4.0 (Fig. 1).

* The copy numbers were calculated relative to that of each plasmid's poc* copN22 parent plasmid. For each experiment, duplicate samples of at least four dilutions were applied to the filter.
applied to nitrocellulose filters, and the DNA was immobilized as described by Shields et al. (21). The repA gene was used as the P1-specific probe. A 900-base-pair HindIII-BamHI fragment from pEU90.19 (12) was labeled with [α-32P]dATP (Amersham) by the method of Feinberg and Vogelstein (11). The hybridization conditions, washing, and exposure of the filter were as described by Froehlich and Scott (12). The relative amount of probe hybridizing to each sample was measured by two-dimensional scanning of the autoradiogram by using a Bio-Rad video densitometer 620.

Table 1 shows that each of the poc mutations reduces the copy number to 0.2 to 0.3 times that of the poc+ copN22 parent plasmid.

The poc-1 mutation was localized to the 1.2-kb HindIII fragment of P1 (Fig. 1) by replacing the poc+ copN22 HindIII fragment of pEU10 (a coelectate containing the 6.7-kb BglII fragment of pEU3.0 inserted into the BamHI site of pBR322 [Fig. 1]) with the poc-1 copN22 HindIII fragment from pEU3.01. Under polA- conditions, the copy number of the poc-1 copN22 coelectate (pEU10.1) relative to its poc+ copN22 parent was 0.15. Since the ratio of the copy number of pEU10.1 relative to that of pEU10 was the same as the ratio of the copy number of pEU3.01 relative to that of its parent (pEU3.0 [Table 1]), we conclude that the only mutation needed for the poc phenotype lies within the HindIII fragment.

To map the poc mutations, DNA sequence analysis of the complete P1 replicon region (the 1.2-kb HindIII fragment and the P1 origin [Fig. 1]) was performed on supercoiled plasmid DNA by using a modification (23) of the dideoxy chain-termination method (18) as described by Froehlich and Scott (12). Because all six poc mutations retained the copN22 lesion (results not shown), they were all second-site mutations. For each of the six, a single base in the repA gene differed from that of the copN22 parent (Table 2). Furthermore, all six poc mutations were in the same codon of repA, the one that specified the seventh amino acid.

Among the six independently isolated poc mutants there were only three different lesions (Table 2). The same thing was found when the original P1 cop mutants were selected—each independently derived mutation was isolated twice (5). This is probably because there are very few ways in which RepA can be changed to alter copy number while still maintaining both its replication function and its autoregulatory function. If the replication function were affected, the mutation would be lethal to the plasmid; if the autoregulatory function had a severe defect, runaway plasmid replication would kill the cell (22).

In all three poc mutants, a serine, which has a relatively small side chain, is replaced by an amino acid with a very bulky side chain which contains a ring structure (Pro, Tyr, or Phe [Table 2]). Such a substitution would be expected to alter the conformation of RepA and probably results, either directly or indirectly, in a change in the interaction of the protein with the P1 DNA. This change may occur as a result of alteration of the interaction of RepA with itself, with some other protein, or with the P1 DNA.

This work was supported by Public Health Service grant AI17696 from the National Institutes of Health. The Bio-Rad video densitometer was purchased with support from BRSG grant S07 RR05364.

We thank Lisa Coffield, Susan Guritz, Allison Groff, Richard Pollack, and David Beattie for assistance in performing these experiments. We are grateful to D. Stalker for pIF11 and S. Austin for pALA284.

### LITERATURE CITED


#### Table 2. Changes found by DNA sequence analysis of the poc mutants

<table>
<thead>
<tr>
<th>poc mutation</th>
<th>Codon*</th>
<th>Amino acid</th>
</tr>
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<tbody>
<tr>
<td>poc+</td>
<td>TCC</td>
<td>Ser</td>
</tr>
<tr>
<td>poc-1</td>
<td>TAC</td>
<td>Tyr</td>
</tr>
<tr>
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<td>Tyr</td>
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<td>Phe</td>
</tr>
<tr>
<td>poc-8</td>
<td>CCC</td>
<td>Pro</td>
</tr>
</tbody>
</table>

* The single base change found in all six independent poc mutants was in the codon coding for the seventh amino acid of RepA.

FIG. 1. Map of P1 copN22 miniplasmids pEU3.0 and pEU4.0. In pEU4.0, the complete P1 par region (map shown below the line [2]) has been inserted at the BamHI site of pEU3.0. The P1-derived DNA is represented by the thin line; the pIF11 DNA is represented by the thick line. The open box represents the P1 origin; the shaded box represents the incA region (3). The arrows show the extent of the open reading frame and the direction of transcription of repA, parA, parB, and the kanamycin/neomycin resistance gene, aprA-1 (2, 3, 14). The last amino acid of the ParA protein is missing in del parA, the extent of which is delineated by the arrow.