The ParB protein encoded by the RP4 *par* region is a Ca²⁺-dependent nuclease linearizing circular DNA substrates

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The *parCBA* operon, which together with the *parDE* operon constitutes an efficient stabilization system of the broad-host-range plasmid RP4, encodes a 20 kDa polypeptide (ParB), which exhibits sequence homology to nucleases. The ParB protein was overexpressed by means of an inducible *tac*-promoter system. Plate assays with herring sperm DNA as substrate provided evidence for nuclease activity. The ParB nuclease shows specificity for circular DNA substrates and linearizes them regardless of the presence *in cis* of parts of the RP4 partitioning region. The nuclease activity *in vitro* is stimulated by the presence of Ca²⁺ ions. EDTA (5 mM) completely inhibits nuclease activity. By restriction analysis of the ParB-linearized products, cleavage of circular DNA substrates taking place preferentially at specific sites was demonstrated. Run-off sequencing and primer extension analysis of ParB-linearized plasmid DNA revealed a specific target for ParB action adjacent to an AT-rich region containing palindromic sequence elements on a pBR322-derived plasmid.

Keywords: plasmid stability, RP4, endonuclease, circular DNA

INTRODUCTION

The broad-host-range plasmid RP4 (identical to RK2) is stably maintained in almost all Gram-negative bacteria (Thomas & Helinski, 1989). Deletions extending into a region termed the partitioning region (*par*) lead to segregational instability (Saurugger *et al.*, 1986). Cloning of the *par* region into different replicons revealed that it represents an extremely efficient system for stabilization of plasmids in a variety of Gram-negative bacteria (Saurugger *et al.*, 1986; Gerlitz *et al.*, 1990; Roberts *et al.*, 1990). Motallebi-Veshareh *et al.* (1990) described a further region, which includes *incC* and *korB*, showing homology to the partitioning loci of plasmids F and P1.

The partitioning region (*par*) comprises two operons, *parCBA* and *parDE*, which are transcribed from divergent promoters (Gerlitz *et al.*, 1990; Eberl *et al.*, 1992; Davis *et al.*, 1992). The *parDE* operon specifies a growth inhibition function mediating plasmid stabilization by cell killing associated with plasmid loss (Roberts *et al.*, 1994; Jensen *et al.*, 1995; Johnson *et al.*, 1996). The *parCBA* operon appears to encode a complex

stabilization function including a site-specific recombination system. The *parA* gene encodes two forms of a resolvase which mediates resolution of plasmid multimers by site-specific recombination between specific sites (*res*) within the promoter region of the *parCBA* operon (Eberl *et al.*, 1994). The *parC* gene encodes a 10 kDa protein of unknown function.

The *parB* gene codes for a 177 amino acid protein with an apparent molecular mass of 20 kDa. The ParB polypeptide is a highly positively charged protein with a calculated isoelectric point of 11.0. The deduced amino acid sequence of ParB shows significant homology to the extracellular nuclease of *Staphylococcus aureus* (Shortle, 1983), the *nuc* gene of plasmid pSa (Close & Kado, 1992) and to ORFA of the *tra* region of plasmid R100 (Yoshioka *et al.*, 1990).

Enzymes with DNase activity have a variety of biological roles. Their functions may be nutritional (e.g. pancreatic enzymes), protective (e.g. restriction enzymes) or involved in various steps of processing DNA as in the course of processes such as DNA repair, recombination and transport. A nuclease involved in a process ensuring high segregational stability of plasmids has not been hitherto described. Some indications of involvement of the ParB nuclease in the stabilizing process exerted by the RP4 *par* system have been reported. It was demon-

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strated that pBR322 derivatives containing the RP4 *par* region deleted for both *parB* and *parC* but leaving the other functions intact showed reduced stability (Gerlitz *et al.*, 1990). Using a mini-RP4 replicon as a test system, Roberts *et al.* (1990) noticed a somewhat decreased plasmid stability when 4 bp were inserted into the *Sall* site, located within the coding region for *parB*. Detailed studies revealing the contribution of the *parB* gene to the stabilizing ability of RP4 *par* are in progress.

In this work we report on the functional characterization of the ParB nuclease. Overexpression of the *parB* gene under the control of a strong inducible promoter resulted in cell extracts exhibiting strong nuclease activity, which could be assigned to the ParB protein by partial purification. Basic enzymic features of the ParB nuclease were studied *in vitro*, revealing a specific action on circular DNA molecules.

METHODS

Bacterial strains and plasmids. *Escherichia coli* SURE (Stratagene) was used as a host strain for the construction of recombinant plasmids and for overexpression of ParB. *E. coli* K-12 C600 (ATCC 33525) was employed as a host strain for preparations of plasmid DNA. pGMA30 is a pBR322-based plasmid containing the complete RP4 *par* region with the intact coding regions for *parA*, *parB*, *parC*, *parD* and *parE* (Gerlitz, 1990). pMS470Δ8 (Balzer *et al.*, 1992) was used as expression vector for ParB. pMS470Δ8 is a derivative of pJF119EH (Fürste *et al.*, 1986) with the *rop* gene deleted, resulting in an increased copy number. In addition, the ribosome-binding site of gene 10 of phage T7 was inserted into the polylinker of pMS119EH. For cloning into pMS470Δ8 the 1.4 kb stuffer insert between *NdeI* and *SphI* was replaced by the *parB* sequence. For control purposes, the insert-less derivative (pMS4K) was constructed by cutting out the 1.4 kb fragment in pMS470Δ8 with *NdeI* and *SphI* and religating the vector after blunting the ends. pEG4, an expression plasmid for overexpression of the *parB* gene, was constructed as follows. The 997 bp *BssHII*–*SphI* fragment from pGMA30, comprising the coding region for *parB* and small parts of the *parA* gene, was inserted with the help of an adapter, consisting of oligo parB1, 5'-CGCGCAGCATGGCGTAGCTTCGGC-GCTTCA3' (30mer), and oligo parB2, 5'-TATGAAGCGC-

CGAAGCTACGCCATGCTG3' (28mer), with compatible ends to *BssHII* and *NdeI*, respectively, into *NdeI*/*SphI*-cut pMS470Δ8, generating pEG3. To remove the remaining sequences of the *parA* gene, a proper fragment containing the C-terminal part of *parB* upstream of the *Sall* site was prepared by cloning the 301 bp *Sall*–*BssHII* fragment from pGMA30 after filling in the *BssHII* site with the Klenow fragment of DNA polymerase I into the *Sall*–*EcoRV* sites of pGEM 5Zf(+) (Promega). The 771 bp *Sall*–*SphI* fragment from pEG3 was removed by *Sall*–*SphI* restriction and exchanged for the 301 bp *Sall*–*SphI* fragment, which was reisolated from pGEM 5Zf(+). The sequence of the *parB* insert in the expression plasmid pEG4 was verified. pGMA60 (Eberl *et al.*, 1992) is a pUC18-based plasmid containing the intact coding regions for *parA*, *parB*, *parC* and *parD*. pHS33 (Saurugger *et al.*, 1986) is a *par*⁺ RP4 deletion derivative, pOU82 (Gerdes *et al.*, 1985) a mini-R1 replicon. pOU82–*parABCDE* (Grohmann, 1994) is a pOU82-based plasmid containing the intact *parA*, *parB*, *parC*, *parD* and *parE* genes. pEG5 (Grohmann, 1994) based on the expression plasmid pMS470Δ8 was used for the overexpression of *parC*. A list of plasmids used is shown in Table 1.

Media and growth conditions. LB medium (Bertani, 1951) was used for routine growth of bacteria and for overproduction of ParB. For solid media agar was supplemented to a concentration of 1.5% (w/v). For overproduction of the ParC protein, supplemented M9 medium (Miller, 1972; modified) was used, containing M9 salts (g l⁻¹: Na₂HPO₄, 6; KH₂PO₄, 3; NaCl, 0.5; NH₄Cl, 1), 1 mM MgSO₄·7H₂O, 0.1 mM CaCl₂·2H₂O, amino acids/vitamin solution (mg l⁻¹: thiamin, 2; L-leucine, 20; L-proline, 20; L-threonine, 20), trace element solution SL6 (mg l⁻¹: ZnSO₄·7H₂O, 0.2; MnCl₂·4H₂O, 0.06; H₃BO₃, 0.6; CoCl₂·6H₂O, 0.4; CuSO₄·5H₂O, 0.02; NiCl₂·6H₂O, 0.04; Na₂MoO₄·2H₂O, 0.06), supplemented with 10 g glucose l⁻¹, 5 g Casamino acids l⁻¹ and 5 g yeast extract l⁻¹. Antibiotics were added as required to final concentrations of 100 mg l⁻¹ for ampicillin and 20 mg l⁻¹ for tetracycline.

DNA manipulations, sequencing and primer extension experiments. Routine techniques used for plasmid isolation, construction and transformation were as described by Sambrook *et al.* (1989). For the isolation of plasmid DNA the Qiagen Plasmid Midi kit (Qiagen) was used. Restriction endonucleases, DNA polymerase I Klenow fragment, T4 polynucleotide kinase and T4 DNA ligase were obtained from and used as recommended by New England Biolabs or

Table 1. Plasmids investigated for ParB nuclease cleavage

Plasmid	RP4 <i>par</i> components	Vector (replicon)	Size (kb)	Reference
pGMA30	<i>parABCDE</i> , <i>res</i>	pBR322 (pMB1)	6.1	Gerlitz (1990)
pGMA60	<i>parABCD</i> , <i>res</i>	pBR322 (pMB1)	5.1	Eberl <i>et al.</i> (1992)
pGMA44	<i>parAD</i> , <i>res</i>	pBR322 (pMB1)	5.0	Gerlitz <i>et al.</i> (1990)
pMRS19A	<i>res</i>	pUC18 (pMB1)	3.0	Eberl <i>et al.</i> (1992)
pCK155	<i>res</i> , <i>res</i> (direct)	pUC19 (pMB1)	4.9	Eberl <i>et al.</i> (1994)
pUC18	–	pMB1	2.7	Yanisch-Perron <i>et al.</i> (1985)
pLEO26	<i>parABCD</i> , <i>res</i>	pACYC184 (p15A)	6.7	Eberl <i>et al.</i> (1992)
pACYC184	–	p15A	4.2	Chang & Cohen (1978)
pHS33	<i>parABCDE</i> , <i>res</i>	RP4	41	Saurugger <i>et al.</i> (1986)
pOU82- <i>parABCDE</i>	<i>parABCDE</i> , <i>res</i>	pOU82 (R1)	15.3	Grohmann (1994)
pOU82	–	R1	12.6	Gerdes <i>et al.</i> (1985)

Boehringer Mannheim. Synthetic oligonucleotides used for sequencing were the *tac*-promoter primer (5'GCTCGTATA-ATGTGTGG3') corresponding to nucleotide positions -17 to -1 on the *tac* promoter sequence and the pBR322 primers, PBR4 (5'TGCGCCTTATCCGGTA3'), PBR5 (5'GCTGAA-GCCAGTTACC3') and PBR3332c (5'TCGCTGAGAT-AGGTGC3'), corresponding to nucleotides 2819-2834, 3001-3016 and 3332-3315, respectively, of the pBR322 sequence (Bolivar *et al.*, 1977). Sequencing was performed according to the dideoxy chain termination method using either the Sequenase 2.0 DNA sequencing kit (USB) and [α -³⁵S]dATP or [α -³²P]dATP, or using an ABI 373A automated DNA sequencer and the Dye-deoxy Terminator method employing AmpliTaq DNA polymerase (Perkin Elmer). Primer extension reactions were performed following the sequencing protocol of the Sequenase 2.0 kit using 2 μ g of ParB linearized and phenol/chloroform-extracted pGMA30 DNA as template, 25 μ M of each dNTP (omitting the dideoxy terminators) and 50 ng of 5'-labelled primer (T4 polynucleotide kinase and [γ -³²P]ATP). Reactions were incubated for 30 min at 37 °C. Control sequencing reactions were performed with the same primer using untreated DNA of pGMA30 and used for determining the size of the primer extension products.

Overexpression of ParB. A mid-exponential-phase culture of the host strain *E. coli* SURE(pEG4) was diluted 10-fold in fresh LB medium containing ampicillin and was incubated at 37 °C to OD₆₀₀ 0.4-0.5 (Beckman DU-50 spectrophotometer, 10 × 10 × 45). Protein expression was induced by addition of IPTG to a final concentration of 1 mM; induction time was 4 h. The cells were harvested by centrifugation and resuspended in lysis buffer (0.1 M Tris/HCl, pH 7.0, 0.9% NaCl, 5% glycerol, 0.1% Triton X-114, 1 mM DTT); for 350 ml of culture 25 ml lysis buffer was used. Cells were disrupted by two freeze-thaw cycles followed by sonication for 3 min on ice using the microtip of the Branson Sonifier 250 with the duty cycle set to 50% and the output control set to 7. The cell debris fraction (insoluble fraction) and the soluble portion of the lysate were separated by ultracentrifugation at 25000 g at 4 °C for 30 min. For monitoring the ParB production, samples containing the whole lysate, as well as cell debris and soluble fraction on their own, were applied onto 12% (w/v) SDS-polyacrylamide gels. Protein was quantified by the protein-assay system of Bio-Rad. Control lysates of *E. coli* SURE containing no plasmid or containing pMS4K were prepared in the same way.

Partial purification of ParB. The soluble portion of the *E. coli* SURE(pEG4) lysate (10 ml; about 6 mg protein ml⁻¹) was loaded onto a 15 ml column of high-resolution hydroxylapatite (Calbiochem, BSA binding capacity > 10 mg ml⁻¹). Prior to loading of the sample, the hydroxylapatite was equilibrated with four column volumes of 0.01 M NaH₂PO₄ (pH 7.0). The protein was eluted with a linear gradient from 0.05 M to 0.2 M NaH₂PO₄ (pH 7.0); the ParB protein eluted at 0.12 M NaH₂PO₄. The fractions containing the ParB protein were pooled and concentrated to 3 ml (3.60 mg protein ml⁻¹) using an Omegacell (Filtron Technology) with an exclusion limit of 5 kDa. A 1 ml portion of this protein solution was applied to a Merck Fractogel EMD BioSEC column (bed size 16 mm × 60 cm). The column was run with 0.25 M NaCl, 0.01 M Tris/HCl (pH 7.3). The fractions containing the ParB protein were pooled, desalted and concentrated by ultrafiltration as described above. This resulted in 500 μ l of a solution containing 0.25 mg protein ml⁻¹.

The chromatographic purification steps were performed with a Pharmacia FPLC system at flow rates of 3 ml min⁻¹ for the hydroxylapatite and 1 ml min⁻¹ for the SEC step; the fraction

volume was 2 ml. The outflow was monitored with a UV detector at 280 nm. The ParB protein was monitored by SDS-PAGE (12%). The protein concentration was determined using the Bio-Rad protein microassay according to the manufacturer's instructions.

The molecular mass of the purified protein was determined using laser desorption mass spectroscopy. The spectra were acquired in a linear mode with a time-of-flight Kompact MALDI II instrument (Kratos), operating at +20 kV acceleration voltage and equipped with a nitrogen laser (337 nm, pulse duration 3 ns). The *m/z* values were calibrated externally. The samples were dissolved in 10% acetonitrile, 0.1% trifluoroacetic acid. Alpha-cyano-4-hydroxycinnamic acid dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid was used as a matrix. For sample preparation a 1:1 mixture of protein solution and matrix solution was deposited onto the target and air dried.

N-terminal sequencing of purified ParB was performed by automated Edman degradation analysis using an Applied Biosystems 476A sequencer (ABI).

Preparation of ParC lysates. Cultivation of *E. coli* SURE(pEG5) and fractionation were performed as described above for *E. coli* SURE(pEG4), except that supplemented M9 medium was used for cultivation and cells were harvested 16 h after IPTG induction.

Metachromatic agar diffusion test. A slightly modified version of the toluidine blue O/DNA agar plate assay described by Lachica *et al.* (1971) and Shortle (1983) was used for detecting nuclease activity in ParB-containing lysates. In contrast to the published method, the toluidine blue solution contained 300 mg herring sperm DNA l⁻¹ (DNA hspm type IV from Sigma) and 160 mg toluidine blue O dye (TB) l⁻¹. The samples were applied as raw lysates onto the TB plates, which were incubated at 37 °C for 0.5-12 h.

ParB treatment of plasmid DNA (*in vitro* nuclease assay). We used a modification of the enzyme assay described by Puyet *et al.* (1990). Plasmid DNA was treated in nuclease buffer containing 50 mM Tris/HCl, pH 8.0, 3 mM β -mercaptoethanol and 1 mg RNase A from bovine pancreas in a final reaction volume of 20-100 μ l. Optionally divalent cations were added at 0.5, 1, 2.5, 5 or 10 mM. After addition of the appropriately diluted ParB preparation or control lysate the reaction mixtures were incubated at 37 °C; the reactions were stopped after appropriate times by addition of 4-10 mM EDTA and/or by heat inactivation at 68 °C for 10 min.

RESULTS

Overexpression and partial purification of *parB*

The coding region of the *parB* gene (nucleotide positions 1523-990; Gerlitz *et al.*, 1990) was inserted with the help of two oligonucleotides into the *tac*-promoter-based expression vector pMS470Δ8 (Balzer *et al.*, 1992) to generate the expression plasmid pEG4. Expression of *parB* from this construct should result in an authentic ParB protein. The *parB* gene exhibits three possible translational initiation sites. As the second one at nucleotide position 1523 is part of the arrangement of the overlapping translational stop (*parC*) and start (*parB*) signals, it was chosen for *parB* overproduction.

Overexpression of *parB* was most efficient at 37 °C in LB medium upon induction with 1 mM IPTG. Ultrasonic

Fig. 1. Overexpression and partial purification of the ParB nuclease. Samples from the purification steps were separated in 12% SDS-polyacrylamide gels and stained with Coomassie blue. Lane 5, LMW electrophoresis standard (Pharmacia) with bands corresponding to molecular masses of 94, 67, 43, 30, 20.1 and 14.4 kDa; lane 1, pEG4 without induction (soluble fraction, 42.9 µg protein); lane 2, pEG4 after 4 h induction (soluble fraction, 29.8 µg protein); lane 3, after hydroxylapatite (18 µg protein); lane 4, after SEC (3.8 µg protein).

lysates of *E. coli* SURE(pEG4) were analysed on SDS-polyacrylamide gels. A specific band of the expected size for ParB could be detected upon induction with IPTG whereas uninduced cultures did not show such a band (Fig. 1). The lysates of induced *E. coli* SURE(pEG4) cultures were subjected to ultracentrifugation to separate the debris fraction from the soluble fraction. SDS-PAGE analysis of the different protein fractions revealed that about 60% (w/w) of ParB could be detected in the debris fraction and approximately 40% (w/w) was present in the soluble fraction (data not shown). Both fractions exhibited nuclease activity using the toluidine blue O/DNA agar plate assay. Large pink haloes could be detected within 30 min of incubation when applying 5 µl of each lysate. Control lysates of plasmid-free or pMS4K containing *E. coli* SURE did not give rise to halo formation under the same conditions (data not shown).

The ParB protein was partially purified from the soluble fraction using hydroxylapatite and size exclusion chromatography. In this way ParB could be enriched about 10-fold and a purity of at least 80% could be achieved as estimated from SDS-PAGE analysis (Fig. 1). The molecular mass of the ParB protein estimated by SDS-PAGE was about 17 kDa. Exact determination by laser desorption mass spectroscopy resulted in a molecular mass of 17.535 kDa. These results indicate that the ParB protein purified from the soluble fraction represents the processed form with the signal sequence cleaved off. Attempts to determine the N-terminal amino

acid sequence by Edman degradation analysis failed as the N-terminal amino acid was blocked.

The endonuclease activity of ParB specifically linearizes circular DNA

The action of ParB on circular pGMA30 DNA was investigated by an *in vitro* nuclease assay (Puyet *et al.*, 1990; slightly modified) containing appropriate amounts of the soluble fraction of *E. coli* SURE(pEG4) lysates or partially purified ParB protein. Agarose gel electrophoresis revealed rapid conversion of CCC DNA to OC DNA, which is then linearized. During longer incubation times the linearized plasmid DNA was further degraded, indicating the presence of exonuclease activity of the purified ParB protein. No difference between unpurified lysates and partially purified ParB could be observed (Fig. 2). Linear plasmid DNA, for example pGMA30 DNA, cut at a single site by restriction enzymes prior to ParB treatment is only progressively degraded to smaller products without the appearance of distinct bands. A further indication that the ParB endonuclease could only act efficiently on circular DNA molecules was provided by the fact that a mixture of CCC and OC forms of dimeric pBR322 DNA treated with ParB in a standard *in vitro* nuclease assay yielded exclusively linear pBR322 dimers (data not shown). These findings indicate that circular plasmid DNA seems to be the preferred substrate for the ParB endonuclease activity.

The ability of ParB to linearize circular plasmid DNA is not limited to pGMA30, a plasmid which contains the entire partitioning region of RP4. From a selection of plasmids belonging to different incompatibility groups and some containing different parts of the RP4 *par* region, all were efficiently linearized by ParB (Table 1).

Effects of divalent cations, ATP and other Par proteins on nuclease activity

By adding various divalent cations to the *in vitro* nuclease assay we could detect stimulating and inhibitory effects on ParB activity. A strong stimulation of 2.5 mM Ca^{2+} on ParB reactivity was most significant (Fig. 3). In subsequent experiments different concentrations of Ca^{2+} in the range from 0.5 mM to 10 mM were applied. Strong stimulation was found with all tested concentrations, but was highest at 10 mM (data not shown). 2.5 mM Co^{2+} or Mn^{2+} added to the purified ParB protein slightly increased ParB activity, while the addition of 2.5 mM Mg^{2+} or Zn^{2+} did not show any significant effect (Fig. 3). However, when mixtures of 0.5 mM Ca^{2+} and 5 mM of the above mentioned divalent cations were applied, Zn^{2+} showed a clear inhibitory effect on the nuclease activity (data not shown). Considering the ratio between the circular DNA substrate and the added ParB protein, Ca^{2+} ions accelerated the ParB reaction at least 10-fold (Fig. 3; Fig. 4, lanes 4 and 5).

Addition of chelating agents such as EDTA at concentrations of 5 mM totally abolished ParB activity in *in*

Fig. 2. Time-course experiment. Approximately 2 µg pGMA30 DNA and either 1.8 µg protein of the ParB lysate (a) or 0.13 µg SEC-purified ParB protein (b) were incubated in nuclease buffer containing 2.5 mM Ca^{2+} at 37 °C in a 20 µl reaction. At indicated time-points the reaction was stopped by addition of 10 µl EDTA (50 mM)/gel dye solution and heat-inactivated at 68 °C for 10 min. The samples were analysed by electrophoresis through 0.7% agarose gels. The different forms of pGMA30 DNA are marked as follows. CCC, supercoiled form; OC, open circular DNA; Linear, linearized plasmid DNA. Lane S, molecular mass standard, *Hind*III-digested λ DNA; lane K, 8.6 µg protein of an *E. coli* SURE lysate (no ParB nuclease activity), incubation time 8 min; lane 1, pGMA30 untreated, no ParB; lanes 2–6, pGMA30 treated with ParB at incubation times of 30 s, 1, 2, 4 and 8 min; lane 7, *Sa*I-linearized DNA of pGMA30 (no ParB).

Fig. 3. Effects of different divalent cations on ParB activity. Approximately 2 µg pGMA30 DNA and 0.15 µg of the SEC-purified ParB protein were incubated in nuclease buffer containing either no divalent cation or 2.5 mM Ca^{2+} , Co^{2+} , Mg^{2+} , Mn^{2+} or Zn^{2+} at 37 °C in a 20 µl reaction for 2, 4 or 8 min. At these time-points the reaction was stopped by addition of 10 µl EDTA (50 mM)/gel dye solution and heat-inactivated at 68 °C for 10 min. The samples were analysed by electrophoresis on 0.7% agarose gels. The different forms of pGMA30 DNA are marked as follows. CCC, supercoiled form; OC, open circular DNA; Linear, linearized plasmid DNA. Lane S, molecular mass standard, *Hind*III-digested λ DNA; lane 1, pGMA30 untreated, no ParB; lanes 2–19, pGMA30 treated with ParB at incubation times of 2, 4 and 8 min (left to right within triplets of lanes); lanes 2–4, no divalent cation. Divalent cations were added at a concentration of 2.5 mM as follows: Ca^{2+} , lanes 5–7; Co^{2+} , lanes 8–10; Mg^{2+} , lanes 11–13; Mn^{2+} , lanes 14–16; Zn^{2+} , lanes 17–19.

Fig. 4. Restriction analysis of the linearized reaction product generated by ParB treatment of pGMA30 DNA. pGMA30 DNA was linearized by treatment with ParB protein in nuclease buffer (lane 4) or in nuclease buffer containing 2.5 mM Ca^{2+} (lane 5). The amount of ParB and the time of incubation were adjusted to give optimal amounts of linearized DNA not too much further degraded by exonucleolytic activity. For the reaction in presence of 2.5 mM Ca^{2+} the ParB solution was diluted 10-fold to obtain equal linearization. The reactions were terminated by addition of EDTA (50 mM)/gel dye solution, heat-inactivated at 68 °C for 10 min and aliquots were subsequently digested with the indicated restriction endonucleases. The samples were analysed by electrophoresis through 0.8% agarose gels. Lanes 1 and 15, molecular mass standard, 1 µg 1 kb DNA ladder (Gibco-BRL); lanes 2, 3, 6, 9 and 12, DNA of pGMA30 not treated with ParB; lanes 4, 7, 10 and 13, ParB-treated DNA of pGMA30 in the absence of Ca^{2+} ; lanes 5, 8, 11 and 14, ParB-treated DNA of pGMA30 in presence of 2.5 mM Ca^{2+} ; lane 3, pGMA30 DNA digested with *SalI*; lanes 6–8, DNA digested with *HincII*; lanes 9–11, DNA digested with *DraI*; lanes 12–14, DNA digested with *NdeI*.

Fig. 5. Schematic map of pGMA30. The line represents pBR322 sequences; the large box indicates the RP4 sequences containing the *par* genes; the arrows indicate the direction of transcription of the two operons. The region around the identified ParB cleavage site is shown as duplex DNA sequence; the coordinates at the ends refer to the published sequence of pBR322 (Bolivar *et al.*, 1977). The two palindromic *DraI* sites located within this region are marked by horizontal lines. The putative ParB cleavage site between nucleotide positions 3230 and 3231 on the pBR322 part of pGMA30 is marked by a dotted vertical arrow below the sequence. The end-points of the extension products obtained from the primer extension experiments are marked by vertical arrows above the sequence. Ap, ampicillin-resistance gene; Tc, tetracycline-resistance gene, interrupted by the insertion of the *par* genes (Tc^r); oriV, origin of DNA replication. Additional relevant restriction sites are indicated as follows: D, *DraI*; H, *HincII*; N, *NdeI*.

vitro nuclease assays. A slight decrease (approximately 25%) in ParB activity seemed to occur in the presence of ATP (1 mM). Furthermore, no significant influence on *in vitro* ParB activity could be observed by the addition of different amounts of purified ParA protein (Eberl *et al.*, 1994) and/or a ParC-containing lysate (data not shown).

Linearization of circular DNA by ParB occurs preferentially at specific sites

ParB-linearized plasmid DNA was further digested with the restriction endonucleases *HincII*, *DraI* and *NdeI*, respectively. The result of the restriction analysis of ParB-treated pGMA30 DNA is shown in Fig. 4. Except

for *DraI*, all the restrictions of ParB-linearized pGMA30 DNA resulted in additional DNA fragments. We thereby located a preferred ParB cleavage region in the vicinity of the two *DraI* sites present in the pBR322 part of pGMA30 (see Fig. 5).

We also analysed several other plasmids (see Table 1) by restriction analysis following linearization by ParB. Plasmids pGMA60 and pUC18 showed at least two distinct preferred cleavage sites, while with the R1-based plasmid pOU82-parABCDE at least eight such sites could be observed (data not shown).

Run-off sequence analysis and primer extension experiments were performed to map exactly the ParB cleavage site on pGMA30. Two primers, PBR4 (annealing at nucleotide positions 2819–2834) and PBR5 (annealing at positions 3001–3016; numbers refer to the pBR322 sequence), were used in independent sequence reactions. With both primers a specific breaking off in signal intensity could be observed within the expected AT-rich region near the *DraI* sites with the last strong signal at nucleotide position 3230 (CG pair) on the pBR322 part of pGMA30 (Fig. 5). This result was confirmed by primer extension analysis with ParB-linearized pGMA30 DNA and primer PBR3332c (nucleotide positions 3332–3315 of the pBR322 sequence). However, additional extension products of higher intensity terminating at positions 3231, 3259, 3263 and 3268 could also be observed, as indicated in Fig. 5.

DISCUSSION

In this study we demonstrated that the *parB* gene, part of the *parCBA* operon of plasmid RP4, encodes a protein exhibiting endonuclease activity which is highly specific for circular DNA substrates. In addition, ParB progressively degrades linearized plasmid DNA, indicating the presence of exonuclease activity. This progressive degradation could also result from single-strand specific endonuclease activity on partially melted ends of linear DNA. We analysed a plasmid consisting of pBR322 and

the entire *par* region of RP4 (pGMA30) in more detail and demonstrated that ParB endonucleolytic cleavage of circular DNA occurs preferentially at a distinct site. Detailed mapping revealed that this site is within the pBR322 part of pGMA30, adjacent to an AT-rich region between the translational termination signal for the ampicillin-resistance gene and the transcriptional start site for the replication primer RNAII (Balbas *et al.*, 1986). Ca^{2+} was identified as a cofactor for ParB nuclease (see Fig. 3), as is the case with the *S. aureus* nuclease to which ParB shows sequence homology (see Fig. 6).

Sequence analysis of ParB revealed a strongly hydrophobic N-terminus which fits to the criteria of potential signal sequences (von Heijne, 1983). The signal sequence seems to be efficiently cleaved upon overexpression in *E. coli*. On SDS polyacrylamide gels of the soluble fraction, only protein bands of the calculated size of the processed ParB polypeptide of approximately 17 kDa can be seen (see Fig. 1). This was confirmed by exact molecular mass determination using laser desorption mass spectroscopy which gave a molecular mass of 17·535 kDa, indicating that the first 25 N-terminal amino acids are cleaved off and the protein would be located in the periplasm.

In contrast, preliminary experiments on determining alkaline phosphatase activities of translational *phoA* fusions with *parB* gave indications of a possible association of ParB to the inner membrane, as low *PhoA* activities could be seen with ParB::PhoA fusions (Eberl, 1992). As we found a larger part of the overexpressed ParB protein in the insoluble fraction, parts of ParB could be membrane-associated. At this stage of our studies we are not able to discuss the cellular location of ParB in more detail.

Nucleases seem to be encoded by a variety of plasmids of Gram-negative bacteria and examples were reported for different incompatibility groups including IncFII, IncH, IncI, IncM, IncN, IncP, IncQ and IncW. Several of these nucleases share sequence homologies with ParB (Fig. 6), the highest being with that encoded by plasmid pSa (Close & Kado, 1992) and the putative nuclease encoded

ParB	1	MKRRSYAMLR	AAAAVLVV	ASPAWAE	RG	EVVRI	IDGDT	IDVLVDKQPV	RVRLVDIDAP	60
RuvC		AI	ILGIDPGSRV	TGYGVIRQV	RG	RQLSYLGSGC	IRTKVDDLPS	RLKLIYAGVT		
Psa		MKSALAA	LR	AVAAAVLIV	SVPWADF	RG	EVVRI	IDGDT	IDVLVNRQTI	RVRLADIDAP
OrfA		MRK	YIPLVLFIFS	WPVLCADI	HG	RVVRVLDGDT	IEVMSRKA	V	RIRLVNIDAP	
Stanuc			AT	STKKLHKEPA		TLIKAIDGDT	V	KLMYKQPM	TFRLLLVDTP	
						*		*		
ParB	61	EKRQA		HGERARQAL	AGMVFRHVL	VDEKD	T	DRYGRTLGTV	WVNMEASR	109
RuvC		ELITQ		HDPDYFAI	EQVEMAKNAD	SALK		LGQARGVAIV	AAVNQELPVF	
Psa		ESGQA		HGSRARQRL	ADLTFRQEVQ	VTEKE	V	DRYGRTLGVV	YALPQYGG	
OrfA		EKKQD		YGRWSTDM	KSLVAGTKVT	VTYFQ	R	DRYGRILGQV	YALPQYGG	
Stanuc		ETKHPKKGVE	KY	GPEASAF	KKMVENAKKI	EVEFDKGQRT		DRYGRGLAYI	YALPQYGG	
		*				*		*		
ParB	110	PPQPRNVNAA	MVHOGMAWAY	RFHGRAADPE	MLRLEQ	EAARG	KRVGLWSDPH	AVEPWKWRRE		169
RuvC		EYAARQVKQT	VVGIGSAEKS	QVQHMVRLTLL	KLPANP	QADA	ADALAIATH	CHVSQNAQMOM		
Psa		QTQLTNINAI	MVQEGMAWAY	RYYGKPTDAQ	MYEYER	EAARR	QRLGLWSDPN	AQEPWKWRRA		
OrfA		PDGMNVNQF	MVRAGAAVY	EQYNTDPLVLP	VLQN	EAARQ	QKRGLWSDAD	PVPPWIWRHR		
Stanuc		..DGKMYNEA	LVRQGLAKVA	YVYKPNNTHE	QHLRKS	EAARQ	KKEKNIWSE	DNADSGQ		
						*		*		
ParB	177	SNNRRDEG								
RuvC		SESRLNLARG	RLR							
Psa		SKNATN								
OrfA		K								
Stanuc										

Fig. 6. Amino acid sequence alignment of nucleases. ParB, nuclease of plasmid RP4 (Gerlitz *et al.*, 1990); RuvC from *E. coli* (Sharples *et al.*, 1991); Psa, nuclease from plasmid pSa (Close & Kado, 1992); ORFA from plasmid R100 (Yoshioka *et al.*, 1990); Stanuc, *Staphylococcus* nuclease (Shortle, 1983). Regions of high homology are enclosed in boxes and amino acids identical in at least four of the five sequences are in bold letters. Amino acids located in the catalytic centre of *Staphylococcus* nuclease and involved in Ca^{2+} binding, respectively, are marked by asterisks. Additionally, amino acids identical only in ParB and RuvC are underlined. The numbering corresponds to the amino acid sequence of ParB (RP4).

by ORFA of plasmid R100 (Yoshioka *et al.*, 1990). But these nucleases are not yet characterized in detail.

Weak but significant overall homology of the ParB protein can also be recognized to the nuclease from *Staphylococcus aureus* and to RuvC from *E. coli*, both well-characterized enzymes. However, blocks of high sequence conservation can be defined with all these nucleases (Fig. 6). The structure of the *Staphylococcus* nuclease has been determined at high resolution (Cotton *et al.*, 1979, Loll & Lattman, 1989). The amino acids Arg-35, Glu-43 and Arg-87 of the *Staphylococcus* nuclease (numbering refers to processed polypeptide) were proposed to form the catalytic centre of the enzyme. In addition, the carboxylate groups of Asp-21 and Asp-40 are involved in Ca^{2+} binding.

The RuvC protein is able to resolve synthetic Holliday junctions (Dunderdale *et al.*, 1991, 1994; Bennett *et al.*, 1993, 1995), recombination intermediates generated by RecA (Dunderdale *et al.*, 1991, Shah *et al.*, 1994) and cruciform structures extruding from supercoiled plasmids (Iwasaki *et al.*, 1991). Recently the atomic structure of RuvC and thereby the amino acids possibly representing the active site were determined. The proposed active site residues Asp-7, Glu-66, Asp-138 and Asp-141 have been demonstrated to be essential for DNA repair activity *in vivo* and located at the bottom of the putative DNA-binding cleft (Ariyoshi *et al.*, 1994). However, counterparts of these residues are not present in the ParB sequence. On the other hand, a further residue (Arg-104), shown to be indispensable for DNA repair activity of RuvC and located adjacent to a higher conserved block, can be found at the same position in ParB.

Interestingly, the residues corresponding to Arg-35, Glu-43 and Arg-87 of the active site of staphylococcal nuclease are conserved in all investigated nucleases, with the only exception that in RuvC a Lys residue is located at the position of Arg-35. The two Asp residues involved in Ca^{2+} binding (Asp-21 and Asp-40) are highly conserved among the plasmid-encoded nucleases including ParB, but Gly residues are present at these positions in RuvC. This may reflect a specific difference in cofactor requirement. The nicking activity of RuvC requires divalent cations such as Mg^{2+} . Mn^{2+} acts with slightly lower efficiency, but Ca^{2+} is a poor substitute.

At this stage of our studies we can only speculate on a possible role of the ParB nuclease in a process aimed at ensuring high segregational stability of plasmids. Sobecky *et al.* (1996) clearly demonstrated that the *parCBA* operon acts as an independent stabilization function. The post-segregational killing system encoded by the *parDE* operon seems to represent a back-up system which predominantly contributes to the overall stability at conditions of reduced copy number.

Recent research on chromosome partitioning reflects the involvement of site-specific recombination (Leslie & Sherrat, 1995). We have demonstrated that site-specific recombination of the RP4 *parA-res* system is mainly involved in resolution of plasmid dimers. In addition,

the resolution process itself is not sufficient to reach the high stabilization level mediated by the intact *par* system (Gerlitz *et al.*, 1990). We have suggested a model for plasmid partitioning that includes dimer resolution in the segregation process (Gerlitz *et al.*, 1990, Eberl *et al.*, 1994). According to this model, instead of plasmid pairs prepared via specific proteins, as suggested for the F and P1 *par* systems (Austin, 1988), a true plasmid dimer enters the partitioning complex. Resolution of this dimer at a specific stage of the partitioning process would release two separate copies ready to be segregated into the daughter cells. The ParB nuclease could play a role in finalizing the resolution process. McCulloch *et al.* (1994) have found that site-specific recombinase (Xer) action *in vivo* generates Holliday junctions and does not completely separate molecules. RuvC nuclease was initially supposed to be involved in finalizing the resolution process but it was found that the generation of Holliday junctions and recombinant products is equal in RuvC⁻ and RuvC⁺ cells and in cells containing a multicopy RuvC⁺ plasmid.

The possible periplasmic location of the processed ParB protein makes it difficult to explain its involvement in such a process. However, one could discuss a possible interaction of periplasmic enzymes at junctions between the outer membrane and inner membrane formed during the cell division process.

As nothing is known about the function of the ParC protein, a specific role or possible interactions with other components of the RP4 *par* system can not be seriously proposed at this stage of our studies. A missing link would be a protein mediating a connection of plasmid dimers to the segregation machinery of the bacterial cell.

With this work, we have demonstrated that the *parB* gene of RP4 encodes a nuclease with distinct functions. These initial *in vitro* studies demonstrated efficient Ca^{2+} -dependent endonuclease activity acting preferentially at specific sites on circular DNA molecules. However, more extended studies on the functionality and cellular location of the ParB nuclease are necessary to clarify its role in the stabilization process. In addition, detailed *in vitro* and *in vivo* studies on interactions of all components of the RP4 *parCBA* operon will provide a better understanding of the encoded, highly efficient stabilization function.

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