Bacterial partitioning proteins affect the subcellular location of broad-host-range plasmid RK2

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Received 22 March 2008
Revised 28 May 2008
Accepted 5 June 2008

It has been demonstrated that plasmids are not randomly distributed but are located symmetrically in mid-cell, or $\frac{1}{4}$, $\frac{3}{4}$ positions in bacterial cells. In this work we compared the localization of broad-host-range plasmid RK2 mini-replicons, which lack an active partitioning system, in *Escherichia coli* and *Pseudomonas putida* cells. In *E. coli* the location of the plasmid mini-replicon cluster was at the cell poles. In contrast, in *Pseudomonas* cells, as a result of the interaction of chromosomally encoded ParB protein with RK2 centromere-like sequences, these mini-derivatives were localized in the proximity of mid-cell, or $\frac{1}{4}$, $\frac{3}{4}$ positions. The expression of the *Pseudomonas parAB* genes in *E. coli* resulted in a positional change in the RK2 mini-derivative to the mid-cell or $\frac{1}{4}$, $\frac{3}{4}$ positions. Moreover, in a *P. putida parAB* mutant, both RK2 mini-derivatives and the entire RK2 plasmid exhibited disturbances of subcellular localization. These observations raise the possibility that in certain bacteria chromosomally encoded partitioning machinery could affect subcellular plasmid positioning.

INTRODUCTION

Spatial organization, localization and appropriate distribution of genetic material are essential for all cellular organisms. Major advances in the understanding of DNA dynamics in bacteria have been achieved by using fluorescence microscopy to visualize the intracellular location of plasmids and bacterial chromosomes. These studies have shown that plasmids are not randomly distributed but are located at distinct sites within bacterial cells. The low-copy-number *Escherichia coli* plasmids P1 (Gordon *et al.*, 1997; Li & Austin, 2002) and F (Gordon *et al.*, 1997, 2004; Niki & Hiraga, 1997) as well as *Salmonella enterica* plasmid R27 (Lawley & Taylor, 2003) are found at the mid-cell of newborn cells or symmetrically at the $\frac{1}{4}$ and $\frac{3}{4}$ positions in older, pre-divisional cells. For some plasmids, it was proposed that plasmid particles could form clusters. Plasmid R1, with a copy number of four to five per cell, also forms symmetrically located clusters, although in older cells, R1 clusters are shifted towards the cell poles (Jensen & Gerdes, 1999; Weitao *et al.*, 2000). The mid-cell, $\frac{1}{4}$ and $\frac{3}{4}$ cellular positioning of plasmid clusters has been observed for the broad-host-range plasmid RK2 (60 kb in size) in *E. coli*, *Pseudomonas aeruginosa* and *Vibrio cholerae* (Pogliano *et al.*, 2001; Ho *et al.*, 2002). This plasmid belongs to the IncP-1 incompatibility group, is present at five to eight copies per chromosome, and is stably maintained in almost all

Gram-negative bacteria (Rosche *et al.*, 2000; Thomas *et al.*, 1980, 1984; Wilson *et al.*, 1997). When plasmids RK2, F and P1 were localized simultaneously, the majority of plasmid foci were close to but separated from each other, demonstrating that compatible bacterial plasmids are in independent cellular locations in *E. coli* (Ho *et al.*, 2002). The subcellular localization of these plasmids is thought to be mediated by the partitioning (Par) systems they encode. Plasmid partitioning loci function as a cassette containing genes for two *trans*-acting proteins and a *cis*-acting centromere-like site. The first gene of a *par* operon encodes a ParA-like protein, which is a Walker box or actin-like ATPase (Ebersbach & Gerdes, 2005; Funnell, 2005). The product of the second gene, a ParB-like protein, binds as a dimer to specific sequences within the *cis* site (Schumacher & Funnell, 2005). The formation of a partitioning complex and oscillations of the filament-forming ParA ATPase were proposed to result in localization of the plasmid to the mid-cell or the $\frac{1}{4}$ and $\frac{3}{4}$ positions, which represent the middle of the next cell generation (Ebersbach & Gerdes, 2004; Ebersbach *et al.*, 2006). For plasmid RK2, the partitioning cassette consists of the genes encoding IncC, a Walker box ATPase, and KorB, a protein that interacts with a palindromic operator (O_B) found 12 times in RK2 DNA (Kostelidou & Thomas, 2000; Rosche *et al.*, 2000; Williams *et al.*, 1998). The rules governing plasmid positioning in bacterial cells are not fully understood. It is not known, for example, what bacterial factors affect plasmid cluster formation, localization and partitioning. In this work we demonstrate that

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization.

chromosomal partitioning genes affect the subcellular location of a plasmid mini-replicon.

METHODS

Plasmids and bacterial strains. The plasmids and bacterial strains used in this work are listed in Table 1. Plasmids were introduced into *E. coli* and *Pseudomonas* cells by transformation (Sambrook *et al.*, 1989).

Media and growth conditions. Bacteria were grown at 30 °C in LB or M63/glucose media. Antibiotics were added to the following final concentrations: ampicillin 50 µg ml⁻¹ for liquid media or 100 µg ml⁻¹ for agar plates, chloramphenicol up to 150 µg ml⁻¹, kanamycin 30 µg ml⁻¹ for liquid media or 60 µg ml⁻¹ for agar plates, gentamicin up to 20 µg ml⁻¹, tetracycline up to 50 µg ml⁻¹.

Fluorescence *in situ* hybridization (FISH). Mini-RK2 specific Cy3-labelled probes for FISH were prepared essentially as described by Pogliano *et al.* (2001). The procedure was modified as follows. After incubation with the blocking solution, the cells were treated with lysozyme (1 mg ml⁻¹ in PBS) for 10 min at room temperature. The next step was the same as described by Jensen & Shapiro (1999). Chromosomal DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 0.5 µg ml⁻¹.

Fluorescence microscopy. *E. coli* S17-1 and *Pseudomonas* strains were prepared for microscopy as described previously (Ho *et al.*, 2002; Pogliano *et al.*, 2001). The expression of *tetR-eyfp* from pKO10 and pKO15 was induced with 0.4% arabinose for 1 h. The expression of *parA*, *parB* and *tetR-eyfp* from pLAK15 in *E. coli* cells was induced with 0.4% arabinose for 1.5 h. The expression of *tetR-eyfp* in *E. coli* MGTRY was induced as described by Verheust & Helinski (2007). The cells were stained with FM4-64 (0.5 µg ml⁻¹), immobilized on a poly-L-lysine-treated slide coverslip and observed using an Olympus BX51 fluorescence microscope with F-View-II CCD camera. Measurements and image analysis were conducted with AnalySIS software.

Gel filtration assay. Column gel filtration was used to fractionate the DNA-ParB complexes as described previously (Konieczny & Helinski, 1997). The reaction mixture (100 µl), containing *P. putida* ParB (4 µg) and the indicated supercoiled plasmid DNA (2 µg) in 40 mM HEPES/KOH, pH 8.0, 40 mM potassium glutamate, 10 mM magnesium acetate, 4% sucrose, 4 mM dithiothreitol, and 2 mM ATP, was incubated for 20 min at 32 °C and then run through a Sepharose CL-4B (0.5 × 12 cm) column, equilibrated at room temperature with the incubation buffer and 0.01% Brij 58. Fractions (80 µl) were collected and a portion of each (35 µl) was analysed by SDS-PAGE followed by silver staining, while another portion (35 µl) was analysed on agarose gel stained with ethidium bromide.

Formaldehyde cross-linking and chromatin immunoprecipitation. Cells were grown at 30 °C in LB medium. Samples were taken during exponential growth (OD₆₀₀ 0.6) and prepared as described by Lin & Grossman (1998). Protein-DNA complexes were immunoprecipitated with polyclonal anti-ParB antibodies (C. M. Thomas, University of Birmingham, UK), followed by incubation with 30 µl of a 50% Protein A-Sepharose slurry (Amersham Pharmacia). PCR was performed with Taq DNA polymerase (Fermentas) and oligonucleotide primers (O_B³, 5'-TCGCCGTTGCGAACCACCTTC-GG-3', 5'-AAATCGGGAGTGCGAAAAGCATCACC-3'; O_B¹⁰, 5'-GATTATGGCTCATATCGAAAAGTCTC-3', 5'-GAGCACACGAAGGATGTTGGTG-3'; *oriV*, 5'-AAGCCGTGTGCGAGACACCGC-3', 5'-AAAGACAGGTTAGCGGTGGC-3'; IR-2, 5'-CCGCTTCAGCTCA-

TCAACCCAGAC-3', 5'-CTTGCGAAGCTGGGTCAAGTGACC-3'). PCR products were separated on agarose gels and stained with ethidium bromide.

Purification of ParB. The *P. putida* ParB protein was expressed from plasmid pETK1 (Table 1). The bacterial strain, the conditions of *parB* expression and the protein purification were the same as described for the P1 plasmid ParB homologue (Davis & Austin, 1988) with the exception that only a phosphocellulose column was used. Peak fractions were pooled and dialysed against P buffer (150 mM NaCl). The final product yielded 8 µg µl⁻¹ of >95% homogeneous protein.

Plasmid stabilization assays. The cells carrying a mini-RK2 (pCVI) or RK2 (pZZ15) plasmid were diluted from overnight cultures into LB medium containing appropriate antibiotics (see above, Media and growth conditions) and incubated to OD₆₀₀ 0.5. The cells were then diluted into medium without antibiotics to allow free growth of RK2 and mini-RK2 segregants and maintained in exponential growth for at least 120 generations by sequential dilutions. The samples removed during this period were plated on agar plates and, from each plating, ≥400 colonies were patched onto agar with antibiotics to score retention or loss of plasmids. The number of generations was estimated from optical density measurements.

RESULTS AND DISCUSSION

The subcellular position of RK2 mini-replicons depends on the host bacterium

To test for the possibility that a host cell can affect plasmid positioning, a dual plasmid system was used allowing identification of the location of clusters of the mini-derivative pCVI of broad-host-range plasmid RK2 in the cells of different host bacteria. pCVI, which lacks partitioning genes but contains two centromere-like sites (O_B³ and O_B¹⁰), the gene for plasmid replication initiator protein (*trfA*), the origin for vegetative DNA replication (*oriV*), the origin for conjugational transfer (*oriT*), antibiotic resistance determinants and a *tetO* array, was tagged by the TetR-EYFP fusion protein expressed from pKO10, an RK2-compatible dual-origin broad-host-range plasmid. The subcellular location of pCVI was examined after arabinose induction of the *tetR-eyfp* gene and staining of the cell membrane with the fluorescent dye FM4-64. When both plasmids were introduced into *E. coli* cells, arabinose induction resulted in fluorescent signals mainly localized at one of the cell poles (Fig. 1A i, ii, iii; D). Fluorescent foci were observed in only approximately 50% of the cells, the majority of which had only one focus. A small fraction of *E. coli* cells had two foci (Fig. 1A iv, E). A similar subcellular distribution of pCVI was obtained when the TetR-EYFP protein was expressed from the *tetR-eyfp* gene on the chromosome of *E. coli* MGTRY (data not shown). Polar positioning of pCVI was confirmed by FISH in *E. coli* cells containing only this plasmid (Fig. 1B). Plasmid fluorescent signals were observed on the edge of DAPI-stained *E. coli* nucleoids, suggesting that the plasmid was not located in the mid-cell or 1/4, 3/4 positions but at the cell poles. Similarly as we observed for pCVI, it was previously reported that F and R1 mini-derivatives lacking

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Resistance	Reference or source
Strains			
<i>E. coli</i> S17-1	<i>pro hsdR recA</i> with integrated TraI region of plasmid RP4 (Tc::Mu-Spc::Tn7)	Spc ^R	MacKenzie <i>et al.</i> (1995)
<i>E. coli</i> MGTRY	MG1655 (F λ^- <i>ilvG rfb-50 rph-1</i>) derivative containing gene for fusion protein TetR-EYFP under the control of P _{BAD} promoter	Spc ^R	Verheust & Helinski (2007)
<i>P. aeruginosa</i> PAO1161	[PAO001] <i>pyrB49 chl-2</i>	–	Isaac & Holloway (1968)
<i>P. putida</i> KT2440	pWW0 [–] Res [–] derivative of <i>P. putida</i> mt-2	–	Franklin <i>et al.</i> (1981)
<i>P. putida</i> KT2440 Δ <i>parAB</i>	<i>P. putida</i> KT2440 derivative, Δ <i>parAB</i> kan	Kan ^R	Godfrin-Estevenson <i>et al.</i> (2002)
Plasmids			
RK2	Broad-host-range IncP plasmid	Amp ^R , Kan ^R , Tet ^R	Meyer <i>et al.</i> (1975)
pZZ15	RK2 derivative containing <i>lacO</i> array and gene for fusion protein LacI-GFP	Amp ^R , Tet ^R	Ho <i>et al.</i> (2002)
pCVI	Mini-RK2 (pFF1 derivative) containing <i>tetO</i> array, <i>tfrA</i> gene, <i>oriV</i> and <i>oriT</i>	Cm ^R , Gent ^R	Verheust & Helinski (2007)
pLAU53	pUC18 derivative containing genes for fusion proteins LacI-ECFP and TetR-EYFP	Amp ^R	Lau <i>et al.</i> (2003)
pLAK53	pUC18 derivative containing gene for fusion protein TetR-EYFP, constructed by <i>NcoI</i> deletion of plasmid pLAU53	Amp ^R	This work
pLAK15	pLAK53 derivative containing gene for fusion protein TetR-EYFP and genes for ParA and ParB partitioning proteins from <i>P. putida</i> under the control of P _{BAD} promoter	Amp ^R	This work
pLAK16	pLAK15 derivative containing ' <i>parAparB</i> ' fragment from <i>P. putida</i> and deletion of gene for fusion protein TetR-EYFP	Amp ^R	This work
pBBR1MCS-2	Broad-host-range cloning vector	Kan ^R	Kovach <i>et al.</i> (1995)
pKO10	Dual-origin (pUC-pBBR1MCS-2) broad-host-range cloning vector; entire pLAK53 inserted into <i>HindIII</i> site of vector pBBR1MCS-2	Kan ^R , Amp ^R	This work
pKO15	Dual-origin (pUC-pBBR1MCS-2) broad-host-range cloning vector; ' <i>gidBparB</i> ' <i>parA</i> ' fragment (2792 bp) from <i>P. putida</i> (Godfrin-Estevenson <i>et al.</i> , 2002) inserted into <i>HindIII</i> and <i>BamHI</i> sites of pKO10	Kan ^R , Amp ^R	This work
pKO16	Dual-origin (pUC-pBBR1MCS-2) broad-host-range cloning vector, constructed by <i>ClaI</i> deletion of pKO15	Kan ^R	This work
pUC18-IR2	pUC18 derivative; insertion of fragment with IR-2 sequence from <i>P. putida</i> chromosome and <i>Mph1103</i> site (5'-ctagAGGTTCCACGTGGAACAATGCATC; 5'-ccggGATGCATTGTTCACGTGGAACCT) into <i>XbaI</i> and <i>Eco88I</i> sites of vector pUC18	Amp ^R	This work
pUC18-O _B ³	pUC18 derivative; insertion of fragment with O _B ³ sequence from RK2 plasmid and <i>Mph1103</i> site (5'-ctagACCCCTTTTAGCCCGCTAAACTCTATGCAT; 5'-ccggGATGCATAGAGTTTTAGCGGGCTAAAA-AGGGT) into <i>XbaI</i> and <i>Eco88I</i> sites of vector pUC18	Amp ^R	This work
pUC18-O _B ¹⁰	pUC18 derivative; insertion of fragment with O _B ¹⁰ sequence from RK2 plasmid and <i>Mph1103</i> site (5'-ctagAAGAACTTTAGCGGGCTAAATTTATGCATC; 5'-ccggGATGCATAAATTTAGCCCGCTAAAGTTCTT) into <i>XbaI</i> and <i>Eco88I</i> sites of vector pUC18	Amp ^R	This work
pETK1	pET22b (Novagen) derivative with deletion of His-tag sequence and insertion gene for ParB protein from <i>P. putida</i> into <i>NdeI</i> and <i>BamHI</i> sites	Amp ^R	This work

Fig. 1. Subcellular localization of the RK2 mini-derivative pCVI in *E. coli* S17-1 and *P. putida* KT2440, grown in LB medium at 30 °C. The subcellular position of pCVI was determined by tagging with TetR-EYFP (A, F) or FISH (B, G). The gene for TetR-EYFP was expressed from pKO10. Cell membranes were stained with FM4-64 (red) (A, Ci, ii, F, Hi, ii). Chromosomal DNA was stained with DAPI (blue) (B, G). Representative *E. coli* S17-1 and *P. putida* KT2440 cells with one or two foci of pCVI are shown in panels A, B, F and G. Panels D, E, I and J demonstrate the subcellular distribution of pCVI in *E. coli* (D, E) and *P. putida* (I, J) for cells containing one focus (D, I) or two foci (E, J). Each point represents the relative position of a single focus measured as the distance to the pole in relation to the cell length. The pole was chosen randomly. Cell length is plotted against position of foci given as a fraction of cell length. Negative controls for tagging with TetR-EYFP and FISH are shown in panels C and H: representative *E. coli* S17-1 and *P. putida* KT2440 cells containing just pCVI (C i, H i), pKO10 (C ii, H ii) or no plasmid DNA (C iii, H iii).

the partitioning segments are distributed at the cell poles (Ebersbach & Gerdes, 2004; Niki & Hiraga, 1997). Polar localization of RK2 mini-replicons in *E. coli* cells has also been reported recently by Verheust & Helinski (2007), who showed that insertion of the RK2 *par* locus into pCVI restores the mid-cell, or $\frac{1}{4}$, $\frac{3}{4}$ positions of plasmid foci.

When we examined the localization of pCVI in *P. putida*, the results differed substantially from those obtained in *E. coli*. Instead of a polar location, we observed that the plasmid formed foci at approximately mid-cell and the $\frac{1}{4}$, $\frac{3}{4}$ positions (Fig. 1Fi, J). Also FISH and DAPI co-staining demonstrated plasmid foci inside DAPI-stained *P. putida* nucleoids (Fig. 1G). Overall, the location of pCVI foci in *Pseudomonas* cells was similar to the location of intact RK2 (data not shown), although with a broader distribution in the case of the mini-derivative clusters. Also, as observed in *E. coli*, foci of the mini-derivative were detected in only a

Fig. 2. ParB protein from *P. putida* forms a complex with plasmid RK2 and its mini-derivative *in vitro*. The reaction mixture contained the indicated supercoiled DNA (RK2, pCVI, pUC18, pUC18-OB³(RK2), pUC18-OB¹⁰(RK2), pUC18-IR2(*P. putida*)) and ParB protein in the amounts and with the incubation conditions described in Methods. After incubation, the reaction mixtures were loaded on a Sepharose CL-4B column. Fractions were collected and analysed by SDS-PAGE followed by silver staining. Agarose gels were stained with ethidium bromide.

Fig. 3. ParB interacts in live *P. putida* KT2440 cells with centromere-like sites of plasmid RK2 and its mini-derivative. The PCR-amplified products of immunoprecipitation (IP) with anti-ParB antibodies and lysates of *P. putida* KT2440 (A lane 2; B lane 2, C lane 1), *P. putida* KT2440(pCVI) (A lane 1, B lane 1) or *P. putida* KT2440(RK2) (C lane 2). Lanes 3, control reactions with isolated DNA (A, B) and negative control of immunoprecipitation with anti-ParB antibodies and *E. coli* S17-1(RK2) lysates (C). The PCR-amplified products were separated on 1% agarose gels and stained with ethidium bromide. Primers used in reactions were designed for RK2 plasmid sequences containing O_B^3 , O_B^{10} and *oriV* regions and the IR-2 sequence of *P. putida* KT2440 chromosomal DNA as indicated. All primer sequences are listed in Methods.

fraction of the *Pseudomonas* cells (60%). Our experiments were carried out in both LB and M63/glucose media and no substantial difference was observed regarding plasmid positioning and plasmid foci number. We have also analysed RK2 mini-derivative localization in *P. aeruginosa* (data not shown). Using either TetR-EYFP tagging or FISH experiments we demonstrated that pCVI localization in *P. aeruginosa* is similar to that in *P. putida*.

In control experiments, the expression of *tetR-eyfp* in *E. coli* and *P. putida* cells without pCVI resulted in yellow fluorescence of the whole cells (Fig. 1Cii, H ii). When *tetR-eyfp* was not expressed in the cells, no signal was detected (Fig. 1Ci, H i). Also FISH and DAPI co-staining demonstrated no signal when bacterial cells did not contain pCVI (Fig. 1Ciii, H iii).

***P. putida* ParB protein interacts with RK2 DNA by binding centromere-like sites**

The fluorescence microscopy experiments indicated that the subcellular localization of an RK2 mini-derivative that lacks partitioning genes depends on the host bacterium.

This raised the possibility that the subcellular position of an RK2 mini-derivative could be affected by host factors, including the host-encoded partitioning machinery. Some similarities between the RK2 and *Pseudomonas* partitioning systems have been described (Hayes, 2000). In addition, RK2 and *Pseudomonas*, but not *E. coli*, have a type I *par* locus (Mohl & Gober, 1997; Ogasawara & Yoshikawa, 1992). The ParB protein from *P. putida* shows high similarity to the RK2 KorB protein (Lin & Grossman, 1998): based on amino acid sequence, it is 27% identical with KorB. It has also been demonstrated that KorB of RK2 specifically interacts with the *P. putida* chromosomal *cis* site (Chiu & Thomas, 2004). We asked if the *P. putida* ParB protein could interact with RK2 DNA. Size-exclusion chromatography was used to investigate this possibility. Purified ParB protein was incubated with or without plasmid DNA and then fractionated using a Sepharose CL-4B column. The results demonstrated that *P. putida* ParB formed nucleoprotein complexes with RK2 DNA (Fig. 2). When the entire RK2 plasmid or its mini-derivative pCVI were present in the incubation mixture, the ParB protein was detected in the column void volume, indicating the

formation of a nucleoprotein complex. In the absence of DNA or in the presence of pUC18 DNA, no ParB protein was found in void volume fractions (Fig. 2). This result indicated interaction of ParB with RK2 plasmid DNA. The pCVI plasmid used in our study did not contain *incC* or the *korB* genes; however, two RK2 centromere-like sites, O_B^{10} and O_B^3 , were still present adjacent to the *trfA* and *oriT* sequences. To investigate whether those sites are capable of interacting with *P. putida* ParB protein, DNA fragments containing O_B^{10} , O_B^3 or *P. putida* centromere site IR2 were introduced into pUC18 plasmid DNA. When incubations were carried out with these plasmid constructs, ParB was detected in the void fractions (Fig. 2). Since no ParB complex formation was observed with pUC18, we concluded that the observed nucleoprotein complexes are the result of ParB protein interaction with RK2 centromere-like sites. The RK2 O_B motifs differ from the *Pseudomonas* IR2 sequence; therefore we could not exclude the possibility that ParB possesses two domains for interaction with DNA.

To test the possibility that this interaction also occurs in *P. putida* cells, a chromatin immunoprecipitation with polyclonal anti-ParB antibodies followed by a PCR reaction with O_B^3 -, O_B^{10} -, *oriV*- or IR2-specific oligonucleotide primers was performed. The interaction of the ParB protein with RK2 O_B^3 and *P. putida* chromosomal IR2 was clearly detected when RK2 mini-derivative pCVI (Fig. 3A, B, lane 1 and Fig. 4 lane 1) was present in *P. putida* cells. Less intensive bands of O_B^{10} -specific PCR product detected in immunoprecipitation experiments suggest that ParB interaction with this site on RK2 mini-derivatives could be less efficient (Fig. 3A, lane 1 and Fig. 4 lane 1). When *oriV*-specific primers were used, we did not detect any signal, indicating that a ParB complex is not formed with the RK2 origin (Fig. 3A, lane 1 and Fig. 4 lane 1). When the RK2 mini-derivative was not present in *P. putida* cells, we were able to detect ParB interaction only with the chromosomal IR2 sequence (Fig. 3B, lane 2). Similar results indicating *P. putida* ParB interaction with both O_B^3 and O_B^{10} sites were obtained when the entire RK2 plasmid was present in *P. putida* cells (Fig. 3C lane 2). When RK2 plasmid was present in *E. coli* cells, no signal was detected, indicating that anti-ParB antibodies did not interact with RK2 KorB protein (Fig. 3C lane 3). These data showed that in *P. putida* cells chromosomally encoded ParB protein interacts with centromere-like O_B sites of plasmid RK2 DNA.

P. putida ParAB affects the position of RK2 replicons

To further explore the possibility that the *Pseudomonas* partitioning machinery could affect the subcellular position of RK2 mini-derivatives, we investigated whether the expression of *P. putida* *parAB* could change the asymmetrical polar distribution of the RK2 mini-derivative in *E. coli* cells. Plasmid pLAK15 containing both the gene for TetR-EYFP and *P. putida* *parAB* under the control of the P_{BAD}

Fig. 4. *P. putida* ParB protein interacts in live *E. coli* S17-1 cells with centromere-like sites of the RK2 mini-derivative. PCR-amplified products of immunoprecipitation (IP) with anti-ParB antibodies and lysates of *E. coli* S17-1(pCVI, pKO10) (lane 3), *E. coli* S17-1(pCVI, pKO15) (lane 4) or *E. coli* S17-1(pCVI, pLAK15) (lane 5). pKO15 and pLAK15 are vectors for expressing *P. putida* *parAB*. pKO10 is a pLAK15 derivative which does not contain *P. putida* *parAB* (see Table 1). Lanes 1, 2 and 6, control of immunoprecipitation with anti-ParB antibodies and lysates of *P. putida* KT2440(pCVI) (lane 1) and *P. putida* KT2440 Δ *parABΩkan* (pCVI) (lane 2), and control reactions with isolated DNA (lane 6) on 1 % agarose gels stained with ethidium bromide. Primers used in reactions were designed for RK2 plasmid sequences containing O_B^3 , O_B^{10} and *oriV* regions. All primer sequences are listed in Methods.

promoter was constructed and introduced into *E. coli* cells containing the RK2 mini-derivative pCVI. In control experiments, in *E. coli* cells, instead of pLAK15 we used pKO10, which expresses only *tetR-eyfp*. After arabinose induction, when only the *tetR-eyfp* gene was expressed, the pCVI localization was polar and asymmetric (Fig. 5A–C). Approximately 40 % of the cells contained fluorescent plasmid foci and multiple signals were detected in only 3 % of the cells (Fig. 5M). In contrast, when TetR-EYFP and both ParA and ParB *P. putida* proteins were expressed, the distribution of pCVI in *E. coli* cells was more or less symmetrical, with plasmid foci located in the proximity of the mid-cell or the $\frac{1}{4}$, $\frac{3}{4}$ positions (Fig. 5D–F). Also, the proportion of the cells containing plasmid foci increased (Fig. 5M). We detected plasmid foci in 60 % of the cells and among them approximately 30 % had more than one fluorescent signal. These data were very similar to those obtained in a control experiment where pCVI number and location were analysed in wild-type *P. putida* cells (Fig. 5G–I, M, N). Further, we asked if *P. putida* ParB protein interacted with pCVI centromere-like sites when both ParA and ParB

Fig. 5. ParA and ParB proteins from *P. putida* alter the location of RK2 plasmid. The subcellular position of pCUI (A–L, N, P) was determined by tagging with TetR-EYFP in *E. coli* S17-1 (A–F), *P. putida* KT2440 (G–I, N) and *P. putida* KT2440 Δ parAB Ω kan (J–L, P). The subcellular position of pZZ15 was determined by tagging with LacI-GFP in *P. putida* KT2440 (O) and *P. putida* KT2440 Δ parAB Ω kan (Q). Bacteria were grown in LB medium at 30 °C. Genes for TetR-EYFP, ParA and ParB were expressed from pLAK15, whereas only *tetR-eyfp* was expressed from pKO10 and *lacI-gfp* from pZZ15. Cell membranes were stained with FM4-64 (red). Representative cells with one (i, ii) and two (iii) foci of pCUI are shown in panels C, F, I and L. Panels A, D, G and J demonstrate the subcellular distribution of pCUI for cells containing one focus. In order to facilitate comparison, histograms have been used in which the distance of the plasmid focus to the nearest cell pole is shown as a fraction of cell length. Panels B, E, H and K demonstrate subcellular distribution of mini-RK2 for cells with two foci. Cell length (μ m) is plotted against position of foci given as a fraction of cell length. (M) Table showing the percentage of cells containing no, one or two and more fluorescent foci when ParA and ParB proteins are or are not present in the cells.

were expressed from pLAK15 or pKO15 in *E. coli* cells. Chromatin immunoprecipitation demonstrated that indeed under those experimental conditions promoting the mid-cell or the $\frac{1}{4}$, $\frac{3}{4}$ positions of pCVI in *E. coli* cells (Fig. 5D–F), both O_B^3 and O_B^{10} sites were bound by *P. putida* ParB (Fig. 4, lanes 4 and 5). In a control reaction, no PCR products were observed when *E. coli* cells contained plasmid pKO10, without the *P. putida* *parAB* region (Fig. 4, lane 3).

We also analysed the subcellular location of pCVI in a *P. putida* $\Delta parAB\Omega kan$ mutant. The lack of functional ParA and ParB proteins changed the localization of the pCVI foci from the approximately mid-cell and $\frac{1}{4}$, $\frac{3}{4}$ positions to positions near the cell poles (Fig. 5J–L, P). We did not observe differences in the number of cells containing plasmid foci; however, the number of cells with a single signal was slightly increased compared to the wild-type *P. putida* (Fig. 5M). No O_B^3 - and O_B^{10} -specific PCR products were observed after immunoprecipitation of *P. putida* $\Delta parAB\Omega kan$ (pCVI) extract with anti-ParB antibodies (Fig. 4, lane 2). In the control, when *oriV*-specific primers were used (Fig. 4, lane 2), no signal was detected either.

Interestingly, when the localization of the entire RK2 plasmid (pZZ15) was analysed in the *P. putida* $\Delta parAB\Omega kan$ mutant, dispersed signals were observed compared with the localization of pZZ15 in *P. putida* wild-type cells (Fig. 5O, Q). This indicates that in *P. putida* disturbances in the chromosomally encoded partitioning system affect the subcellular position of RK2 plasmid.

Stability of RK2 and its mini-derivative pCVI in *E. coli* and *P. putida* strains

The data presented above clearly show that the expression of *P. putida* *parAB* genes affects the position of RK2 mini-replicons. In *E. coli*, the expression of *P. putida* *parAB* genes compensates for the lack of the RK2 partitioning system with regard to the cellular location of the RK2 mini-derivative pCVI. In *P. putida*, the lack of chromosomal partitioning genes results in a shift of the position of pCVI towards the cell poles and disturbances of the localization of the entire RK2 plasmid. To test if the observed subcellular localizations of the RK2 plasmids affect their stabilities, we analysed the maintenance of pZZ15 and pCVI in the strains used in the course of this work. pZZ15 was stably maintained in both *E. coli* S17-1 and *P. putida* KT2440 (Fig. 6A, E). Although we observed disturbances in pZZ15 localization in *P. putida* KT2440 $\Delta parAB\Omega kan$ (Fig. 5Q), stability tests revealed that the plasmid is stably maintained in this mutant (Fig. 6A). This result could be explained by the compensating effect of the dispersed plasmid localization. Stabilities of RK2 mini-derivative pCVI in *E. coli* S17-1 and *P. putida* KT2440 were significantly reduced compared to the stabilities of pZZ15 in these strains (compare Fig. 6E with Fig. 6A and B). It must be pointed out that pCVI stability in *P. putida* KT2440, where the plasmid was found at approximately mid-cell and the $\frac{1}{4}$, $\frac{3}{4}$ positions (Fig. 1G), was better

when compared to pCVI stability in *E. coli* S17-1, where it was located at the cell poles (Fig. 1B and compare Fig. 6E and B). The disruption of the *P. putida* *parAB* locus (mutant KT2440 $\Delta parAB\Omega kan$) resulted in only a very limited reduction of pCVI stability (Fig. 6B). A similar effect was observed when pCVI stability was tested with pKO10 also present in the cell. The pCVI stability observed in *P. putida* KT2440 $\Delta parAB\Omega kan$ was slightly reduced in comparison with its stability in *P. putida* KT2440 (Fig. 6C). Similarly, only a limited effect on chromosome stability was previously observed in *P. putida* KT2440 $\Delta parAB\Omega kan$ (Godfrin-Estevenson *et al.*, 2002). Also, during our experiments the pCVI stability loss rate tested in *E. coli* in the presence of pKO15 or pKO10 was very similar, regardless of the expression of *P. putida* *parAB* genes (Fig. 6D). However, at the beginning of the experiment more cells contained pCVI in the strain where *P. putida* *parAB* was expressed (Fig. 6D).

Fig. 6. Stability of pCVI and pZZ15 in *E. coli* S17-1, *P. putida* KT2440 and *P. putida* KT2440 $\Delta parAB\Omega kan$ cells. Stability assays were performed as described in Methods. Stability of pZZ15 in *E. coli* (E) and *P. putida* (A). Stability of pCVI when the mini-derivative was present alone in the cells (B, E) or in the presence of another plasmid, pKO10 or pKO15, as indicated (C, D).

A limited effect of the *parAB* locus on RK2 mini-derivative stability could be a result of ParA and ParB, which may not support plasmid segregation as efficiently as IncC and KorB. It is also possible that the full stabilization of the plasmid mini-derivatives requires other factors such as additional O_B sites, or post-segregation killing (*psk*) and multimer resolution (*msr*) systems that are missing in RK2 mini-derivatives. Accordingly, when the RK2 *par* locus containing *korB* and *incC* genes was introduced into pCVI DNA, it restored the mid- and quarter-cell positions of the plasmid but did not stabilize the plasmid during cell growth (Verheust & Helinski, 2007).

In summary, the experiments reported here demonstrate, we believe for the first time, that the subcellular position of broad-host-range plasmid RK2 is affected by *Pseudomonas* partitioning machinery. The *Pseudomonas* partitioning module plays a role in chromosomal segregation (Godfrin-Estevenon *et al.*, 2002; Lewis *et al.*, 2002); however, it has also been speculated that it is a remnant of RK2-like plasmid integration (Chiu & Thomas, 2004). That may explain cross-talk between chromosomal and plasmid partitioning systems. Our results at least raise the possibility that in certain bacteria the chromosomally encoded partitioning machinery could affect the subcellular positioning of a specific plasmid element.

ACKNOWLEDGEMENTS

We are grateful to Christopher Thomas for providing anti-ParB antibodies, to Donald Helinski and David Sherratt for providing pCVI and pLAU plasmids and to David Lane for supplying the strain *P. putida* KT2440 Δ *parAB* Ω *kan*. We also thank Donald Helinski for critically reading the manuscript. This work was supported by the Polish State Committee for Scientific Research Grant 2P04A02730, The Foundation for Polish Science and the EMBO/HHMI Young Investigator Programme. Katarzyna Kolatka and Marcin Pierechod are recipients of stipends from the Foundation for Polish Science.

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Edited by: L. Jannière