Differential effects of Kid toxin on two modes of replication of lambdoid plasmids suggest that this toxin acts before, but not after, the assembly of the replication complex

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Kid is a small protein that is encoded by plasmid R1. It is a toxin that belongs to a killer system that ensures the stability of the plasmid in host cells. The results of previous studies have suggested that Kid is an inhibitor of DNA replication, possibly acting at the onset of initiation. Here, the authors tested the effects of Kid on oriλ-initiated and oriJ-initiated replication, which may be driven by both the newly assembled replication complex and the heritable complex. It was found that Kid inhibits only replication that is driven by the newly assembled replication complex. The authors also report that Kid inhibits ColE1-like plasmid replication in vivo, in agreement with the previously reported inhibition of ColE1 during in vitro replication. It is proposed that the Kid toxin acts at the level of replication either by preventing de novo assembly of the replication complex or by impairing the functional interactions of the replication complex at the initiation stage.

Keywords: plasmid R1, λ plasmids, oriJ-based plasmid, inheritance of the replication complex

INTRODUCTION

Plasmid R1 contains the parD region, which is responsible for the stabilization of the plasmid in host cells. This stabilization is based on the mechanism involved in the killing of plasmid-free cells. Two proteins are encoded by the parD system, Kid and Kis. Kid is a toxin that is responsible for killing cells and Kis neutralizes the lethal activity of Kid (Bravo et al., 1987, 1988).

Previous studies have revealed that Kid inhibits the in vitro replication of a DnaB-dependent replicon, ColE1, at an early stage and that this toxin also prevents the induction of the λ prophage. Moreover, the overproduction of the DnaB protein (a helicase) results in a considerable increase in the viability of cells expressing the kid gene (Ruíz-Echevarría et al., 1995). These results led to the original proposal that Kid could target DnaB, thus poisoning the replication complex at the initiation stage (Ruíz-Echevarría et al., 1995). Alternatively, Kid could interact with another component of the replication complex in a manner that could be disrupted by an excess of DnaB in the cell. However, it remains unknown whether this toxin acts before or after the assembly of the replication complex.

To test at which step Kid inhibits the replication process, we used in vivo systems based on two phage-derived replicons, λ and oriJ. There are two ‘pathways’ of replication for both of these replicons. After a round of replication, the replication complex is inherited by one of two daughter plasmid copies and it may function in future rounds of replication; a new replication complex has to be assembled in the second plasmid copy (Węgrzyn & Taylor, 1992; Potrykus et al., 2000). The heritable replication complex contains λ-encoded O and P replication proteins and two bacterial proteins: DnaB and DnaK (a molecular chaperone) (Węgrzyn & Węgrzyn, 1995, 2001; Węgrzyn et al., 1995a, b, 1996; Zylilcz et al., 1998; Potrykus et al., 2002).

It is possible to restrict the replication of plasmids λ and oriJ solely to the pathway that is based on the heritable replication complex (Fig. 1). Amino-acid starvation prevents the synthesis of new replication proteins, thus...
the assembly of new replication complexes is inhibited. Therefore, any replication of the plasmids observed under these conditions is dependent on the activity of the heritable replication complexes (Węgrzyn & Taylor, 1992; Węgrzyn et al., 1992; Potrykus et al., 2000; Baranśka et al., 2002). Amino-acid starvation leads to the rapid relA-dependent synthesis of guanosine 3′-diphosphate 5′-diphosphate (ppGpp; Cashel et al., 1996). Due to the ppGpp-mediated inhibition of pR promoter activity, and the resulting impairment of the transcriptional activation of the origin are necessary for triggering a new round of replication (see review by Węgrzyn & Węgrzyn, 2001).

**METHODS**

**Bacterial strains and plasmids.** *Escherichia coli* strains MG1655 (wild-type) and BM711 (supE44 hisD− bsdR thi−1 bvr−1 leuB6 lacY1 tonA21Δrac slr−1::Tn10 relA251::kan), described by Jensen (1993) and Potrykus et al. (2000), respectively, were used in this study. The plasmids that were used in this study are listed in Table 1. The *kid* gene was amplified from MG1655 by PCR using the primers pkidB (5′-CGGGGATCC-GTCAGGAGGAATTCTGAC-3′) and pkidE (5′-CAAGAA-TTCTGTTTCAAGTCAATGTGGAGA-3′). The PCR product was cloned into the low-copy-number cloning vector pNMD220 (Gotfredsen & Gerdes, 1998), which contains lacI8 and the LacI-regulated pMLM96 promoter (Lanzer & Rujard, 1988), by using the BamHI and EcoRI restriction sites, to generate pSS100. Plasmid pSS100 was then digested with *Aat*II and *Hind*III. The 4895 bp fragment containing pMLM96−kid and lacE was ligated to the 2846 bp *Aat*II−*Hind* III fragment of pACYC177, which contains oriP15A and bta, to yield pMLM96. Plasmid pKP96, a pMLM96 derivative conferring resistance to chloramphenicol but not ampicillin, was constructed by digestion of pMLM96 with *Aat*II and *Bgl*II. Plasmid pKP2000, a pKP96 derivative conferring resistance to chloramphenicol and erythromycin, was constructed by the digestion of pKP96 with EcoRI. The linearized fragment was ligated to a 1800 bp fragment bearing the erythromycin-resistance gene. The 1800 bp fragment was obtained by the digestion of pVA8912 with ClaI and BamHI.

**Culture media and amino-acid starvation.** LB medium (Sambrook et al., 1989) was used for molecular cloning procedures. Minimal medium 2 (Węgrzyn et al., 1991) supplemented with 100 µg 1-leucine ml−1 and 100 µg threonine ml−1 was used for all experiments. Amino-acid starvation was provoked by the addition of *t*-valine (to a final concentration of 1 mg ml−1) to a bacterial culture growing in minimal medium 2, as described previously (Węgrzyn et al., 1991).

**Estimation of plasmid DNA replication.** The replication of plasmid DNA in host cells was investigated as described previously (Herman et al., 1994a; Szalewska-Pałasz et al., 1994). Briefly, 5 ml samples of bacterial cultures were withdrawn at 30 min intervals for 4 h. The samples were centrifuged (4000 g, 5 min), and the bacterial pellets were used for the isolation of plasmid DNA by alkaline lysis (Sambrook et al., 1989). Following its linearization via a restriction digest, the plasmid DNA was subjected to agarose-gel electrophoresis. (Enzymes that cut a given plasmid at a unique site) After staining the gel with ethidium bromide, the intensity of the plasmid bands, which corresponded to the relative amounts of plasmid DNA, was measured by densitometry, using the UVP E.A.S.Y. densitometry system. To assess whether this assay was quantitative, different volumes of a culture of plasmid-harbouring bacteria were withdrawn and used for plasmid DNA isolation. In addition, an equal amount of an external control (purified DNA from another plasmid) was added to each sample. Following electrophoresis and densitometry, we estimated that the values obtained from the test samples differed from the expected values by <10%.

**Measurement of plasmid DNA synthesis.** Plasmid DNA synthesis was measured as described by Herman et al. (1994b) and Potrykus et al. (2000). Briefly, the method was similar to that for the ‘Estimation of plasmid DNA replication’, but samples of equal cell mass (1 OD unit) were used. Following the suspension of the pellets in fresh medium (0.5 ml per sample), 5H thymidine was added up to a final concentration of 20 µCi ml−1 (740 kBq ml−1). After 5 min of pulse-labelling, Na2O, was added to a concentration of 20 mM. The cells were then lysed, and the plasmid DNA was isolated and linearized with restriction enzymes and separated by agarose-gel electrophoresis. The amounts of plasmid DNA present in the gel were estimated by densitometry. Plasmid bands were then excised from the gel, and the agar blocks were dissolved in 2 M HCl at 60 °C overnight. The radioactivity of each sample was then measured in a scintillation counter.
RESULTS
Kid inhibits the replication of ColE1-like plasmids, but not of plasmids \( \lambda \) and ori, in amino-acid-starved cells

Ruiz-Echevarria et al. (1995) reported that Kid inhibited the in vitro synthesis of ColE1 plasmid DNA when it was added before the initiation of replication, but that this toxin was ineffective 10 min after the initiation of replication. These results suggest that Kid inhibits DNA replication at an early stage of the process. One possible hypothesis to explain these observations was that Kid acts before the assembly of the replication complex, and that the target of Kid is unavailable to this protein once the complex is formed. To test this hypothesis, we investigated the replication of \( \lambda \) plasmids in an isoleucine-starved relA mutant. Under these starvation conditions, \( \lambda \) plasmid replication is due exclusively to the activity of replication complexes that were assembled before the onset of amino-acid starvation and that were inherited by one of two daughter plasmid copies after each round of replication (Węgrzyn & Taylor, 1992; Szalewska-Palasz et al., 1994; Węgrzyn et al., 1995a, b; Węgrzyn & Węgrzyn, 2001). Therefore, if the hypothesis was true, we should have observed no significant effect of Kid on \( \lambda \) plasmid replication in amino-acid-starved relA cells.

Since amino-acid starvation inhibits protein synthesis, the induction of Kid expression by the addition of IPTG was provoked 10 min before the induction of isoleucine starvation. ColE1-like plasmids have been shown previously to replicate in isoleucine-starved relA mutants, though by a mechanism different from the inheritance of the replication complex (Wrobel & Węgrzyn, 1997, 1998). Hence, the putative target for Kid is not protected in ColE1-like plasmids. By monitoring the replication of a ColE1 replicon, we found that the induction of Kid production 10 min before the onset of amino-acid starvation was sufficient to inhibit the replication of plasmid DNA (Fig. 2). In these experiments, equal volumes of the culture were withdrawn at the times indicated in Figs 2–4, from which plasmid DNA was isolated and linearized by a restriction digest. After separation of the DNA by agarose-gel electrophoresis, the amount of plasmid DNA was estimated by densitometry. Thus, any increase in the amount of plasmid DNA present would indicate replication, whereas the

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**Table 1. Plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
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<tbody>
<tr>
<td>pBR328</td>
<td>oriMB1, amp(^R), cm(^R), tet(^R)</td>
<td>Bolivar &amp; Backman (1979)</td>
</tr>
<tr>
<td>pCB104</td>
<td>ori(^R), cm(^R)</td>
<td>Boyd &amp; Sherratt (1995)</td>
</tr>
<tr>
<td>pLG1</td>
<td>oriColE1, amp(^R)</td>
<td>Diaz &amp; Pritchard (1978)</td>
</tr>
<tr>
<td>pLG2</td>
<td>ori(^R), amp(^R), cm(^R)</td>
<td>Diaz &amp; Pritchard (1978)</td>
</tr>
</tbody>
</table>
| pNB       | oriMB1, amp\(^R\), \( p_{

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**Fig. 2.** Replication of the ColE1 plasmid (pLG1) in amino-acid-starved E. coli BM711 cells (a) and bacterial growth under these conditions (b). Isoleucine starvation was provoked by the addition of L-valine (final concn 1 mg ml\(^{-1}\)) to the bacterial culture growing in minimal medium (\( \odot \)) at the time indicated by the open arrow. In addition, IPTG was added to half of the culture (\( \bullet \)), to a final concentration of 0.5 mM, at the time indicated by the solid arrow, to induce the expression of Kid from pKP96. In (b), the growth of a non-starved culture (\( \odot \)) is also shown. In both (a) and (b), the mean results from three experiments are presented. Error bars ± SD are shown in (a); in (b), in all cases the SD was < 10%.
constant value in Fig. 2(a) shows inhibition of plasmid replication. When the replication of \( \lambda \) plasmid DNA was investigated under the same conditions, the amount of plasmid DNA increased both in the bacteria devoid of Kid and in the cells producing this protein (Fig. 3).

Another replicon that uses the mechanism of inheritance of the replication complex is a plasmid bearing oriJ as its only origin of replication (Potrykus et al., 2000). We found that, as with the \( \lambda \) plasmid, the replication of the oriJ-based plasmid was not inhibited by Kid in amino-acid-starved cells (Fig. 4). Therefore, it seems that once assembled the replication complex is protected from Kid-mediated inhibition.

**Effects of Kid on the replication of the oriJ-based plasmid in non-starved cells**

If the hypothesis that Kid acts as a toxin only before the assembly of the replication complex was true, one should be able to observe some effects of \( \text{kid} \) expression on the replication of plasmids employing the inheritance of the replication complex mechanism in non-starved cells, i.e. when both ‘pathways’ of plasmid DNA replication operate (Fig. 1). Indeed, we found that the replication of the oriJ-based plasmid was inhibited by Kid in cells cultivated under standard growth conditions, but that this inhibition was moderate (Fig. 5). These results are compatible with the tested hypothesis.

**Kid-mediated inhibition of \( \lambda \) plasmid DNA replication based on the newly assembled replication complexes**

We aimed to verify the hypothesis that the inhibitory action of Kid operated solely before the assembly of the replication complex by using another approach. Again, we used the \( \lambda \) plasmid system, but this time the synthesis of plasmid DNA, rather than the amount of plasmid present, was measured in non-starved cells (Fig. 6). Our assumptions were as follows. In non-starved \( E. \ coli \) cells, there are two pathways of \( \lambda \) plasmid replication.
Mechanism of action of the Kid toxin

Fig. 5. Replication of the oriJ plasmid (pLG2) in E. coli BM711 cells (a) and bacterial growth in the minimal medium (b) either without additional treatment (○) or after the induction of kid expression (●) from pKP2000. Kid expression was induced by the addition of IPTG to the culture (final concn 0·5 mM) at the time indicated by the solid arrow. Error bars ± SD are shown in (a); in (b), in all cases the SD was <10%.

Fig. 6. Synthesis of λ plasmid DNA in E. coli BM711 cells growing in a minimal medium (○), after the addition of rifampicin (final concn 0·2 mg ml−1) (▲) and after the induction of kid expression (●) from pMLM96. Kid expression was induced by the addition of IPTG to the culture (final concn 0·5 mM) at the time indicated by the solid arrow. Equal amounts of plasmid DNA were used for the measurements of [3H]thymidine incorporation during 5 min pulses. The mean results from three experiments are presented; error bars ± SD are indicated. The curves (dashed lines) indicate the theoretical results expected if: (a) replication was totally unaffected; (b) only half of the plasmid molecules appearing after each round of replication entered the next rounds of replication; and (c) replication was completely inhibited after the induction of kid expression.

(see Fig. 1). Regardless of the ‘source’ of the replication complex (heritable or newly assembled), the initiation of λ plasmid DNA replication occurs at constant time intervals, which roughly corresponds to a generation time of untreated cells growing in a given medium at a given temperature (Węgrzyn & Taylor, 1992; Węgrzyn et al., 1996). We investigated the synthesis of plasmid DNA by measuring the incorporation of [3H]thymidine into the DNA. Samples of a bacterial culture were pulse-labelled; the radioactivity derived from equal amounts of plasmid DNA was then analysed. From such an experimental system, we could predict three possibilities. (i) If λ plasmid DNA replication was totally insensitive to Kid, overproduction of this toxin should not affect this replication. Thus, the incorporation of radioactive thymidine into the DNA should be constant over time [dashed line (a) in Fig. 6], exactly as in the control experiment where Kid synthesis was not induced (open circles in Fig. 6). (ii) If Kid inhibited DNA replication irrespective of the stage of the replication complex (i.e. before and after its assembly), we should observe strong inhibition of λ plasmid DNA synthesis, since both of the replication pathways would be inhibited [dashed line (c) in Fig. 6]. Such a scenario – i.e. the dramatic inhibition of λ plasmid DNA replication – is represented by the results of the control experiment in which rifampicin, an antibiotic that inhibits transcription and thus indirectly but strongly inhibits λ plasmid replication initiation (see review by Węgrzyn & Węgrzyn, 2001), was added to the bacterial culture (open squares in Fig. 6). (iii) If Kid acted only before the assembly of the replication complex, and the formation of such a complex protected the target from Kid-mediated inhibition, then one pathway (that based on the heritable replication complex) should be functional and some synthesis of plasmid DNA should be observed. In such a case, because an equal amount of plasmid was analysed in each sample and the number of plasmid molecules would increase while the number of active replication complexes, unaffected by Kid, would be constant (compare Fig. 1), the measured amounts of [3H]thymidine in the plasmid DNA should decrease over time, as depicted by the dashed line (b) in Fig. 6. In fact, experimental results (closed circles in Fig. 6) indicate that after the induction of Kid production the curve for the incorporation of [3H]thymidine into plasmid DNA resembles theoretical curve (b) rather than curve (a) or (c). Moreover, under the same experimental conditions, the replication of ColE1-like plasmid DNA was strongly inhibited by kid expression (data not shown), indicating that Kid production was sufficiently high to halt DNA synthesis in the sensitive replication system. Therefore, these results suggest that Kid prevents the formation of an active λ replication.
complex either by impeding the assembly of the replication complex or by blocking its activity.

**DISCUSSION**

Previously, it was reported that the R1-encoded Kid toxin inhibited the in vitro replication of ColE1 plasmid DNA, and that overexpression of kid prevented the induction of the λ prophage (Ruiz-Echevarría et al., 1995). However, a direct in vivo evaluation of the effects of Kid on the replication of ColE1-like and λ replicons was not done. In this study, we have addressed this and can report that the Kid toxin can inhibit the replication of both of these replicons to different extents. We also examined whether Kid is able to act before or after the assembly of the replication complex. For this purpose, we employed experimental systems which allowed us to distinguish between the activities of already assembled replication complexes and those of newly assembled complexes. These systems were based on plasmids λ and oriJ, which replicate according to two ‘pathways’: the first pathway functions due to activity of the heritable replication complex and the second pathway requires assembly of a new replication complex (Fig. 1).

Four lines of evidence support the hypothesis that the Kid toxin acts before, but not after, the assembly of the replication complex. First, Kid loses its inhibitory potential in the in vitro DNA replication of the ColE1 plasmid when it is added several minutes after the initiation of replication (Ruiz-Echevarría et al., 1995). Second, Kid does not inhibit the replication of plasmids λ and oriJ in amino-acid-starved cells, i.e. under conditions that only allow replication based on previously assembled heritable replication complexes (Figs 3 and 4). Under the same conditions, the replication of a ColE1-like plasmid, which does not form a heritable replication complex, was inhibited by the expression of kid (Fig. 2). Third, oriJ plasmid replication was impaired by Kid in non-starved cells; however, the inhibition of replication was not complete, indicating that a significant number of plasmids could still replicate (Fig. 5). Fourth, experiments that measured the incorporation of [3H]thymidine into λ plasmid DNA in non-starved cells expressing kid revealed that the kinetics of plasmid DNA synthesis agree only with the model when it is assumed that after each round of replication only half of the plasmid molecules enter the next round of replication (Fig. 6).

Our experiments show that λ plasmid replication based on newly assembled replication complexes, but not that carried out by heritable replication complexes, is inhibited by the Kid toxin. However, these results do not answer the question as to what is the specific target for Kid. Previous studies have indicated that overexpression of dnaB alleviates some of the effects of Kid (Ruiz-Echevarría et al., 1995). We have also found that the overproduction of DnaB, but not of DnaC, partially suppresses Kid-mediated inhibition of bacterial growth, and that an excess of DnaB in cells does not influence Kid production in our experimental system (data not shown). Therefore, one possibility is that DnaB may be a target for Kid. However, DnaB-mediated alleviation of Kid toxicity might be indirect; hence, the hypothesis that a factor other than DnaB is a real target for Kid is equally possible. Clearly, additional experiments are necessary to specifically address this problem.

It also remains to be elucidated whether Kid has access to its target (DnaB or another component of the replication complex) once the replication complex has been assembled or whether the target protein becomes resistant to Kid after binding to DNA and/or to other proteins. In fact, the protection of particular target proteins from other proteins has been reported previously. For example, the λO protein is rapidly degraded by the ClpP/ClpX protease, but it is protected from this protease after the formation of the λ replication complex (Węgrzyn et al., 1992, 1995a; Zyllicz et al., 1998).

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