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Indole inhibition of ColE1 replication contributes to stable plasmid maintenance

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ABSTRACT

In the absence of active partitioning, strict control of plasmid copy number is required to minimise the possibility of plasmid loss at bacterial cell division. An important cause of multicopy plasmid instability is the formation of plasmid dimers by recombination and their subsequent proliferation by over-replication in a process known as the dimer catastrophe. This leads to the formation of dimer-only cells in which plasmid copy number is substantially lower than in cells containing only monomers, and which have a greatly increased probability of plasmid loss at division. The accumulation of dimers triggers the synthesis of the regulatory small RNA, Rcd, which stimulates tryptophanase and increases the production of indole. This, in turn, inhibits *Escherichia coli* cell division. The Rcd checkpoint hypothesis proposes that delaying cell division allows time for the relatively slow conversion of plasmid dimers to monomers by Xer-*cer* site-specific recombination. In the present work we have re-evaluated this hypothesis and concluded that a cell division block is insufficient to prevent the dimer catastrophe. Plasmid replication must also be inhibited. *In vivo* experiments have shown that indole, when added exogenously to a broth culture of *E. coli* does indeed stop plasmid replication as well as cell division. We have also shown that indole inhibits the activity of DNA gyrase *in vitro* and propose that this is the mechanism by which plasmid replication is blocked. The simultaneous effects of upon growth, cell division and DNA replication in *E. coli* suggest that indole acts as a true cell cycle regulator.

1. Introduction

For a plasmid to persist in a bacterial host, at least one copy must be inherited by both daughter cells at division. It is often assumed that multicopy plasmids of *Escherichia coli* such as ColE1 are distributed randomly throughout the cell (Summers and Sherratt, 1984) such that the probability (P) of one daughter inheriting all n copies of the plasmid at cell division, and the other none, is:

$$P = 2^{(1-n)}$$

Plasmid dimers can arise through homologous recombination between monomers (reviewed in Smith, 1988). A dimer contains two active replicons and is consequently perceived by the copy number control system as two plasmids. Thus the formation of a single dimer effectively reduces the plasmid copy number by 1 and doubles the probability of plasmid loss. For plasmid ColE1, each origin on a dimer can fire independently to initiate replication, and the product of replication is two dimers (Summers et al., 1993). Consequently once the first dimer has been formed by recombination, dimers accumulate by replication twice as fast as plasmid monomers. The dimer catastrophe hypothesis (Summers et al., 1993) describes how, as a consequence of the over-replication of dimers, dimer-only cells emerge within a few generations. These cells have approximately half the plasmid copy number of monomer-only cells and thus seriously threaten plasmid stability.

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Two factors counteract the accumulation of plasmid dimers. Firstly, ColE1 contains the 240 bp *cer* site, which significantly increases plasmid stability by allowing multimer resolution (Summers and Sherratt, 1984), although this recombination process is thought to be relatively slow (Oram et al., 1997). Site-specific recombination at *cer* requires the host-encoded proteins ArgR (Stirling et al., 1988), PepA (Stirling et al., 1989) and recombinases XerC (Colloms et al., 1990) and XerD (Blakely et al., 1993). The second factor limiting dimer proliferation is the slower growth of dimer-containing cells which limits their presence in the population (Summers et al., 1993). Recently, when the dimer catastrophe was re-examined *in silico*, the main features of the hypothesis were confirmed, although the effect of dimers on plasmid stability was found to be somewhat less than originally proposed. In consequence it was suggested that the deleterious effect of dimers may be due more to the reduction they cause in host fitness (growth rate) than their threat to plasmid stability (Field and Summers, 2011).

In addition to its role in dimer resolution, the *cer* site also encodes a 70nt RNA, Rcd, whose transcription is driven by the P_{cer} promoter that lies within the site (Patient and Summers, 1993). The complex formed by ArgR, PepA, XerCD and a fifth host-encoded protein, Fis, allows Rcd expression only from plasmid dimers (Blaby and Summers, 2009). Rcd binds the enzyme tryptophanase and stimulates the reaction that converts tryptophan to pyruvate, ammonia and indole (Chant and Summers, 2007). Indole inhibits the growth and cell division of *E. coli* at concentrations of 3 mM and above (Chant and Summers, 2007; Piñero-Fernandez et al., 2011). The Rcd checkpoint hypothesis asserts that Rcd is transcribed from dimers in order to stall cell division and give the multimer resolution system time to convert the dimers into monomers and ensure plasmid stability.

In this work, the Rcd checkpoint hypothesis is critically examined and found to be incomplete because the proposed mechanism would be insufficient to prevent the rapid accumulation of plasmid dimers. It is proposed that, to arrest the dimer catastrophe, the checkpoint must also inhibit plasmid replication. It is shown experimentally that this is indeed the case and evidence is presented that inhibition is exerted by the effect of indole on DNA gyrase.

2. Results and discussion

2.1. Re-evaluation of the Rcd checkpoint hypothesis

The Rcd checkpoint hypothesis suffers from a critical flaw. Imagine that the accumulation of dimers triggers Rcd production and cell division is prevented while all other cellular processes, including growth, continue normally. The increase in cell volume dilutes the repressor of replication, RNAI (Tomizawa et al., 1981), such that plasmid replication continues at the same rate as before. Most significantly, in a cell with both monomers and dimers, dimers continue to out-replicate monomers. Thus the dimer catastrophe would continue unchecked by the inhibition of cell division. Under these circumstances, blocking cell division makes the eradication of plasmid dimers by Xer-*cer* recombination no more effective than when cells are growing normally.

Previous work has shown that indole does more than just prevent cell division; it also inhibits growth at a concentration of 3 mM or higher (Chant and Summers, 2007; Piñero-Fernandez et al., 2011). If slower growth could reduce the rate of plasmid replication by reducing the dilution rate of RNAI, then the accumulation of dimers might be halted. However, a variety of studies of plasmid copy number have suggested that the rate of plasmid replication is only marginally affected by slower growth of the host cell. Consequently, copy number increases in slower-growing cells. Engberg and Nordström (1975) report that the copy number of plasmid R1 increased from 2 to 6 when the growth rate decreased from 1.8 to 0.4 doublings per hour. Atlung et al. (1999) similarly reported that the copy number of plasmid pBR322, a ColE1 derivative, increased 3- to 4-fold when the generation time was increased from 20 to 80 min.

Increased plasmid copy number is also associated with slower growth as broth culture enters stationary phase. This was quantified by Stueber and Bujard (1982), who reported that the copy number of pBR322 increases 4-fold as the growth rate slows during the transition from exponential to stationary phase. Finally, it has been known for many years that the addition of chloramphenicol to a culture in late exponential phase increases the yield of ColE1-like plasmids (Clewell, 1972; Frenkel and Bremer, 1986). In the presence of chloramphenicol, protein synthesis is inhibited but RNA and DNA synthesis continue for as long as the relevant proteins remain intact. This leads to continued expression of RNAII, which initiates ColE1 replication (Itoh and Tomizawa, 1980), and continued plasmid replication even though the chloramphenicol-treated cells stop growing.

These pieces of evidence all indicate that reducing the cell growth rate will not prevent plasmid replication. At best, it might reduce the rate of dimer proliferation, but the difference is unlikely to be dramatic enough to allow the dimer resolution system to arrest the dimer catastrophe. If the inhibitory effects of indole on cell division and growth are not sufficient to prevent the accumulation of dimers, how does the Rcd–indole mechanism stabilise the plasmid? We hypothesised that indole must have an additional, as yet unknown, effect. The dimer catastrophe could be mitigated either by increasing the rate of dimer resolution or decreasing the rate of dimer creation. Stimulation of site-specific recombination by indole seemed less plausible, so the possibility of a direct effect of indole on plasmid replication was investigated.

2.2. The effect of indole on plasmid replication

In order to establish whether indole has a direct effect upon plasmid replication, it was necessary to control for its effects on cell division and growth. As mentioned above, the antibiotic chloramphenicol prevents cell division and growth but allows continued plasmid replication. Therefore our first approach was to investigate whether indole