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Isolation and Characterization of a *priB* Mutant of *Escherichia coli* Influencing Plasmid Copy Number of Δ*rop* ColE1-Type Plasmids

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The lethality induced by the overproduction in *Escherichia coli* of a heterologous protein was used to select bacterial mutants. In one of these, the mutation responsible was mapped to *priB*. We describe the isolation of this mutant, the sequencing of the mutated gene, and its in vivo effect on plasmid replication.

The product of *priB*, initially called the n protein, was identified biochemically as one of the proteins necessary for the assembly on the DNA of the primosomal protein complex (1, 14, 16, 19). The proposed role of this element in DNA replication is to eventually provide an entry point for the DNA polymerase III holoenzyme. The assembly of the primosome requires six proteins, PriA, PriB, PriC, DnaB, DnaC, and DnaT, the functions of which have mostly been explored by in vitro experiments (2, 13, 16). PriA initiates primosome assembly by binding to a specific site on the DNA, the *pas* sequence (2, 16, 20). The ensuing structure is recognized by proteins PriB and PriC, to generate the preprimosome complex. However, no direct evidence for an in vivo role of PriB in replication had been obtained until now, probably because no *priB* mutant had yet been described. The results described below on the effect on plasmid copy number of a *priB* mutant that we have isolated suggests that PriB may indeed be involved in replication of ColE1-related plasmids in vivo.

Selection of hGM-CSF-tolerant mutants. In plasmid pEMR727 (3, 4), the gene coding for the human granulocyte-macrophage colony-stimulating factor (hGM-CSF) lies downstream of the *tac* promoter, so that in strain JS219 (6), its synthesis is efficiently repressed by the LacI protein encoded by both the plasmid and the chromosome. Addition of isopropyl-β-D-thiogalactopyranoside (IPTG) inactivates the repressor to allow transcription to proceed from the *tac* promoter. For the bacterium, the result of inducing hGM-CSF expression is severe growth inhibition (IPTG^s phenotype): the pEMR727-containing strain forms colonies on medium supplemented with 1 mM IPTG at a frequency of only 3.5×10^{-6} (IPTG^r phenotype). Independent cultures of strain JS219 were treated with nitrosoguanidine (17), transformed with plasmid pEMR727, and plated on Luria-Bertani (LB) medium (17) supplemented with 1 mM IPTG and ampicillin. Of 40 IPTG^r clones, only 1 displayed an increase, by a factor of 2, in the level of recombinant protein recovered in the soluble fraction of the periplasm (data not shown). We focused on the physiological and genetical characterization of this mutant, which turned out to be affected primarily in the regulation of plasmid copy number.

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Genetic mapping to the *priB* gene of the mutation of strain OFB3085. The original mutant strain was first cured of plasmid pEMR727, to give strain OFB3085. Insertions of mini-Tn*10* genetically linked to the mutation in OFB3085 were then selected (11, 17). Two, Tet^r-7 and Tet^r-26, were retained because bacteriophage P1 cotransduction data suggested they were derived from independent insertions events. To determine more precisely the position of the Tet^r insertions on the chromosome of *Escherichia coli*, genomic DNA from the strains carrying insertions Tet^r-7 and Tet^r-26 was digested with the eight restriction endonucleases used by Kohara et al. (12) to establish the detailed chromosomal restriction map of strain W3110. The products of the single- and double-digestion reactions were separated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with a radiolabelled DNA fragment corresponding to the *tetA* and *tetR* genes present in mini-Tn*10*. The analysis of the autoradiographs allowed reconstitution of the restriction maps of the two regions in which the insertions events occurred. Comparison of these maps with that of the entire chromosome revealed a correspondence with two neighboring regions, centered around coordinate 4426 kb for the site of insertion Tet^r-26 and around coordinate 4470 kb for that of Tet^r-7, on the revised map established by Rudd (17) (Fig. 1). These sites are therefore 44 kb apart, and the mutation, being linked to both (about 17 kb from insertion 7 and 37 kb from the other), must be located in between them at around coordinate 4458 kb (Fig. 1). The chromosomal segment containing the Tet^r insertions and the IPTG^r mutation has been entirely sequenced (5a). Comparison of that sequence with the restriction maps around the two sites of mini-Tn*10* insertion confirmed the previous assignment: the probable position of the IPTG^r mutation was calculated to be at bp 116,700 (±5,000 bp). Among the 12 open reading frames in that 10-kb region, only 4 of them, forming the *rpsF priB rpsR rplI* operon, code for proteins of known functions. We focused on *priB* because the properties of its product made it a likely candidate for an effect on plasmid copy number (1, 16, 19). The *priB* genes from the wild-type and mutant strains were amplified with a thermostable DNA polymerase and cloned into the *Bam*HI and *Eag*I sites of a plasmid, pACYC184 (9), compatible with pBR322 derivatives. The plasmid containing the wild-type allele was introduced into the mutant strain OFB3085(pEMR727): this restored the IPTG sensitivity. From these results we concluded that either (i) the mutation present in OFB3085 is located in the *priB* gene and is recessive or (ii) it is not in *priB* but is suppressed by the amplification of *priB*$^+$. The sequencing of

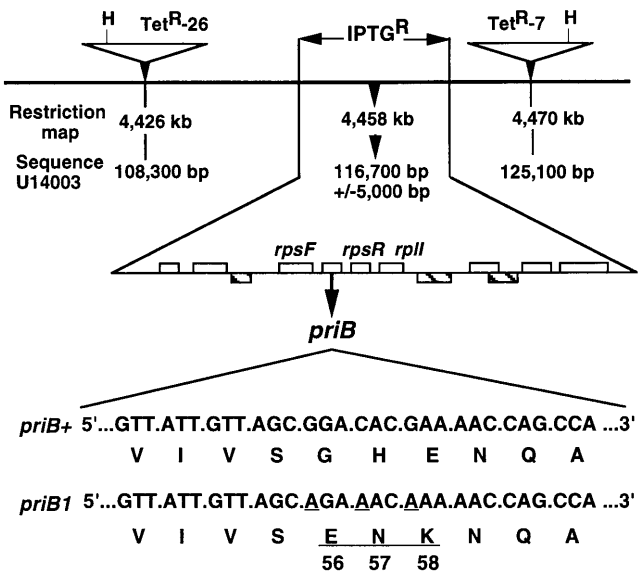

FIG. 1. Localization on the *E. coli* chromosome of the mutation conferring the IPTG^r phenotype to strain OFB3085 and sequence of its mutated *priB* gene. A library of random insertions of mini-Tn*10*, which confers resistance to tetracycline (Tet^r), was generated in the mutant strain OFB3085, using λ1098 as the transposon donor (11, 17, 18). Total DNA from two of these, Tet^r-7 and Tet^r-26, was submitted to a detailed restriction analysis as described in the text to identify the chromosomal region where each transposon inserted. The upper part of the figure shows the location, and orientation (H indicates the position of the *Hin*dIII site in mini-Tn*10*), of the Tet^r-7 and Tet^r-26 insertions on the physical map of the chromosome (17) and in the sequence of the region (GenBank accession no. U14003). The known and potential genes, existing in the 10-kb region containing the mutation conferring the IPTG^r phenotype, are represented. The bottom part of the figure presents the sequence of the part of the *priB* gene in which changes were found in the OFB3085 mutant strain. A 360-bp fragment containing the entire *priB* gene was PCR amplified (with AmpliTaq polymerase [Perkin-Elmer]) from chromosomal DNA of the wild-type (JS219) or mutant (OFB3085) strain. The two PCR primers were defined by using the published sequence of the *priB* region (GenBank accession no. U14003): the first, CGCGGATCCGCTGAAGCTCGGGGATTCTG, corresponds to the region upstream of the translation initiation signals of *priB*, and the second, CATCG GCCCGTGCCATATGGCTAGTCTC, overlaps the 3′ end of the gene. As underlined in the sequence, a *Bam*HI restriction site is introduced by the first oligonucleotide and an *Eag*I restriction site is brought in by the second. Plasmids expressing the wild-type or mutant allele of *priB* were made by cloning the PCR-amplified fragments into the pACYC184 vector (9) downstream from the *tet* gene promoter. In both cases, three independent plasmids were sequenced (Vent$_R$ sequencing kit [New England Biolabs]) over the entire length of the cloned PCR fragments. The only changes found in *priB* are those indicated in the figure.

the entire *priB* genes isolated from the mutant and wild-type strains showed the first alternative to be correct. The results of the sequence analysis, shown in Fig. 1, allowed us to identify in strain OFB3085 a mutant *priB* allele, the first described and hence termed *priB1*, which contains three mutations, leading to three amino acid changes: a glycine-to-glutamic acid substitution at position 56, a histidine-to-asparagine substitution at position 57, and a glutamic acid-to-lysine substitution at position 58. A notable effect of these substitutions is that although a normal length protein can be made, the mutations cause important modifications in the nature and positions of charged amino acids. Strain OFB3085(pEMR727) was also transformed with the compatible plasmid carrying the *priB1* allele. Surprisingly, the transformants all recovered the IPTG^s phenotype. Thus, the increased gene dosage of the *priB1* allele, provided by its cloning in a multicopy plasmid vector, allows the full suppression of its effect. This suggests that the mutated PriB1 protein retains activity. That it is not however fully active

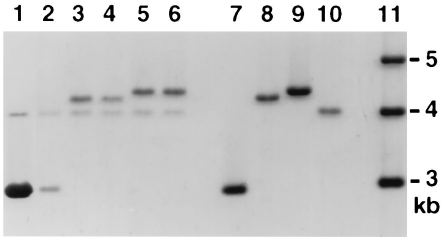

FIG. 2. Effect on plasmid copy number of the *priB1* mutant allele of strain OFB3085. Plasmid DNA was purified, using the alkaline lysis method as described by Maniatis et al. (15), from log-phase cultures (3 optical density units at 600 nm) prepared in LB medium supplemented with appropriate antibiotics (17). A portion of each preparation was digested with *Bam*HI, a unique cutter in the four plasmids used, and electrophoresed in an agarose gel, which was subsequently stained and photographed. Lanes 1 to 6 were loaded, respectively, with DNA from JS219(pGB2)(pBluescript-SK$^+$), OFB3085(pGB2)(pBluescript SK$^+$), JS219(pGB2)(pACYC184), OFB3085(pGB2)(pACYC184), JS219(pGB2)(pBR322), and OFB3085(pGB2)(pBR322). Lanes 7 to 10 contain *Bam*HI-digested DNA from plasmids pBlueScript-SK$^+$, pACYC184, pBR322, and pGB2, respectively, and lane 11 was loaded with size markers (1-kb ladder from Gibco-BRL).

was shown by altering the strength of the promoter, allowing expression of the cloned *priB* gene: the 17-nucleotide spacing between the −35 and −10 boxes was reduced to 14 nucleotides by digestion with *Cla*I and *Hin*dIII, followed by ligation after repair of the ends with the Klenow fragment of DNA polymerase I. The modified *priB1* plasmid does not anymore confer the wild-type phenotype (i.e., IPTG sensitivity) to strain OFB3085 (pEMR727), whereas the modified *priB*$^+$ plasmid still does.

The *priB1* mutant allele affects plasmid copy number. The growth characteristics of strain OFB3085 were examined: they proved indistinguishable from those of the starting wild-type strain, JS219, when assayed for plating efficiency or growth rate in rich medium (LB) with or without IPTG. However, a marked difference appeared when plasmid copy number was examined after retransformation with plasmid pEMR727 or other plasmids based on the same vector (3, 4): there is about four- to fivefold less plasmid DNA (per optical density unit of culture) in the mutant strain than in the corresponding wild-type derivatives. We then used four different plasmids in order to define which types of replicons are affected: (i) pBR322 (5), the ColE1-like plasmid used to generate our cytokine-expressing plasmids (3, 4); (ii) pBluescript-SK$^+$ (Stratagene), which also has the origin of replication of pBR322 but is closer to our expression vector in that it does not contain the *rop* regulatory gene and is therefore of higher copy number; (iii) pACYC184 (9), a plasmid similar to, but compatible with, pBR322 and its derivatives because its origin of replication (ori15A) has a sequence sufficiently divergent to preclude interference (in addition pACYC184 is naturally Δ*rop*); and (iv) pGB2 (10), whose pSC101 replication system is unrelated to that of ColE1 and p15A. The copy number of only the first two plasmids is affected by the mutation of strain OFB3085, thus suggesting an effect specific for Δ*rop*-ColE1 or p15A plasmids. To quantitate it more precisely, we therefore transformed the wild-type and mutant strains with two plasmids, pGB2 and one of the three others. Plasmid DNA, prepared from exponentially growing cultures of the resulting six strains, was digested with *Bam*HI and separated by agarose gel electrophoresis. The ethidium bromide-stained gel was photographed (Fig. 2), and the negative was scanned to determine the relative intensity of the two plasmid bands present in each sample. Using the pGB2 signal for normalization, it appears that in the mutant strain the copy number of pBluescript-SK$^+$ is reduced fourfold (Fig. 2, lanes 1 and 2) and that of pACYC184 is reduced 1.8-fold (lanes 3 and

4); pBR322 remains unaffected (lanes 5 and 6). Therefore, the mutation carried by strain OFB3085, by a direct or indirect effect on DNA replication, significantly reduces the copy number of plasmids using the control mechanism of ColE1-like plasmids, provided that the *rop* regulatory gene is absent, or, to a lesser extent, that of p15A-derived plasmids.

Possible role of PriB in control of plasmid replication. Given the complex mechanism of replication control of ColE1-type plasmids (7, 8, 16), how might the mutations we have found in the *priB* gene reduce the copy number of Δ*rop* plasmids? The site of action of PriB, the *pas* sequence, is located on the same strand as the promoter of the RNA I primer, about 600 bp downstream of the position where RNA I transcription is initiated. It becomes available for the sequential binding of PriA, PriB, and PriC once the primer has been elongated on the opposite strand by DNA polymerase I. In a Δ*rop* strain, the partly active PriB1 protein might have a reduced affinity for the PriA-*pas* complex and would thus frequently delay the organization of the primosome long enough to abort the entire initiation process. Fewer plasmid molecules would be replicated per generation than in the *priB*$^+$ background, leading to a reduction in plasmid copy number. When the plasmid is *rop*$^+$, the product of that gene probably lowers the copy number to a level compatible with the residual capacity of the PriB1 protein and thus no additive effect of the *priB* mutation can be seen. Further biochemical studies will be necessary to determine if the product of the *priB1* allele indeed affects primosome assembly and how this bears on the control of plasmid replication in vivo.

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