Chromosome loss from *par* mutants of *Pseudomonas putida* depends on growth medium and phase of growth

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The proteins encoded by chromosomal homologues of the *parA* and *parB* genes of many bacterial plasmids have been implicated in chromosome partitioning. Unlike their plasmid counterparts, mutant phenotypes produced by deleting these genes have so far been elusive or weakly expressed, except during sporulation. Here the properties of *Pseudomonas putida* strains with mutations in *parA* and *parB* are described. These mutants do not give rise to elevated levels of anucleate bacteria when grown in rich medium under standard conditions. However, in M9-minimal medium different *parA* and *parB* mutations gave between 5 and 10% anucleate cells during the transition from exponential phase to stationary phase. Comparison of the DNA content of bacteria at different stages of the growth curve, in batch culture in L-broth and in M9-minimal medium, suggests that the *par* genes are particularly important for chromosome partitioning when cell division reduces the chromosome copy number per cell from two to one. This transition occurs in *P. putida* during the entry into stationary phase in M9-minimal medium, but not in L-broth. It is proposed that the partition apparatus is important to ensure proper chromosome segregation primarily when the bacteria are undergoing cell division in the absence of ongoing DNA replication.

Keywords: bacterial chromosome, active partitioning, *parA*, *parB*, stationary phase

INTRODUCTION

With the exception of enteric species like *Escherichia coli*, most bacterial chromosomes investigated to date possess genes similar in sequence to the *parA* and *parB* genes originally recognized by Motallebi-Veshareh *et al.* (1990) as archetypes of two large families of proteins possessing active partitioning functions. These genes have been clearly shown to play a key role in active rather than random partitioning of plasmids in dividing cells (reviewed by Williams & Thomas, 1992; Bignell & Thomas, 2001). The ParB homologues are capable of both dimerization and DNA binding (Davis *et al.*, 1988; Mori *et al.*, 1989; Lin & Grossman, 1998; Surtees & Funnell, 1999; Lobočka & Yarmolinsky, 1996; Rodionov *et al.*, 1999; Watanabe *et al.*, 1989; Balzer *et*

al., 1992; Williams *et al.*, 1993). They recognize the *cis*-acting centromere-like sequence, the third known element of the partitioning apparatus (Jensen & Gerdes, 1997; Sharpe & Errington, 1996, 1998; Lin & Grossman, 1998; Glaser *et al.*, 1997). ParA homologues possess, or are predicted to possess, ATPase activity (Motallebi-Veshareh *et al.*, 1990; Davis & Austin, 1992; Watanabe *et al.*, 1992; Lin & Mallavia, 1998; Jensen & Gerdes, 1999) and are required for organization and symmetrical distribution of the ParB/DNA complex (Bignell *et al.*, 1999; Marston & Errington, 1999; Quisel *et al.*, 1999). They have also been implicated in transcriptional regulation (Bouet & Funnell, 1999; Jagura-Burdzy *et al.*, 1999; Cervin *et al.*, 1998; Quisel *et al.*, 1999; Quisel & Grossman, 2000). However, mutational analysis of chromosomally encoded *par* genes has so far failed to provide a strong partitioning phenotype other than during sporulation.

Attempts to obtain deletion mutants of the *par* genes of *Caulobacter crescentus* were unsuccessful, perhaps due

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Abbreviation: DAPI, 4',6-Diamidino-2-phenylindole.

Fig. 1. (a) Schematic diagram illustrating the organization of the *P. putida* *oriC* region (Ogawasara & Yoshikawa, 1992). DnaA boxes are shown in black and the *par* genes are patterned. The direction of transcription is indicated by arrows above the genes. (b) Amino acid sequences and putative functional motifs of ParA and ParB and the mutations introduced into them in the course of this study. Motifs I and II of ParA correspond to the 'Walker A' and 'Walker B' motifs, respectively, characteristic of proteins possessing ATPase activity (Williams & Thomas, 1992). Motifs III and IV of ParA are thought to be involved in protein-protein interactions and interaction with the plasma membrane, respectively (Williams & Thomas, 1992). The point mutations introduced into the motifs of ParA are indicated, as is the in-frame deletion of ParA. The 15 bp intergenic region is indicated by *****. The predicted helix-turn-helix region of ParB is indicated and the alternative amino acid sequence produced as a result of the frameshift mutation, which disrupts the motif and prematurely terminates ParB, is given below the native sequence.

to their involvement in processes other than chromosome partitioning (Mohl & Gober, 1997). However, overexpression of ParA resulted in filamentation of cells and mislocalization of ParB, while overexpression of ParB resulted in 5 % of cells within the population being anucleate. Deletion of the *parA* homologue (*soj*) in *Bacillus subtilis* produced no detectable chromosomal partitioning phenotype during vegetative growth (Ireton *et al.*, 1994), whereas deletion of the *parB* homologue

spo0J gave a phenotype characterized by 1–2 % of the bacterial population being anucleate. Anucleate cell production was also observed in *soj/spo0J* double mutants (Webb *et al.*, 1998). Both *soj* and *spo0J* deletant strains possess more marked phenotypes with regard to chromosome partitioning during sporulation (Ireton *et al.*, 1994; Sharpe & Errington, 1996; Quisel & Grossman, 2000). However, studies with mutants containing *Soj* or *Spo0J* fusions with *Gfp* did indicate

protein localization and movement during vegetative growth, consistent with them being part of an active partitioning apparatus (Sharpe & Errington, 1998; Marston & Errington, 1999). In *Streptomyces coelicolor*, inactivation of *parA* and *parB* led to no effect during vegetative growth, but did result in production of anucleate spores during differentiation of aerial mycelia (Kim *et al.*, 2000).

As all of these studies have involved bacterial species that have relatively complicated survival strategies, we have investigated the *par* genes in *Pseudomonas putida* because of its simpler life cycle, similar to many other Gram-negative rod-shaped bacteria. As a model system it is useful because its *oriC* region has an organization similar to that of most other bacteria whose genomes have been sequenced, including striking similarity to *B. subtilis* (Fig. 1a) (Ogasawara & Yoshikawa, 1992), but with clear differences from that of the enteric bacteria. We have therefore generated mutants defective in *parA* and *parB* and report their properties. The results suggest that, as in other systems studied so far, the products of these genes are most important under specialized circumstances when the bacteria are undergoing a transition from one survival mode to another, during the entry into stationary phase. We propose that the function of the *par* system is to tether chromosomes at the cell quarters when partitioning is required in the absence of reinitiation of replication.

METHODS

Bacterial strains and growth conditions. The *E. coli* K-12 strains used were TG1 [$\Delta(lac-pro)$ *supE thi hsd Δ 5/F'traD36 proA⁺B⁺ lacI^q lacZAM15*] and S17-1 (*pro hsdR⁻ hsdM⁺ recA⁻ T^pR Sm^R Ω RP4-Tc::Mu-Kn::Tn7*). The *Pseudomonas putida* strain used was PaW1, containing plasmid pWWO. Bacteria were grown in L-broth or in M9-minimal medium (Sambrook *et al.*, 1989). For solid media, 1.5% (w/v) agar was added. Antibiotic resistance was selected by addition of ampicillin (100 μ g ml⁻¹) or kanamycin sulphate (50 μ g ml⁻¹) to the appropriate medium. Overnight cultures were diluted 1:100 into either L-broth or M9-minimal medium and growth of the cultures was followed as OD₆₀₀, determined using a Hewlett Packard 8452A diode array spectrophotometer. At appropriate time points, bacteria from 1.5 ml samples of culture were harvested by centrifugation and frozen in liquid nitrogen before being stored at -20 °C until required to prepare slides for fluorescence microscopy.

Recombinant DNA technology. All restriction and modification enzymes were from MBI (Fermentas) with the exception of T4 DNA Ligase (Gibco). All were used according to the manufacturers' instructions. Oligonucleotides for DNA sequencing and PCR were from Alta Bioscience (Birmingham University). DNA sequencing was done by using the BigDye terminator sequencing kit (Amersham Life Sciences). Prepared samples were run on gels by Alta Bioscience (Birmingham University). All electrophoresis equipment and reagents were as previously described (Bignell *et al.*, 1999).

Construction of suicide plasmids containing deletions of the *par* genes. A new *P. putida* suicide vector was constructed by ligation of the linker 5'-ATCCCCGGGTCGACCATGGCTGCAGAAGCTTTCTAGAGAATTCA-3' and its comple-

mentary sequence, with 5' overhangs of 5'-CCGG-3' at each end, into the *Xma*I site of pEX100T (Schweizer & Hoang, 1995) to create the recombinant plasmid pAKE600 carrying sites for *Bam*HI, *Sma*I, *Sal*I, *Nco*I, *Pst*I, *Hind*III, *Xba*I and *Eco*RI as a multiple cloning site with blue/white selection (El-Sayed, 2001). A DNA fragment from pCAW5.3 (Whatling, 1993) containing the *aph* gene from Tn5, conferring kanamycin resistance, was ligated into the *Hind*III/*Xba*I sites of pAKE600 to create pCOL500. The *par* region was amplified from *P. putida* PaW1 chromosomal DNA using the oligonucleotides *parA*(b) (5'-GGCGGATCCCATATGGCTAAGGTATTCGCAATCG-3') and *parB* (5'-GCGTCGACAAGCTTTCAGCGGATGTGAGCGAG-3'), designed using previously determined sequence data (Ogasawara & Yoshikawa, 1992), as primers in a PCR. The PCR product was cloned into pUC18 using the *Eco*RI and *Sal*I sites to receive the insert to give pCOL5. The *par* genes were also transferred from pCOL5 into pCOL500 using the *Hind*III and *Bam*HI sites to receive the insert to create the control plasmid pCOL502. The *Eco*RI site was removed from pCOL500 by *Eco*RI cleavage, treatment with Klenow DNA Pol I fragment plus dNTPs and religation to give pCOL503, and the *par* genes were inserted using the same sites as for pCOL502 to create pCOL504. A *parB* frameshift mutation was created by cleavage of pCOL504 with *Eco*RI followed by treatment with Klenow fragment and religation to produce pCOL505. The *par* insert was transferred from pCOL5 using *Eco*RI and *Sal*I to pCOL5.1 a version of pUC18 that had the *Eco*O1091 site removed by cleavage, treatment with Klenow fragment and religation. An internal deletion mutation was created in *parA* of the pCOL5.1 insert by cleavage with *Eco*O1091, treatment with Klenow fragment and religation. The *par* insert was transferred to pCOL500 using *Hind*III and *Bam*HI to create pCOL501. pCOL501 (*parA*), pCOL505 (*parBA*) and pCOL504 (control) were transformed into *E. coli* S17-1 by the method of Hanahan *et al.* (1991) and used in a mating with *P. putida* PaW1. The presence of mutant genes in the chromosome was verified by PCR (as described below) on chromosomal DNA produced from possible recombinant cells. The size of the PCR products was used as a test for *parAA* strains or cleavage of the product with *Eco*RI as a test for *parBA* strains. To make a *parABA* suicide plasmid, the *parAA* insert was transferred from pCOL501 to replace the *par* insert in pCOL504. The resultant plasmid, called pRAL581, was cleaved with *Eco*RI, treated with Klenow fragment and religated to introduce a frameshift mutation into *parB*. This double mutant plasmid was called pRAL58.

Construction of suicide plasmids containing amino acid substitutions in *parA*. To create the K16A mutation, a 1.07 kbp fragment corresponding to the first 0.5 kb of the *parA* gene and an equivalent amount of upstream DNA were amplified by PCR using the primers CM3 (5'-GGAATTCTGCTCGACAGCCTTAGC-3') and CM4 (5'-CGGGATCCCGTTGTTTCAGGCTCAGACG-3'). The PCR product was ligated into pGEM-T easy vector (Promega) to create pGC3-4, which was used as a template in a second PCR. The primers used in this PCR were CM1 (5'-CAGGTGGTTGTGGCGCCCACACCA-3'), which contains changes from the native sequence to introduce a K16A substitution, and CM3. The resultant PCR product was used as a primer in a third round of PCR with CM4 as the other primer and pGC3-4 as template. The product of this PCR was ligated into the suicide vector pAKE604 (El-Sayed, 2001) to give pWZ1. To create the L138V139-R138R139 mutation a 1.08 kbp fragment, corresponding to about 0.5 kb upstream and downstream of codons 138/139 in *parA*, was amplified by PCR using the primers

CM7 (5'-CGGGATCCCGATGAACTGGTAGCATTG-3') and CM8 (5'-GCTCTAGAGCGAGGCTGATACTTGCCGC-3'), and the product was ligated into pGEM-T easy vector to create pGC7-8. This was used as a template in a second PCR with the primers CM8 and CM6 (5'-GCTGAATGCTCGGCGCGCTTCCG-3') which contain changes from the native sequence to introduce the amino acid changes. The resultant PCR product was used as a primer together with CM7 and pGC7-8 as template in a third PCR and the product of this, containing a mutant version of *parA*, was ligated into pAKE604 to create pWZ2.

Suicide mutagenesis of the *P. putida* chromosome. Saturated cultures of *E. coli* S17-1 carrying the appropriate derivative of pCOL500 (see above) were mixed with a 10-fold excess of *P. putida* PaW1 and filtered onto a sterile 0.45 µm nylon membrane which was then placed on an L-agar plate and incubated at 30 °C overnight. The bacteria were then resuspended in 0.85 % (w/v) saline before being spread on M9-minimal medium, to select against *E. coli*, and with kanamycin to select integrants in which the suicide plasmid had recombined with the *P. putida* chromosome. Putative integrant colonies were purified by streaking and then transferred to L-agar plates containing 5 % sucrose, as the suicide vector contains a copy of the *sacB* gene which is lethal in the presence of sucrose. Colonies which grew should arise from bacteria in which the suicide vector was excised from the chromosome and lost, and thus may have exchanged the mutant copies of the *par* genes for the wild-type genes. The presence of wild-type or mutant copies of the *par* genes was checked by restriction enzyme digestion of PCR products generated from the *par* region.

PCR. A Hybaid 'Sprint' thermocycler was used to run the PCR and the conditions were as follows: 5 min at 95 °C followed by 2 cycles of 96 °C for 15 s, 50 °C for 30 s and 72 °C for 2 min, then 25 cycles of 96 °C for 15 s, 55 °C for 30 s and 72 °C for 2 min with a final extension step of 72 °C for 10 min. Chromosomal DNA was prepared by picking a colony from an agar plate, washing the cells in 100 µl 0.85 % saline and pelleting them by centrifugation. The cells were then resuspended in 50 µl dH₂O and boiled for 2 min. The lysate was spun in a microfuge for 5 min and the cleared supernatant used as template in the PCR to check for presence of the mutant *par* genes. The reaction mix contained 1 µl *Taq* polymerase (Boehringer), 5 µl *Taq* polymerase buffer (+ 15 mM MgCl₂), 1 µl template DNA, 2.5 pmol each oligonucleotide primer (Alta Bioscience, Birmingham University), 200 µM each dNTP and dH₂O to 50 µl.

Fluorescence microscopy. The cells were fixed as described previously (Bignell *et al.*, 1999), harvested by centrifugation, washed three times in PBS (Sambrook *et al.*, 1989) and finally resuspended in 200 µl PBS. The cells were added to coverslips coated in poly-L-lysine (Sigma) in 5 mm diameter areas, left for 5 min, aspirated and then washed three times in PBS and air-dried. The cells were rehydrated in PBS, left for 5 min then aspirated and washed three times in PBS. The dried coverslips were mounted as described previously (Bignell *et al.*, 1999). The slides were viewed using an Olympus IX70 inverted reflected light fluorescence microscope fitted with a Sensys CCD camera (Photometrics). Images were captured and manipulated on a Macintosh G3 with the Smartcapture I program (Digital Scientific). For each culture at each time point images containing ~ 2000 cells were assayed to determine the number of anucleate cells in the population. Two hundred cells chosen at random for each culture at each time point were measured using the IPlab software (Vysis) to determine their total fluorescence, length and width.

Determination of DNA content per bacterium. The technique used was as described by Burton (1956). Cells from 4.5 ml culture were pelleted by centrifugation and resuspended in 4.5 ml PBS. Then 0.5 ml 0.5 M perchloric acid was added and the mixture was incubated on ice for 3 h. The mixture was then centrifuged, the supernatant discarded, the pellet resuspended in 1 ml 0.5 M perchloric acid and heated to 70 °C for 30 min before being cooled on ice for 15 min. The mixture was again centrifuged and the supernatant decanted into glass tubes and 2 ml diphenylamine reagent was added [1.5 g diphenylamine (Sigma), 1.5 ml conc. sulphuric acid and 1.5 ml aqueous acetaldehyde (16 mg ml⁻¹) in 100 ml glacial acetic acid]. The mixture was incubated at 30 °C in the dark for 20 h, after which the absorbance at 600 nm was determined. The number of cells per millilitre of the cultures used in the DNA assay was determined by examination of diluted culture samples placed in a Thoma chamber and viewed at × 400 magnification with phase-contrast using a Leitz 'Wetzlab' microscope. The two datasets, each with a minimum of five replicates, were used to calculate the DNA per cell of M9-minimal-medium- and L-broth-grown PaW1 cultures. Salmon sperm DNA was used to create a standard curve, but the results are presented as relative DNA content because we are not confident of the absolute values generated.

RESULTS

Construction of *parA* and *parB* mutants in *P. putida*

To introduce specific mutations into the *P. putida* chromosome (see Methods) we used a suicide plasmid that carries penicillin and kanamycin resistance markers that function in both *E. coli* and *P. putida*, an *oriT* region allowing conjugative transfer of the plasmid between *E. coli* and *P. putida*, and *sacB* for selection of subsequent plasmid loss from the *P. putida* chromosome after reversal of the Campbell integration event. The high-copy-number pMB1 replicon in pCOL500 is functional in *E. coli*, but not in *P. putida*. Therefore, any *P. putida* kanamycin-resistant transconjugants generated by a mating with *E. coli* carrying a suicide plasmid are likely to have the plasmid inserted into the chromosome by homologous recombination between the *P. putida*-derived insert and the corresponding sequence of the *P. putida* chromosome. Bacteria in which the suicide vector subsequently excised from the chromosome may have exchanged the mutant copy of the *par* gene for the wild-type gene. Excisants were identified by growth on L-agar plates containing 5 % sucrose, since the product of *sacB*, levan sucrose, converts sucrose to levan in the periplasmic space, which is lethal to Gram-negative bacteria. The presence of mutant copies of the *par* genes was checked by PCR.

The mutagenesis strategy had to take into account the polycistronic nature of the *gid-par* operon of *P. putida* in which *parA* is upstream of *parB*. Fortunately, these are the last two cistrons in the operon. For *parA* we designed both a deletion and two substitution mutations. The deletion removes 96 bp (32 triplets) of the gene, encoding amino acids 180–211 of ParA which is 251 aa long (Fig. 1b). The in-frame nature of the mutation means that polar effects on transcription and translation of *parB* are unlikely. However, to make the study more complete we

Fig. 2. Representative fluorescence micrographs of DAPI-stained wild-type or mutant *P. putida* grown for different times in L-broth or in M9-minimal medium. For clarity an abbreviated code is used in the figure: M9, M9-minimal medium; LB, L-broth; Wt, wild-type; exp, exponential phase; stat, stationary phase. Times from dilution of the overnight culture were 5.5 (a), 13.5 (b), 3 (c), 10.5 (d) or 13.5 h (e–h) based on growth curves similar to those shown in Fig. 4(a) and at approximate times indicated by the arrows. Bars, 5 μm long.

also included two additional mutations affecting motifs, located in the N terminus, which are conserved in the *parA* family. These mutations should have no polar effects at all. Motif I, corresponding to the ‘Walker Box A’ of ATPases, was disrupted by a K16A amino acid

Fig. 3. Growth curves and anucleate cell production for *P. putida* wild-type and *par* mutant strains grown in L-broth (a) and M9-minimal medium (b) based on the combined results of two representative experiments. Approximately 2000 cells were counted for each time point. ●, Wild-type; ■, *parA* mutant; ▲, *parB* mutant. Optical density measurements (connected by solid lines) are presented as $\log_{10}$ of the OD₆₀₀ as a percentage of the plateau value for OD₆₀₀ in stationary phase. Since the growth curves for wild-type and mutants were indistinguishable in L-broth the data were amalgamated and a single line is shown. The growth curves in M9-minimal medium were also very similar, so again they were combined although as shown in Fig. 4 there are minor differences, with wild-type giving slightly higher values during the transition into stationary phase. The percentages of DNA-less bacteria are indicated by dashed lines. Error bars are omitted for clarity. The degree of error in these results is indicated in the text. (c) Mean volume of bacteria at different stages plotted against the measured percentage of DNA-less bacteria for the *parA* mutant. The lefthand vertical dashed line indicates the mean plateau volume of the bacteria in stationary phase after growth in M9-minimal medium, while the righthand vertical dashed line indicates the same figure after growth in L-broth.

change, and Motif IV, which may be involved in interaction with the cell membrane, was disrupted with simultaneous L138R and V139R amino acid changes.

Table 1. Bacterial size dimensions for *P. putida* PaW1 in late exponential and stationary phases when grown in M9-minimal medium and L-broth

Medium	Growth time of culture (h)*	Mean cell length (µm)	Mean cell width (µm)
L-broth	3	2.8 ± 0.6	1.2 ± 0.1
L-broth	6.5	3.0 ± 0.6	1.2 ± 0.1
L-broth	10.5	2.4 ± 0.5	1.1 ± 0.1
M9-minimal	5.5	2.8 ± 0.7	1.2 ± 0.1
M9-minimal	7.5	2.6 ± 0.6	1.1 ± 0.1
M9-minimal	9.5	2.0 ± 0.4	1.1 ± 0.1
M9-minimal	13.5	1.5 ± 0.2	1.1 ± 0.1

* The position of these sampling points on the growth curve can be estimated by inspection of Fig. 4(a).

parB was disrupted by means of a frameshift mutation which was predicted to generate a mutant protein identical to ParB for the first 147 aa fused to the short sequence LIRTHPAAGG before terminating prematurely. The remaining segment of ParB should contain neither the predicted dimerization nor the DNA recognition domains (Fig. 1b) and should therefore be non-functional.

Because knocking out one of a pair of genes that work together may have a different effect from knocking out both, we also created a strain containing both the *parA* deletion and *parB* frameshift. This was achieved by simultaneously introducing mutant copies of both *par* genes into *P. putida* using a suicide vector containing a doubly mutated *par* region.

Determination of the phenotypes of *parA* and *parB* mutants

To assess the effect of these *parA* and *parB* mutations, we first compared the wild-type and deletion/frameshift mutant strains after growth to stationary phase in rich medium (L-broth) at 37 °C. Neither the *parA* nor the *parB* mutant strains showed any chromosome loss. However, when we repeated the experiment in M9-minimal medium we observed significant numbers of anucleate bacteria for all the mutants (Fig. 2e–h). The averaged levels of anucleate bacteria from five experiments were as follows: *parAΔ*, 9.9 ± 0.9% and *parBΔ*, 4.9 ± 0.8%. This compares with 0.9 ± 0.4% observed for the wild-type control, which in fact corresponded to only very rare appearance of anucleate bacteria (Fig. 2a–d). The *parAΔ* phenotype was significantly and reproducibly more severe than the *parBΔ* phenotype. The double *parAparB* mutant gave 11.6 ± 3.4% anucleate cells averaged over two experiments, similar to the single *parA* mutant. The point mutations in *parA*, *parA* K16A and *parA* LV138/9RR, gave 10.9 ± 1.5 and 10.8 ± 2.1%, respectively, averaged over two experiments.

To follow up this observation we carried out time course experiments with *parAΔ*, *parBΔ* and wild-type cultures in which the optical density, proportion of anucleate bacteria and bacterial size were monitored

from dilution into fresh medium to stationary phase. The averaged results from two such experiments are shown in Fig. 3. Growth of the *parAΔ* and *parBΔ* strains measured by culture optical density was indistinguishable from the wild-type strain from which they were derived and so the data are shown as a single growth curve in Fig. 3(a) and (b). In L-broth the *par* mutants generated very few anucleate bacteria, with the *parAΔ* mutant possibly giving a slightly elevated level (Fig. 3a). When the experiments were repeated in M9-minimal medium we observed that the proportions of anucleate bacteria in the mutant cultures were not constant, but rose markedly during the transition from exponential to stationary phase (Fig. 3b). During the transition into stationary phase, bacterial size decreased due to continued cell division (Fig. 2a–d), although the bacteria in L-broth never became as small as those in M9-minimal medium (compare Fig. 2b and d). Measurement of the bacteria showed that in exponential phase the cells were similar in size in the two media, whereas in stationary phase the L-broth bacteria were nearly 90% larger by volume, made up almost entirely by differences in length (Table 1). During the transition into stationary phase the number of colony forming units increased for some time after the OD₆₀₀ of the culture stopped increasing (data not shown). During this transition the appearance of anucleate bacteria in M9-minimal medium mirrored the reduction in cell size (Fig. 3c). Note that the DNA loss is essentially at a basal level in M9-minimal medium when the volume corresponds to the mean size of stationary-phase bacteria in L-broth. During prolonged incubation of the *parBΔ* mutant in stationary phase, the total number of anucleate bacteria decreased, as can be seen in Fig. 3(b), possibly suggesting lysis of these bacteria. The size of bacteria in both media also decreased further during prolonged incubation in stationary phase.

Stationary-phase bacteria in L-broth have a higher DNA content than those in M9-minimal medium

A possible explanation for the production of anucleate bacteria by the *parA* and *parB* mutants only in M9-minimal medium is that cell divisions in L-broth stop before the bacteria reach the stage at which active

Fig. 4. Characterization of wild-type and *par* mutants of *P. putida* with respect to apparent DNA content as estimated by total DAPI fluorescence per cell during exponential phase and stationary phase in M9-minimal medium (M9) and L-broth (LB). Approximately 200 cells were analysed for each time point. Cells were grouped by fluorescence intensity intervals and total numbers in each category plotted. (a) Growth curves: ■, *parA*Δ cells (M9); ▲, *parB*Δ cells (M9); ●, wild-type cells (M9); ○, wild-type cells (LB). (b) DAPI fluorescence intensities of wild-type cells (M9): dashed line, 5.5 h; ○, 7.5 h; ●, 9.5 h; thick line, 13.5 h; thin line, 27 h. (c) DAPI fluorescence intensities of wild-type cells (LB): dashed line, 3 h; ●, 6.5 h; thick line, 10.5 h; thin line, 27 h. (d) DAPI fluorescence intensities of *parA*Δ cells (M9): symbols as for (b). (e) DAPI fluorescence intensities of anucleate cells: solid line, *parA*Δ anucleate cells; ○, *parB*Δ anucleate cells. (f) DAPI fluorescence intensities of nucleate/anucleate cell doublets: solid line, nucleate/anucleate cell doublets; dashed line, mono-nucleate single cells. (g) DAPI fluorescence intensities of nucleate/anucleate cell doublets: solid line, nucleate/anucleate cell doublets; dashed line, mono-nucleate single cells.

Table 2. DNA content of *P. putida* PaW1 in late exponential and stationary phases when grown in M9-minimal medium and L-broth.

Growth time of culture (h)*	Medium	Relative DNA content per cell†
5.5 (late exponential/stationary phase)	L-broth	2.0 ± 0.1
7 (stationary phase)	L-broth	1.7 ± 0.1
8 (late exponential/stationary phase)	M9-minimal	1.2 ± 0.4
11 (stationary phase)	M9-minimal	1.0 ± 0.3

* The position of these sampling points on the growth curve can be estimated by inspection of Fig. 4(a).

† Although a DNA standard was used, absolute levels of DNA are difficult to determine. Therefore, DNA content was related to the final sample in M9-minimal medium which was taken as 1 unit per cell.

partitioning is necessary to prevent production of DNA-less bacteria. The most obvious prediction from this hypothesis is that not only should the size of the bacteria in L-broth remain larger than in M9-minimal medium, but also that the DNA content should remain larger. To test this we therefore repeated the time course experiments described above, determining the total fluorescence in the 4',6-diamidino-2-phenylindole (DAPI) emission spectrum for each of 200 cells at each time point across the transition from exponential to stationary phase. This was carried out for wild-type, *parAΔ* and *parBΔ* *P. putida* strains grown in M9-minimal medium and wild-type *P. putida* grown in L-broth (Fig. 4a). The results (Fig. 4b–e) show that for all cultures the total fluorescence per bacterium decreased as the bacteria progressed from the end of exponential phase towards stationary phase, corresponding to the cell divisions referred to above. The background fluorescence not due to DNA, obtained from the fluorescence intensities of anucleate cells, was subtracted from the values of the nucleate cells' fluorescence. The resulting fluorescence profiles show peaks which are approximately multiples of the value of the lowest intensity peak, suggesting that these may represent bacteria with one, two and more chromosomes. Most significantly, while the majority of wild-type bacteria in M9-minimal medium eventually progress to the lowest intensity peak, the bacteria in L-broth accumulate at a stage corresponding to somewhere between one and two times as much fluorescence signal as the smallest bacteria in M9-minimal medium. Although from the dataset shown it appears that the *parAΔ* strain lags behind the wild-type strain in the timing of the 2–1 chromosome reductive division event, this result was not a consistent observation, whereas the other general characteristics of the data were reproducible. The *par* mutant strains differed from the wild-type strain in that the fluorescence intensity peaks were not so clearly defined as in the control strain.

The appearance of anucleate bacteria was also observed using this fluorescence measurement technique, although their appearance was not so well defined in the *parBΔ* culture as in the *parAΔ* culture, perhaps due to the less severe nature of the anucleate cell phenotype. The *parAΔ* and *parBΔ* anucleate cells possess similar fluor-

escence intensities (Fig. 4f), suggesting that the mutations result in a similar degree of mis-partitioning in both strains. When we measured the intensity of bacterial pairs, resulting from recent cell division, where one of the pair was anucleate, the other bacterium of the pair possessed a level of intensity suggesting the presence of nearly two chromosomes worth of DNA (Fig. 4g).

To rule out the possibility that the results presented in Fig. 4(b–e) represent changes in DAPI permeability, we determined bacterial DNA content by a combination of cell counts and total DNA determination by the diphenylamine reaction as described in Methods. The results (Table 2) are consistent with the differences in DAPI fluorescence intensities observed in *P. putida* grown in the two media types and support the proposal that when grown in M9-minimal medium the chromosome copy number of *P. putida* decreases to one per cell during stationary phase. After growth in L-broth to stationary phase the normal complement appears to be somewhere between one and two chromosomes per cell (Table 2).

DISCUSSION

In this paper we describe the characterization of *P. putida* mutants with defects in the *parA* and *parB* genes. No problems were encountered in creating these mutations, implying that these genes are not essential for bacterial growth. This contrasts with the findings in *C. crescentus* where the genes could not be inactivated (Mohl & Gober, 1997). However, it does fit with observations in *B. subtilis* (Ireton *et al.*, 1994; Sharpe & Errington, 1996) and *S. coelicolor* (Kim *et al.*, 2000) in which bacterial growth was largely unaffected by mutations in equivalent genes. In line with this initial observation, we saw no significant increase in appearance of chromosome-less bacteria in the *parA*, *parB* or *parAB* mutants in L-broth. However, we did see a phenotype in M9-minimal medium. Youngren & Austin (1997) previously observed that a P1 partitioning defect was more severe in L-broth than in M9-minimal medium. They suggested that the different salt concentration or osmotic potential of the two media may affect the function of the partitioning mechanism. Such a link could explain the different responses of *P. putida* to entry into stationary phase in the two media, but does

not remove the significance of the observation that the partitioning genes are only important under a subset of growth conditions.

The Par⁻ phenotype observed in M9-minimal medium was unexpectedly severe, with up to 14% of bacteria being anucleate in some cultures of *parA* mutants, when compared to phenotypes previously observed in *B. subtilis*. The differences in the apparent severity of the Par⁻ phenotype arising from the *parA* and *parB* mutations may reflect pleiotropic effects of the mutations, if ParA and ParB affect other aspects of the bacteria. In the RK2 plasmid system we have noted that deletion of the *parA* equivalent has a greater effect on stability than deleting the *parB* equivalent (Williams *et al.*, 1998; Bignell *et al.*, 1999). We have suggested that the explanation for this is that, in the absence of ParA, ParB can pair the centromere-like sequences, thus actively interfering with the normal partitioning process (Williams *et al.*, 1998; Bignell *et al.*, 1999; Jensen & Gerdes, 1997; Bignell & Thomas, 2001). However, the double mutant strain shows a rate of anucleate cell production more characteristic of *parA* mutants. This indicates that the higher rate of chromosome loss in the *parA* mutant is not simply due to retention of active ParB.

The fact that we do not see anucleate cells in diluted cultures brought back into exponential phase is presumably due to outgrowth by the normal bacteria and possible lysis of the anucleate bacteria. Indeed the proportion of cells that were anucleate in the *parBA* strain decreased during prolonged incubation in stationary phase. Given that the total number of bacteria did not increase during this time suggests the disappearance of these forms. The differences in the apparent severity of the Par⁻ phenotype arising from the *parA* and *parB* mutations may reflect differences in the stability of these forms as well as the rate at which such forms appear.

B. subtilis spo0J mutants exhibit a weak Par phenotype during vegetative, exponential growth (Ireton *et al.*, 1994). A very weak Par phenotype is also seen in *P. putida* for both *parA* and *parB* mutants (Fig. 3). However, the *par* mutations appear to have no effect on exponential growth rate and expression of the Par⁻ phenotype is not as severe as it is in later stages of the growth cycle. Thus the importance of the *par* genes during the transition from exponential to stationary phase in *P. putida*, at least in M9-minimal medium, parallels the importance of the equivalent genes during sporulation in *B. subtilis* (Ireton *et al.*, 1994; Sharpe & Errington, 1996) and *S. coelicolor* (Kim *et al.*, 2000). In *B. subtilis*, the results to date have been interpreted to mean that Soj and Spo0J are needed for chromosome tethering during segregation into the prespore (Sharpe & Errington, 1996). In *S. coelicolor* they are needed to ensure that chromosomes are regularly spaced relative to the septa laid down during differentiation of aerial hyphae into spores (Kim *et al.*, 2000).

The question is therefore, what is the critical process that the *par* genes are essential for? A cartoon of the

divisions occurring during progress to stationary phase is shown in Fig. 5. Fitting our observations to this scheme would have the bacteria grown in M9-minimal medium reaching the stage of a single non-replicating chromosome per cell (Fig. 5c), whereas the L-broth-grown bacteria would have reached the stage of a partially replicated chromosome being present in each cell (Fig. 5b). Why should this make a difference in the observed Par⁻ phenotype?

First, it may be simply that the division of a bacterium that is preparing for stationary phase and with two unit chromosomes is inherently more likely to generate chromosome-less bacteria than division of a cell with two partially replicated chromosomes, due to the shape, bulk or activity of the chromosomes. The supercoiling density of the chromosome may change the volume occupied and the level of gene expression may influence the association with RNA, translation machinery and membranes (for secreted proteins), influencing the chance of chromosome loss (Drlica, 1992; Dorman, 1995; Norris & Fishov, 2001).

Second, it may be that the key difference between cells in rich and minimal medium is whether or not, at the final division before entering stationary phase, the chromosomes are still replicating, i.e. whether they are attached to the replication apparatus. The basis for this hypothesis is that DNA replication appears to take place at membrane-associated replication factories located at mid-cell or quarter/three-quarter positions in both *B. subtilis* (Lemon & Grossman, 1998, 2000) and *E. coli* (Hiraga *et al.*, 1998, 2000; Brendler *et al.*, 2000). Recent work (reviewed by Lewis, 2001) suggests that the process of replication feeds the daughter molecules out to either side of the replication plane, mediated by proteins such as Smc (Britton *et al.*, 1998) and SeqA (Brendler *et al.*, 2000). It has been suggested that the cycle of replication from initiation to termination and reinitiation is more akin to sister chromatid separation during replication than mitosis (Sawitzke & Austin, 2001). The ability of replicating chromosomes to drive the physical process of separation in the absence of a functional partitioning apparatus, perhaps as a simple consequence of their bulk, is also suggested by the observations of Lin & Grossman (1998). They observed that while *soj/spo0J* mutations have a major effect on stability of low-copy-number plasmids whose segregational stability in *B. subtilis* is dependent on the presence in the plasmid of the putative centromere-like sequence from the chromosome, these mutations have little effect on chromosomal partitioning in *B. subtilis*. Thus reinitiation of replication prior to cell division may then tether the replicating chromosomes symmetrically on either side of the division plane. Only if this tethering is not in place, as in cells where replication has ceased in preparation for stationary phase, might it be essential to have a partitioning apparatus to ensure that the chromosomes remain on the correct sides of the septum. Therefore, we suggest that the Par apparatus is redundant in exponentially growing bacteria with overlapping cell cycles (Bignell, 1999).

Fig. 5. Schematic diagram illustrating reductive cell division. Rectangles represent bacteria, black squiggles represent chromosomes; replication complexes are represented by grey ovals and the partitioning complex by white circles; chromosome separation due to DNA replication is indicated by grey arrows and that due to the partitioning complex by black arrows. (a) represents a bacterium during late exponential phase replicating for the final time its chromosomes, which are tethered when division occurs. (b) represents cells in early stationary phase when replication is no longer occurring. We suggest cells grown in L-broth remain in this state because for some reason they do not complete the last cell cycle. (c) represents cells produced as a result of a final reductive division stage only occurring in M9-minimal medium where the chromosomes are not tethered and there is an enhanced probability of mis-partitioning in the absence of the Par proteins.

The above hypothesis makes some testable predictions. For example, it predicts that at very slow growth rates, if reinitiation of replication does not take place before division, then we should also see a Par phenotype. Also, if we can block DNA replication, by for example interfering with DnaA function, we would expect to see the Par phenotype exhibited during the run out to non-growing bacteria. Therefore, further observation of the growth conditions under which we observe chromosome loss from these mutants as well analysis of the location of the *oriC* region in wild-type and *par* mutants of *P. putida* during the transition to stationary phase will allow us to test these hypotheses. These hypotheses focus attention critically on attachment of the nucleoid to the cell membrane and the relationship between initiation of replication for the next cell cycle and completion of division for the last cell cycle. Both these areas are aspects of microbial cell biology that deserve further attention.

ACKNOWLEDGEMENTS

This work was supported by a BBSRC Committee Studentship (for C. R. B.) and a project grant from the BBSRC (6/G10277). Oligonucleotides and DNA sequencing were carried out by Alta Bioscience or in the Genomics Laboratory funded by BBSRC JIF Grant 6/JIF13209. We thank Grazyna Jagura-Burdzy for useful discussions during the course of this work.

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Received 22 June 2001; revised 21 September 2001; accepted 29 October 2001.