

Indole signalling contributes to the stable maintenance of *Escherichia coli* multicopy plasmids

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Summary

The efficient transmission of multicopy plasmids to daughter cells at division requires that a high copy number is maintained. Plasmid multimers depress copy number, thereby causing instability. Various mechanisms exist to counter multimerization and thus ensure stable maintenance. One well-studied example is the multimer resolution system of the *Escherichia coli* plasmid ColE1 which carries a recombination site (*cer*) at which multimers are resolved to monomers by the XerCD recombinase. A promoter within *cer* initiates synthesis of a short transcript (Rcd) in multimer-containing cells. The Rcd checkpoint hypothesis proposes that Rcd delays cell division until multimer resolution is complete. We have identified tryptophanase (which catabolizes tryptophan to pyruvate and indole) as an Rcd binding protein. Furthermore, the stabilization of multicopy plasmids by Rcd is shown to be tryptophanase dependent, and a tryptophanase-deficient strain is resistant to growth inhibition by Rcd overexpression. Rcd increases the affinity of tryptophanase for its substrate tryptophan which causes increased indole production by cells in low-density cultures. Thus Rcd-mediated stabilization of multicopy plasmids is dependent upon indole acting as a signalling molecule. This is an novel role for this molecule which previously has been implicated in quorum sensing-like processes at high cell density.

Introduction

Multimers of multicopy plasmids in *Escherichia coli* are an important cause of instability (Summers and Sherratt, 1984). Although multimers arise initially by recombination they accumulate rapidly by over-replication in a process known as the dimer catastrophe (Summers *et al.*, 1993). Multimer accumulation causes a decrease in plasmid copy number which increases the frequency at which

plasmid-free cells arise. Because of their reduced metabolic load, plasmid-free cells often out-grow their plasmid-containing counterparts (Glick, 1995). The combined effects of the dimer catastrophe and plasmid metabolic load ensure that even a low rate of plasmid multimer formation can cause a rapid increase in the proportion of plasmid-free cells. In response to this threat of instability, plasmids have evolved sophisticated strategies to ensure their stable maintenance. One example is the Xer-*cer* multimer resolution system of the multicopy plasmid ColE1, which contains a site (*cer*) at which four host-encoded proteins (XerC, XerD, ArgR and PepA) mediate site-specific recombination to resolve plasmid multimers to monomers (Stirling *et al.*, 1988; 1989; Colloms *et al.*, 1990; Blakely *et al.*, 1993).

Despite its undoubted importance, the conversion of multimers to monomers by site-specific recombination is not a complete solution to the threat which multimers pose to plasmid stability. Timing is important too, and multimer resolution must be achieved before the cell divides because this is the only time when a plasmid may be lost. A promoter, P_{cer} , located centrally within *cer* (Summers and Sherratt, 1988) directs transcription of a short, untranslated RNA called Rcd (regulator of cell division). Rcd is approximately 70 nt, and its predicted secondary structure is a hairpin loop (Sharpe *et al.*, 1999). Mutations which inactivate P_{cer} or abolish Rcd function reduce plasmid stability, despite having no detectable effect on Xer-*cer* recombination (Patient and Summers, 1993; Balding *et al.*, 2006). Elevated concentrations of Rcd are found in multimer-containing cells, and the presence of the transcript correlates with an increased doubling time. Overexpression of Rcd in cells grown on solid medium severely inhibits colony formation and appears to introduce a bottle-neck in the cell cycle at a point after the replicated nucleoid has been partitioned, but before cell division (Patient and Summers, 1993). Based upon these observations it has been proposed that Rcd is part of a checkpoint that is activated by the presence of plasmid multimers and delays cell division until the multimers have been resolved to monomers (Patient and Summers, 1993).

Attempts to identify anti-sense RNA or DNA targets of Rcd were unsuccessful, and this led to the suggestion that Rcd might interact directly with a protein (Sharpe *et al.*, 1999). Here we report evidence that Rcd interacts with the

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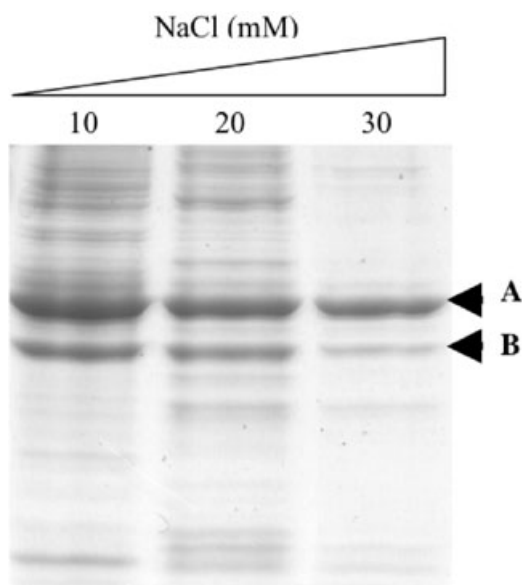


Fig. 1. Rcd-RNA affinity chromatography of an *E. coli* crude cell lysate. Fractions eluted from the column by increasing concentrations of NaCl (10, 20 and 30 mM) were analysed by SDS-PAGE. Mass spectrometry was used to identify protein A as TnaA, the monomer subunit of tryptophanase, and protein B as EFTu.

enzyme tryptophanase, increasing its affinity for tryptophan and stimulating indole production. We propose that cell division delay is mediated by indole acting in a novel role as an intracellular signalling molecule.

Results

Identification of tryptophanase as a candidate target of Rcd

RNA affinity chromatography was used to identify candidate protein targets of Rcd. Cell lysates of *E. coli* DS953 were applied to CNBr-activated sepharose cross-linked to *in vitro* transcribed Rcd. Protein fractions were eluted from the column using a step-gradient of NaCl and analysed by SDS-PAGE. Figure 1 illustrates the profile of proteins eluted at 10, 20 and 30 mM NaCl. One protein band (band A) persisted at the highest salt concentration, making it a strong candidate for an Rcd binding protein. Using MALDI spectroscopy fingerprinting, this protein was identified as TnaA. A tetramer of TnaA constitutes the enzyme tryptophanase, which catalyses the conversion of tryptophan to indole, pyruvate and ammonia (Hopkins and Cole, 1903). A second, somewhat fainter band (band B) was identified as elongation factor Tu (EFTu). EFTu is the most abundant protein in *E. coli*, comprising 5% of total cell protein (Miller and Weissbach, 1977) and it is possible that its retention on the column may be due more to its abundance than a specific affinity for Rcd. We adopted

tryptophanase as our primary candidate for the target of Rcd and designed further experiments to test this hypothesis.

Disruption of the tryptophanase gene (*tnaA*) reduces plasmid stability

In the hyper-recombinogenic host JC8679 (AB1157 *recBC sbcA*) a *cer^r* multicopy plasmid is retained in more than 99% of cells, even after 100 generations of non-selective growth. However, mutations which inactivate Rcd or its promoter cause instability (Patient and Summers, 1993; Balding *et al.*, 2006). Our affinity chromatography data suggest that the plasmid stabilizing effect of Rcd is exerted via tryptophanase. If this is true, a *cer^r* plasmid in a tryptophanase-deficient derivative of JC8679 should be as unstable as one which cannot make Rcd.

Figure 2 compares the stability of plasmids in JC8679 and its tryptophanase-deficient derivative JC8679 Δ *tnaA*. We examined the stability of three related plasmids: pUC8 contains no *cer* site, pKS490 is a pUC8 derivative with a wild-type *cer* site and pKS494 is a derivative of pKS490 in which a mutation has inactivated the Rcd promoter (Summers and Sherratt, 1988). In both strains pUC8 was lost rapidly in the absence of selection and, as reported previously, pKS494 was less stable than pKS490 in JC8679. Consistent with our prediction we found that pKS490 and pKS494 were both unstable in JC8679 Δ *tnaA*. The rates of loss of the two plasmids were indistinguishable. Northern blotting was used to confirm that Rcd was expressed from pKS490 at similar levels in JC8679 and in its Δ *tnaA* derivative (data not shown).

An *E. coli tnaA* deletion mutant is resistant to growth inhibition by Rcd

Overexpression of Rcd severely inhibits *E. coli* colony formation on solid medium, and this property has been exploited in screens for loss-of-function mutants of Rcd (Patient and Summers, 1993; Balding *et al.*, 2006). If Rcd acts via tryptophanase, a *tnaA* deletion mutant should be immune to Rcd overexpression. To test this we used the Rcd expression plasmid pCm(ss)-19 from which Rcd is expressed at 42°C but not 37°C. Figure 3 compares the effect of Rcd expression on the tryptophanase-deficient strain BW25113 Δ *tnaA* and its parent BW25113. As expected, colony formation by BW25113 pCm(ss)-19 was abolished at 42°C. Consistent with our prediction, BW25113 Δ *tnaA* pCm(ss)-19 formed colonies at 42°C, although they grew slightly less vigorously than at 37°C when Rcd was not expressed. The cells in these colonies showed no sign of the extensive filamentation which would result from growth continuing in the absence of cell

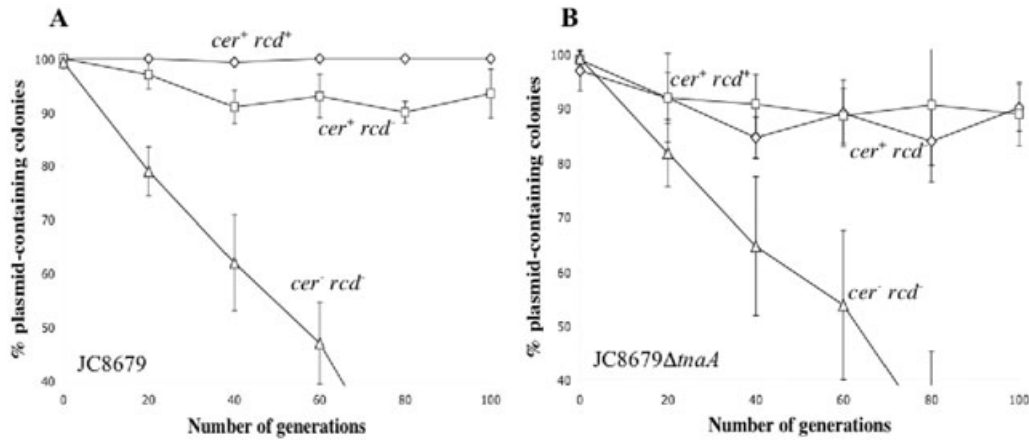


Fig. 2. Plasmid stability in *TnaA*⁺ and *TnaA*⁻ derivatives of *E. coli* JC8679. Plasmid stability in non-selective batch culture was assayed for plasmids pKS490 (*cer*⁺*rcd*⁺; ◇), pKS494 (*cer*⁺*rcd*⁻; □) and pUC8 (*cer*⁻*rcd*⁻; △) in JC8679 and its Δ *tnaA* derivative. Data plotted are the mean of six independent assays.

division (data not shown). Although this experiment used a heat shock to induce Rcd expression, the elevated temperature was not necessary for BW25113 Δ *tnaA* to be Rcd-resistant. The same result was obtained when Rcd was induced with IPTG from a *P*_{lac} promoter on a multi-copy plasmid (data not shown).

Rcd upregulates tryptophanase activity in vitro

Tryptophanase converts L-tryptophan to indole, pyruvate and ammonia. The effect of Rcd on the kinetic parameters of the reaction was measured *in vitro* using a coupled tryptophanase–lactate dehydrogenase assay (Zakomir-

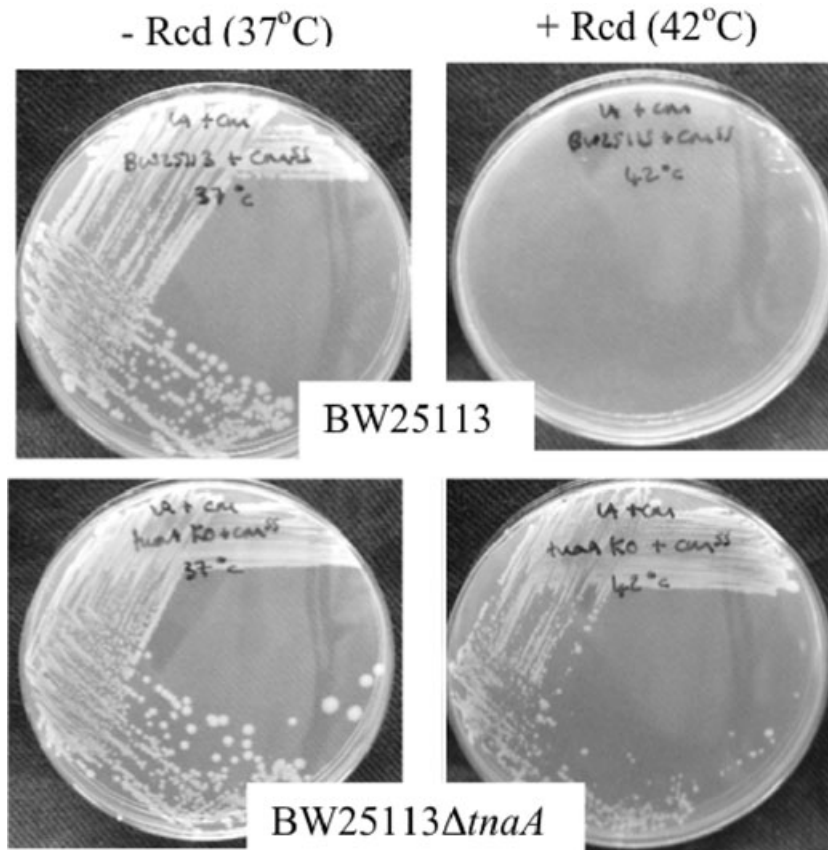


Fig. 3. A tryptophanase-deficient mutant is resistant to growth inhibition by Rcd. BW25113 Δ *tnaA* and its isogenic parent BW25113, both containing the Rcd expression plasmid pCm(ss)-19, were streaked onto on L-agar at 37°C (Rcd expression repressed) and 42°C (Rcd expressed).

Table 1. Wild-type Rcd increases the affinity of tryptophanase for its substrate *in vitro*.

	No Rcd	+Rcd (wild-type)	+Rcd4 (mutant)
K_M^a (μM)	167.3 \pm 17.1	33.4 \pm 13.2	185.1 \pm 37.5
V_{max}^a ($\mu\text{M min}^{-1}$)	2.4 \pm 0.23	2.0 \pm 0.16	2.8 \pm 0.3

a. Data are the mean of three independent assays.

dina *et al.*, 2002). Pyruvate, produced from the breakdown of L-tryptophan, is metabolized to lactate by lactate dehydrogenase. This is associated with the reduction of NADH to NAD⁺. As NADH absorbs light at 340 nm, but NAD⁺ does not, the reaction can be monitored by the decrease in absorbance at 340 nm. Data from the assay were used to calculate the K_M and V_{max} of tryptophanase in the absence of Rcd (Table 1). The assay was then repeated in the presence of either wild-type Rcd or Rcd4, a non-functional mutant derivative (Balding *et al.*, 2006). Both Rcd transcripts were 74 nt and were synthesized by *in vitro* transcription; Rcd4 differs from wild-type Rcd by just one nucleotide. Neither the wild-type nor the mutant Rcd significantly affected the V_{max} of tryptophanase. However, wild-type Rcd decreased the K_M from 167 μM to 33 μM , whereas Rcd4 had no significant effect. Thus wild-type Rcd causes an approximately fivefold increase in the affinity of tryptophanase for tryptophan.

Among the products of tryptophan catabolism, pyruvate is channelled into the TCA cycle, and ammonia is used either as a precursor in amino acid biosynthesis or excreted as a waste product. It is also the sole source of indole *in vivo* (Newton and Snell, 1965). Indole is not further degraded and, in addition to serving as a precursor in the biosynthesis of tryptophan, there is emerging evidence that it has a role as a stationary phase signalling molecule (Hirakawa *et al.*, 2005). It is thus plausible that Rcd exerts its effect on plasmid stability by increasing the concentration of indole in cells containing plasmid multimers.

Rcd stimulates indole production by tryptophanase *in vivo*

As Rcd has been shown to stimulate tryptophanase *in vitro*, expression of the transcript *in vivo* should elevate cellular indole levels. To test this we used an indirect assay for indole which monitors its conversion to indigo by styrene monooxygenase. Indigo is detected spectrophotometrically as OD₆₁₀. Styrene monooxygenase was expressed constitutively from vector pStyABB in *E. coli* BW25113 and Rcd was expressed from vector pG10-cm (from which Rcd expression is repressed by glucose and induced by IPTG). The intracellular indole concentration was measured at different stages of growth, and the data

are presented in Fig. 4 as the OD₆₁₀/OD₆₀₀ ratio (which normalizes indole concentrations for differences in culture density) plotted against OD₆₀₀ (which reflects the culture density at which each sample was taken). In the absence of Rcd, the intracellular indole concentration remained low during exponential phase (OD₆₀₀ = 0.4–1.0), increasing by five- to sixfold as the culture approached stationary phase (OD₆₀₀ > 1.0). When Rcd was expressed from pG10-cm, the intracellular indole concentration at low culture density increased by three- to fivefold. The stimulatory effect of Rcd became less pronounced at higher density as the culture approached stationary phase. When the experiment was repeated in a tryptophanase-deficient strain (BW25113 Δ tnaA), no indole was produced (OD₆₁₀ < 0.007) in the presence or absence of Rcd, confirming that tryptophanase is essential for indole production *in vivo*.

In the light of our discovery that Rcd stimulates indole production by tryptophanase, and the well-established link between plasmid multimerization and Rcd expression (Patient and Summers, 1993), we sought to confirm that plasmid multimerization is sufficient to increase the intracellular indole concentration. Cultures of the hyperrecombinogenic strain JC8679 containing either pUC8 (no *cer* site), pKS490 (wild-type *cer* site) or pKS494 (*cer* site with an Rcd promoter knockout) were grown at 37°C to OD₆₀₀ = 0.7 (approximately). The cells were harvested by centrifugation and intracellular indole assayed using Kovac's test. When the host strain was plasmid-free, or carried pKS494 or pUC8 (plasmid multimers but no Rcd expression), the indole concentrations were indistinguish-

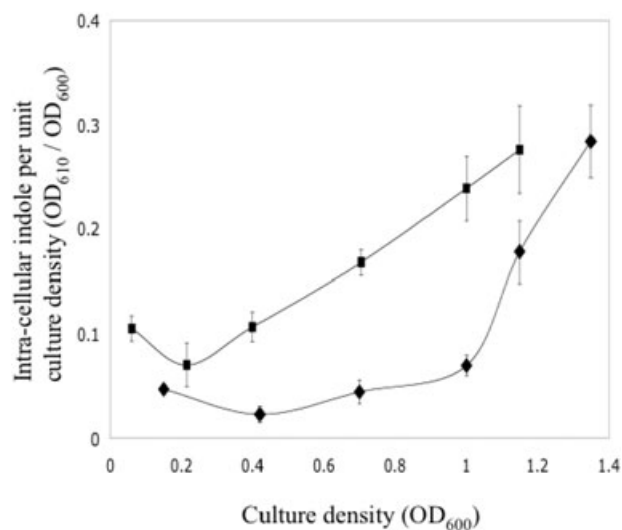


Fig. 4. Rcd causes a growth phase-dependent increase in intracellular indole. *E. coli* strain BW25113 containing pSTYABB24 and pG10-cm was grown under inducing (+ IPTG ■) and repressing (+ glucose ◆) conditions for Rcd expression. Samples were taken hourly and the culture density (OD₆₀₀) recorded. Indole assays (OD₆₁₀) were carried out on each sample and the results were normalized for culture density (OD₆₁₀/OD₆₀₀).

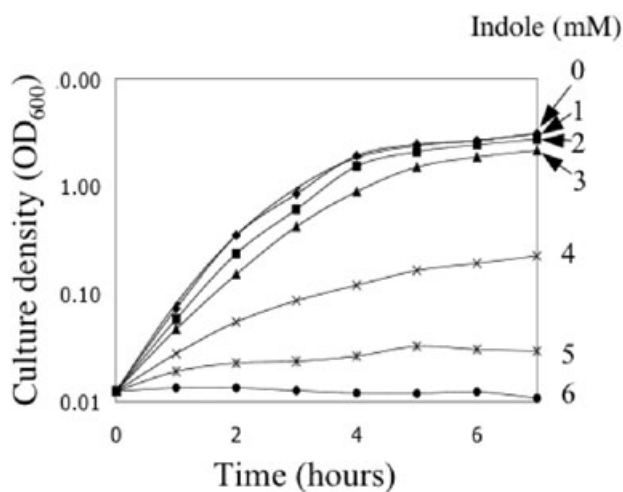


Fig. 5. The effect of exogenous indole on the growth of *E. coli* BW25113. Cultures were grown in L-broth supplemented with 0–6 mM indole. A control culture was grown with DMF (the solvent used to prepare stock solutions of indole) but no indole. Data are the average of three independent repeats.

able and all around 480 μM (470 ± 30 , 478 ± 11 and $484 \pm 36 \mu\text{M}$ respectively). However, for JC8679 containing pKS490 (plasmid multimers and Rcd expression), the concentration was $1690 \pm 212 \mu\text{M}$. Thus plasmid multimerization led to a fourfold increase in intracellular indole, but only if the plasmid contained a *cer* site which was capable of expressing Rcd. Preliminary measurements of the indole concentration in cell-free supernatants of these cultures indicated that in the absence of Rcd (JC8679 containing pUC8 or pKS494), the extracellular indole concentration was slightly higher than inside the cells ($\sim 650 \mu\text{M}$), and it increased approximately twofold in the presence of Rcd (JC8679 pKS490).

Indole inhibits E. coli growth in a concentration-dependent manner

Finally we investigated the effect of exogenous indole on a broth culture of *E. coli* BW25113. Figure 5 shows that indole inhibits growth in a concentration-dependent but non-linear manner. There was little effect on growth when $< 3 \text{ mM}$ indole was present in the medium, but strong inhibition became apparent as the indole concentration increased through the range 3–6 mM. It is clear from Fig. 5 that a threefold increase in indole (e.g. from 2 mM to 6 mM external concentration) can make the difference between no detectable effect on growth and total growth inhibition. This encourages us to believe that the fourfold increase in the intracellular indole observed in response to plasmid multimerization is likely to have a biologically significant effect.

Discussion

This work has demonstrated a novel role for indole in cell signalling, and has greatly advanced our understanding of the link between plasmid multimerization and cell division proposed in the Rcd checkpoint hypothesis (Patient and Summers, 1993; Summers, 1998). Our current understanding of the Rcd checkpoint can be summarized as follows. Transcription from the P_{cer} promoter in *cer* is induced in plasmid multimers, leading to expression of the Rcd transcript. Promoter activation is thought to occur by twisting within the Xer-*cer* synaptic complex (Chatwin and Summers, 2001). The Rcd thus produced interacts with tryptophanase, increasing the affinity of the enzyme for its substrate tryptophan and stimulating indole synthesis. Although the affinity chromatography results are in principle consistent with an indirect interaction between Rcd and tryptophanase, the demonstration that Rcd stimulates tryptophanase in an *in vitro* assay suggests a direct interaction.

We propose that the upregulation of indole synthesis by Rcd is responsible for delaying cell division. This is supported by our recent observation (I. Blaby and D. Summers, unpubl. data) that when 4 mM indole is added to a culture of cells growing exponentially in broth culture they stop dividing. The cells continue to elongate slowly, increasing in length approximately fivefold in 5 h. The mechanism by which indole blocks division remains unknown. Modulation of gene expression is one possibility and precedents exist for indole-dependent regulation of transcription. Wang *et al.* (2001) observed a dose-dependent induction of three genes (*astD*, *tnaB* and *gabT*) involved in the uptake and metabolism of amino acids. More recently, Hirakawa *et al.* (2005) observed transcriptional induction by indole of a variety of xenobiotic exporter genes including *acrD*, *acrE*, *cusB*, *emrK*, *mdtA*, *mdtE* and *yceL*. The effect upon *mdtE* was mediated via the AraC-type transcription factor GadX (Ma *et al.*, 2003; Masuda and Church, 2003). While the binding of indole to a transcription factor offers a plausible mechanism for regulation of gene expression, an alternative possibility is riboswitch modulation. Riboswitches form in mRNA (Winkler and Breaker, 2005) and are bound by metabolites; often pyrrole ring-based molecules like indole. They control gene expression by switching between alternative mRNA conformations. Finally it is possible that indole interacts directly with one or more cell cycle regulator proteins and we find this mechanism attractive as it would be more rapid than one which operates via altered gene expression.

Wang *et al.* (2001) and Hirakawa *et al.* (2005) identified indole as an extracellular signalling molecule involved in quorum sensing in stationary phase or high-density cultures, although initially this proposal was greeted with

caution by others in the field (Winzer *et al.*, 2002). Di Martino and coworkers demonstrated that inactivation of the *tnaA* gene, or inhibition of tryptophanase activity by the competitive inhibitor oxindolyl-L-alanine, led to a decrease in biofilm formation by *E. coli*. They concluded that indole is not a quorum-sensing molecule *per se* but is 'a signalling molecule which shares some modes of action with quorum sensing systems' (Di Martino *et al.*, 2002; 2003). In contrast to these cases where elevated extracellular indole acts as an indicator of high cell density, we have shown that Rcd expression increases indole production in low-density cultures, distinguishing clearly its role in plasmid stability from any involvement in quorum sensing. For Rcd to have the greatest effect in low-density cultures makes biological sense. A failure to resolve plasmid dimers in non-dividing cells in high-density culture presents no serious problem, but rapidly growing cells in low-density culture might divide before dimer resolution is complete, resulting in loss of the plasmid.

In our experiments with the hyper-recombinogenic strain JC8679, the fourfold elevation of intracellular indole caused by multimerization of plasmid pKS490 was accompanied by a twofold increase in extracellular indole. This effect on the extracellular environment is not surprising because, under these atypical conditions, every cell in the culture contains plasmid multimers and exhibits increased indole production. However in a Rec⁺ strain, where plasmid multimerization is relatively rare, an isolated dimer catastrophe is unlikely to cause a significant increase in extracellular indole. The Xer-*cer* multimer resolution system ensures that plasmid multimers exist only transiently and, once they have been restored to the monomeric state, Rcd expression will cease and indole production will decline. Indole is removed from the cell by xenobiotic exporters and, in a low-density culture, the exported indole will be insufficient to increase significantly the extracellular concentration.

In conclusion, we have identified a novel role for indole signalling in *E. coli*. We see common features between the roles of this molecule in high- and low-density cultures. If a major role of indole is to delay cell division, this will be of use both in high-density culture when impending resource limitation makes it prudent to brake the cell cycle, and in low-density culture when cell division must be delayed transiently to allow time for multimers to be resolved to monomers.

Experimental procedures

Bacterial strains and plasmids

DS953 is a *recF lacI^q* derivative of *E. coli* K12 AB1157 (Bachmann, 1972). DH5 α was obtained from Invitrogen. *E. coli* strains BW25113 and BW25113 Δ *tnaA* were obtained from Tomoya Baba (<http://ecoli.aist-nara.ac.jp>). BW25113 Δ *tnaA*

contains a Km^r gene in place of *tnaA* and was generated from BW25113 by the method of Datsenko and Wanner (2000). JC8679 (AB1157 *recBC sbcA*; Gillen *et al.*, 1981) was obtained from A.J. Clark. JC8679 Δ *tnaA* was generated by bacteriophage P1 transduction (Sambrook *et al.*, 1989) of JC8679 with phage grown on BW25113 Δ *tnaA*.

Plasmid pKS490 is a pUC8 derivative with *cer* cloned in the multiple cloning site (Summers and Sherratt, 1988). pKS494 is a derivative of pKS490 in which P_{*cer*} has been inactivated by mutation of the invariant T (Patient and Summers, 1993). pCm(ss)-19 is a pACYC-based vector containing two copies of *rcd* (in tandem repeat) under the control of λ P_R, and the *cts857* temperature-sensitive repressor (Rowe and Summers, 1999). pStyABB (obtained from Paolo Landini, University of Milan) constitutively expresses *styAB* from *Pseudomonas* strain S12 (O'Connor *et al.*, 1997). pCA24N-*tnaA* expresses His-tagged TnaA under the control of P_{*lac*} and was obtained from M. Kitagawa (<http://ecoli.aist-nara.ac.jp>). pRcd-plus is a pUK21-derivative from which Rcd fused to the StreptoTag sequence (Bachler *et al.*, 1999) is transcribed from P_{T7}. pG10-cm is a pUC9 derivative containing the Rcd-coding sequence under the control of P_{*lac*}. p38 is a pUC18-derivative containing the Rcd-coding sequence under the control of P_{T7}. pBAD24-ABI contains a mutant Rcd4-coding sequence under the control of P_{T7}.

Media and bacterial transformation

For the routine growth of bacteria, L-broth (Kennedy, 1971) and Oxoid iso-sensitest agar were used. Where appropriate, the following supplements were added to media: carbenicillin (100 μ g ml⁻¹); chloramphenicol (30 μ g ml⁻¹); IPTG (1 mM); glucose (0.2%). All reagents and antibiotics were obtained from Sigma-Aldrich. Enzymes were obtained from New England Biolabs (unless otherwise stated) and used according to the manufacturer's instructions. Plasmid transformation of CaCl₂-treated cells was by the method of Cohen *et al.* (1972).

Synthesis of RNA by *in vitro* transcription

To prepare templates for transcription, 5 μ g of plasmid DNA was linearized using a restriction endonuclease that cleaves downstream of the sequence to be transcribed. The linearized DNA was purified using a QIAQuick reaction clean-up kit (Qiagen), and eluted in 40 μ l of RNase-free water. *In vitro* synthesis of RNA was carried out using a T7 Large Scale Transcription Kit (Novagen) according to the manufacturer's instructions, with one modification. The transcription reaction mixture was incubated at 37°C for 12 h, after which 5 U of RNase-free DNase I was added, and the mixture incubated for a further 20 min at 37°C. RNA was extracted as described by Sambrook *et al.* (1989) and dissolved in RNase-free water.

Rcd-RNA affinity chromatography

Cell lysates were prepared as follows: 1 l of 2YT medium [16 g Tryptone (Oxoid), 10 g Yeast Extract (Oxoid), 5 g NaCl, pH 7.4] was inoculated with *E. coli* DS953. The culture was

incubated at 37°C with shaking until the OD₆₀₀ reached 0.9. The culture was centrifuged at 4°C and the pellet weighed and washed in 30 ml water. The supernatant was removed, and the pellet resuspended in 10 ml of a solution of lysozyme (1 mg ml⁻¹; Sigma-Aldrich) in Tris-HCl (pH 8). Protease Inhibitor Cocktail (Sigma-Aldrich) was prepared according to the manufacturer's instructions and added to the solution at a final volume of 5 ml per 20 g cells. RNaseOUT (Invitrogen) was added to the mixture at a final concentration of 1000 U per 20 g cells. The mixture was incubated on ice for 30 min, and then at 4°C for 10 min on a rocking platform. Triton X-100 (Sigma-Aldrich) was added to a final concentration of 0.1%, and the mixture left to incubate at 4°C for 10 min with rocking. The lysates were centrifuged for 1 h at 4°C and the supernatants removed and passed through a 0.45 µm Minisart filter (Sartorius).

The affinity column was prepared as follows: 6 ml of CNBr-activated Sepharose 4B (Sigma-Aldrich) was prepared according to the manufacturer's instructions. Three millilitres of a 0.4 M solution of Mes-KOH (pH 6) was added to the Sepharose suspension on ice, followed by 1600 µg of Rcd RNA (synthesized by *in vitro* transcription using pRcd-plus, linearized with XbaI, as a template). The mixture was incubated overnight at 4°C on a rocking platform. The coupled Sepharose-RNA beads were left to settle on ice, and the supernatant removed. The beads were washed three times with 10 ml RNase-free ice-cold water. Any unreacted groups on the CNBr-activated Sepharose were blocked by the addition of 10 ml of 0.1 M Tris-HCl (pH 7.8). The mixture was incubated for 2 h at 4°C on a rocking platform. The beads were left to settle on ice, the supernatant removed and the slurry poured into an FPLC XK 16/20 column (GE Healthcare), which was equilibrated using wash buffer (20 mM HEPES-Na, 2 mM DTT, pH 7.4). The appropriate cell lysate was applied to the column, unbound proteins washed through using wash buffer, and bound proteins eluted using elution buffer (20 mM HEPES-Na, 2 mM DTT, 200 mM NaCl, pH 7.4). Eluted fractions were collected using an ÄKTA™ FPLC high performance liquid chromatography system (GE Healthcare), under the control of the UNICORN™ software package. All buffers used with the system were first vacuum filtered through a 0.22 µm Stericup (Millipore). Eluted fractions were analysed using SDS-PAGE (Sambrook *et al.*, 1989).

Protein identification by protein fingerprinting

Protein identification by MALDI spectroscopy fingerprinting was conducted by the Protein and Nucleic Acid Chemistry (PNAC) facility at the University of Cambridge. Proteins of interest were resolved using SDS-PAGE, submitted to the PNAC facility, where they underwent extraction, digestion, mass spectrometry and analysis using a database of all known *E. coli* protein sequences.

Plasmid stability assays

A single colony of the test strain was picked from a selective plate, inoculated into 2 ml L-broth (containing ampicillin to select against plasmid-free cells) and incubated overnight at

37°C. This culture was diluted 10⁻⁶-fold into antibiotic-free L-broth and grown to stationary phase. The cycle of dilution and growth was performed five times and each cycle constituted approximately 20 generations. From each stationary phase culture, samples were diluted and spread onto antibiotic-free iso-sensitest agar and the resulting colonies were tested for plasmid content using a starch/iodine assay for ampicillin resistance (Boyko and Ganschow, 1982).

Tryptophanase protein purification

Escherichia coli DH5α containing the His-tagged tryptophanase expression vector, pCA24N-*tnaA*, was used to inoculate 1 l of 2YT medium containing chloramphenicol. Cell lysates were prepared as described for Rcd-RNA affinity chromatography, except that expression of TnaA from pCA24N-*tnaA* was induced by the addition of IPTG to the medium.

A HiTrap™ Chelating HP column (GE Healthcare) was used for the purification of His-tagged tryptophanase. The column was charged with Ni²⁺ according to the manufacturer's instructions. The cell lysate was applied to the column, which was washed with wash buffer (20 mM NaPO₄, 500 mM NaCl, pH 7.4) to remove excess protein. Proteins were eluted with elution buffer (20 mM NaPO₄, 500 mM NaCl, 250 mM imidazole, pH 7.4).

In vitro assay of tryptophanase activity

One millilitre of the following solution was added to each of a series of cuvettes: 0.1 M potassium phosphate buffer (pH 7.8), 0.1 mM pyridoxal 5' phosphate, 0.2 mM NADH, 8 U lactate dehydrogenase (Sigma-Aldrich), 16 µg His-tagged tryptophanase. The reaction mixture was warmed to 37°C and L-tryptophan was added to varying concentrations (0.05 mM, 0.1 mM, 0.5 mM, 1 mM and 5 mM). The mixture was shaken, and the OD₃₄₀ measured continuously at 37°C for 2 min to detect the decrease in absorbance of NADH (and hence the activity of tryptophanase). Where appropriate, 10 µg of either wild-type or mutant Rcd RNA was added. Wild-type and mutant Rcd were synthesized by *in vitro* transcription from p38 and pBAD24-ABI templates respectively, each linearized with MluI. From the data, a Hanes-Woolf plot was used to calculate the K_M and V_{max} of tryptophanase.

Styrene monooxygenase indole assay

This method is based on that of Lacour and Landini (2004). *E. coli* BW25113 containing pStyABB and pG10-cm was used to inoculate 50 ml L-broth, which was grown at 37°C with shaking. Expression of Rcd from pG10-cm was induced by the addition of IPTG (1 mM) or repressed by the addition of glucose (0.2%) to the medium. When cultures reached the desired density, the cells were harvested by centrifugation. The pellet was lysed by repetitive pipetting with 1 ml DMF (to dissolve the indigo) in a 2 ml microfuge tube. The mixture was incubated at room temperature for 10 min with shaking. The lysate was centrifuged for 10 min and the supernatant transferred to a glass cuvette. OD₆₁₀ measurements were used to determine the relative amounts of indole between samples.

Kovac's test for indole

To measure the intracellular indole concentration, cells were harvested by centrifugation (Eppendorf Minispin, 12 500 rpm, 10 min). They were lysed by resuspending the pellet in 1 ml of a solution of lysozyme (1 mg ml⁻¹) in Tris-HCl (pH 8). The lysate was centrifuged for 15 min (Eppendorf Minispin, 12 500 rpm), and the supernatant transferred to a glass test tube. The supernatant was mixed immediately with 0.3 ml of Kovac's reagent (10 g *p*-dimethylamino-benzaldehyde; 50 ml HCl; 150 ml amyl alcohol) for 2 min. The reaction mixture forms two phases. 0.1 ml of the upper phase was added to 2 ml of an HCl-amyl alcohol mixture (75 ml HCl and 225 ml amyl alcohol). The absorbance of the solution was measured spectrophotometrically (540 nm) and the corresponding indole concentration calculated using a standard curve. Intracellular indole concentrations were calculated assuming the volume of an *E. coli* cell to be 4.5 µm³ (Black, 1996).

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