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ParABS Systems of the Four Replicons of *Burkholderia cenocepacia*: New Chromosome Centromeres Confer Partition Specificity†

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Most bacterial chromosomes carry an analogue of the *parABS* systems that govern plasmid partition, but their role in chromosome partition is ambiguous. *parABS* systems might be particularly important for orderly segregation of multipartite genomes, where their role may thus be easier to evaluate. We have characterized *parABS* systems in *Burkholderia cenocepacia*, whose genome comprises three chromosomes and one low-copy-number plasmid. A single *parAB* locus and a set of ParB-binding (*parS*) centromere sites are located near the origin of each replicon. ParA and ParB of the longest chromosome are phylogenetically similar to analogues in other multichromosome and monochromosome bacteria but are distinct from those of smaller chromosomes. The latter form subgroups that correspond to the taxa of their hosts, indicating evolution from plasmids. The *parS* sites on the smaller chromosomes and the plasmid are similar to the "universal" *parS* of the main chromosome but with a sequence specific to their replicon. In an *Escherichia coli* plasmid stabilization test, each *parAB* exhibits partition activity only with the *parS* of its own replicon. Hence, *parABS* function is based on the independent partition of individual chromosomes rather than on a single communal system or network of interacting systems. Stabilization by the smaller chromosome and plasmid systems was enhanced by mutation of *parS* sites and a promoter internal to their *parAB* operons, suggesting autoregulatory mechanisms. The small chromosome ParBs were found to silence transcription, a property relevant to autoregulation.

Like all organisms, bacteria must actively segregate their chromosomes before cell division if new cells are to be viable. How they do this is still an open question. The early finding (42) that the replication origin regions of *Bacillus subtilis* and *Pseudomonas putida* contain homologues of the *parAB* loci responsible for active partition of low-copy-number plasmids had suggested that the answer would be straightforward. In plasmids, ParB protein binds to a specific, centromere-like site, *parS*, and the complexes thus formed are presumed to interact to pair sibling plasmid copies: ParA protein, an ATPase, splits the pair and helps drive the plasmid molecules towards each pole (15). It appeared that this process, together with factors needed to facilitate the movement of the much bulkier chromosome such as dispersion of *parS* sites (18, 34) and the DNA-condensing properties of SMC proteins (14, 41), would suffice to explain chromosome partition.

Such has not proved to be the case, at least for the bacteria studied so far. The partition phenotype of chromosomal *parAB* mutants is often weak or conditional. In *B. subtilis*, *parB* (*spo0J*) mutants generate anucleate cells (17), but *parA* (*soj*) mutations do not (50), and positioning of origins is little affected by the deletion of *spo0J* (28). In *P. putida*, *parAB* mutations do generate anucleate cells, but this is restricted largely to cultures undergoing slow or decelerating growth (10, 33). Furthermore, the role of *parAB* homologues in partition may be difficult to analyze, owing to their participation in other activities, such as the regulation of the sporulation cascade in *B. subtilis* (39, 43) or of cell division in *Caulobacter crescentus* (38). It is also notable that certain species, e.g., *Escherichia coli* and *Haemophilus influenzae*, lack *parAB* homologues altogether, implying that bacteria elaborate other types of partition system. Indeed, there have been several proposals and some evidence for other functions playing a major part in chromosome segregation. The motive force could be generated by replication itself (29), by the successive insertion of cotranslated gene products into the membrane ("transertion") (51), or by release from sibling chromosome "cohesion" (3, 48). Active navigation could be provided by factors acting on a non-*parS*, origin-linked centromere (12, 21) or by actin-like MreB proteins (9, 20, 25). The only direct evidence that chromosomal *parAB* loci act in partition is their ability to stabilize unstable vectors carrying cognate *parS* sites (2, 10, 34, 52).

These findings are all based on studies of mitosis in single-chromosome species. We asked whether the need to coordinate the movement and positioning of two or more bulky nucleoids could demand a degree of precision that only *parABS* systems can provide. The study of partition in such bacteria might give a clearer view of the role of these systems. Accordingly, we have investigated the partition function of *parABS* determinants of *Burkholderia cenocepacia*. *Burkholderia* species are β-proteobacterial rods notable for their versatility of metabolism and habitat and for the multipartite nature of their genomes. Like many *Burkholderia* species, the *B. cenocepacia* isolate we have studied is a pathogen associated with cystic fibrosis. Its genome comprises three chromosomes (defined as carrying rRNA genes) and a low-copy-number plasmid.

The multichromosome state raises a potential problem. The chromosomal *parS* sites described so far are remarkably uni-

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† Supplemental material for this article may be found at http://jb.asm.org/.

TABLE 1. Plasmids

Plasmid	Relevant characteristics	Source or reference(s)
pDAG203	Mini-F Δ(*sopOPABC*) *cat*$^+$ (6.67 kb)	30
pDAG123	pRS551 (*rep*$_{pMB1}$ *bla*$^+$ Kan^r *lacZYA*) carrying p*ldc* (12.6 kb)	31, 47
pAM238	*rep*$_{pSC101}$ *aadA*; polylinker of pUC18 (4.3 kb)	8
pBBR1MCS5	*rep*$_{pBBR1}$ *mob*$_{RK2}$ Gen^r (4.77 kb)	24
pDAG551	pDAG203 with single c1 *parS* between AflIII-SexAI	This work
pDAG552	pDAG203 with single c2 *parS* between AflIII-Bsu36I	This work
pDAG553	pDAG203 with single c3 *parS* between AflIII-Bsu36I	This work
pDAG554	pDAG203 with single p1 *parS* between AflIII-Bsu36I	This work
pDAG555	pDAG203 with c2 *parS* cluster between PmlI-Bsu36I	This work
pDAG556	pDAG203 with c3 *parS* cluster between PmlI-Bsu36I	This work
pDAG557	pDAG203 with p1 *parS* cluster between PmlI-Bsu36I	This work
pDAG558	pAM238 with c1 *parAB* fragment (including 91 bp upstream of *parA*) between XbaI-SphI	This work
pDAG559	pAM238 with c2 *parAB* fragment (including 81 bp upstream of *parA*) between XbaI-HindIII	This work
pDAG560	pAM238 with c3 *parAB* fragment (including 141 bp upstream of *parA*) between XbaI-SphI	This work
pDAG561	pAM238 with p1 *parAB* fragment (including 126 bp upstream of *parA*) between XbaI-SphI	This work
pDAG562	pBBR1MCS5 with c1 *parAB* fragment (including 256 bp upstream of *parA*) between SpeI-HindIII	This work
pDAG563	pBBR1MCS5 with c2 *parAB* fragment (including 81 bp upstream of *parA*) between XbaI-BamHI	This work
pDAG564	pBBR1MCS5 with c3 *parAB* fragment (including 267 bp upstream of *parA*) between XbaI-BamHI	This work
pDAG565	pBBR1MCS5 with p1 *parAB* fragment (including 126 bp upstream of *parA*) between XbaI-BamHI	This work
pDAG566	pDAG563 with G→A in central CG of internal *parS**	This work
pDAG567	pDAG565 with C→T in central CG of internal *parS*	This work
pDAG568	pDAG564 with T→A mutation of promoter −10 (TAAAAT[a]) in c3 *parA*	This work
pDAG571	pDAG123 with single c1 *parS* at EcoRI upstream of p*ldc*::*lacZYA*	This work
pDAG572	pDAG123 with single c2 *parS* at EcoRI upstream of p*ldc*::*lacZYA*	This work
pDAG573	pDAG123 with single c3 *parS* at EcoRI upstream of p*ldc*::*lacZYA*	This work
pDAG574	pDAG123 with single p1 *parS* at EcoRI upstream of p*ldc*::*lacZYA*	This work

[a] The mutated base is underlined.

form, with most being minor variants of a canonical inverted repeat sequence, 5′-TGTTNCACGTGAAACA, present in the genomes of representatives of nearly all major bacterial divisions. Maintenance of this uniformity in a multipartite genome might require a mechanism to prevent ParAB from confusing and missegregating chromosomes. The three chromosomes of *B. cenocepacia* could either carry the same *parS* site and be partitioned, with the help of accessory anti-incompatibility functions, by a single, "master" ParAB protein duo or each have a distinct *parS* partitioned independently by its own ParAB. Alternatively, the *parS* sites might allow a degree of cross-reaction to help coordinate partition or be absent from one or more chromosomes that are segregated by a different mechanism. In this paper, we analyze *parABS* systems of *B. cenocepacia* and assess their partition activity in an attempt to determine which of the above strategies *B. cenocepacia* could have adopted to manage mitosis of its genome.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. cenocepacia* isolate used was J2315, gemnovar III, of the ET12 lineage, which serves as the United Kingdom cystic fibrosis reference strain; it was purchased from the Belgian Coordinated Collection of Microorganisms (LMG16656). Its genome was sequenced by the Sanger Centre (ftp://ftp.sanger.ac.uk/pub/pathogens/bc/). *Escherichia coli* K-12 strain DH10B (13) was used as the host for transformation and stabilization tests, and strain MC1061 (4) was used as the host for silencing measurements. Plasmids are listed in Table 1, and maps of plasmid vectors are shown in Fig S1 (see the supplemental material). The vectors for expression of *parAB* were pBBR1mcs5 (high copy number) and pAM238 (pSC101 based). *parS* sites were tested for centromere function following insertion into pDAG203, a mini-F with a deletion of the entire *sop* locus, and for silencing by insertion upstream of the *ldc* promoter in pDAG123 (see below), followed by recombinational transfer to λRS88 (47) and lysogenization of MC1061.

Growth conditions. Bacteria were routinely grown at 37°C in Luria-Bertani medium and, for plasmid stabilization tests, in M9-CSA (M9 salts, 0.4% glucose, 0.2% Casamino Acids). The following antibiotics (in micrograms per milliliter) were added as appropriate: chloramphenicol (Cm), 20; gentamicin, 2.5; spectinomycin, 20; kanamycin, 50.

Plasmid construction. Single *parS* sites were synthesized as complementary 16-mers (Table 2) tailed for insertion into mini-F pDAG203 as described in Table 1. *parS* clusters of c2, c3, and p1 were amplified from J2315 DNA by PCR using oligonucleotide primers (see Table S1 in the supplemental material) and DNA polymerase *Pfu* (Stratagene) or Phusion (Finnzyme) as 1,041-, 1,350- and 469-bp fragments and inserted into pDAG203 (Table 1). *parAB* genes were likewise amplified from J2315 DNA using oligonucleotides (see Table S2 in the supplemental material) designed for insertion downstream of the *lac* promoter in pBBR1mcs5 and pAM238.

parS sites were placed near promoters for silencing assays by amplification from mini-F derivatives (pDAG551, pDAG552, pDAG553, and pDAG554) using flanking sequence primers (5′-CGCAATTGAAACTGGATGGCTTTC TTGC and 5′-CGCAATTGGGATGAATGGCAGAAATTCG) (MfeI sites are underlined) to generate fragments of 1,050 bp (c1) and 708 bp (c2, c3, and p1). After cleavage with MfeI, the fragments were inserted at the EcoRI site upstream of the p*ldc*::*lacZ* fusion in pDAG123, and plasmid products with identically oriented inserts were selected to give the series pDAG571, pDAG572, pDAG573, and pDAG574 (*parS* of c1, c2, c3, and p1, respectively).

Mutagenesis of motifs internal to *parAB*. The internal c3 *parA* promoter −10, the c2 *parS**, and the p1 *parS* were amplified by PCR using one oligonucleotide with the desired mutation (in boldface type below) and another with a site convenient for substituting the product for the wild-type sequence. For c3, K3PR (5′-AAAATTTCTTCACGCATTTTTGTTTTTCCATTGAC) and K3SM (5′-G CAGGATCCCGACCACAACGATCAACAAA) produced a 754-bp fragment that was substituted for the pDAG564 BamHI-DraI fragment to yield pDAG568. For c2, K2SMA (5′-ACATAAAGTCGGGCAGCTG) (the mutant *parS** half-site is underlined) and FOP41 (see Table S2 in the supplemental material) produced a 759-bp fragment that was substituted for the pDAG563 FspAI-BamHI fragment to yield pDAG566. For p1, PLSM (5′-GAACTTCATATGTC TGCCCAATTTCGTACTTCGTGGCACCTTGGCTTGAGCCAACCT) and FOP37 (see Table S2 in the supplemental material) produced a 994-bp fragment

TABLE 2. *parS* oligonucleotides

Replicon	Oligonucleotide name (FOP no.)	Sequence[a] (5′→3′ top, 3′←5′ bottom)	Restriction site(s) for:	
			Insertion	Screening[b]
c1	11	cgtgTGTTTCACGTGAAACA	*Afl*III-	*Pml*I
	12	ACAAAGTGCACTTTGTggacc	-*Sex*AI	
c2	22	cgtgcccGTTTATGCGCATAAACccc	*Afl*III-	*Fsp*AI
	23	gggCAAATACGCGTATTTGgggagt	-*Bsu*36I	
c3	24	cgtgcccGTTGTCACGTGACAACccc	*Afl*III-	*Pml*I
	25	gggCAACAGTGCACTGTTGgggagt	-*Bsu*36I	
p1	26	cgtggggCTTGGCTCGAGCCAAGggg	*Afl*III-	*Xho*I
	27	cccGAACCGAGCTCGGTTCcccagt	-*Bsu*36I	

[a] Capital letters show *parS* sequence; lowercase letters show cohesive ends.
[b] Sites present in the *parS* palindrome used to screen for insertion.

that was substituted for the BamHI-NdeI fragment of pDAG565 to yield pDAG567. The mutations were confirmed by sequencing.

DNA procedures. *B. cenocepacia* DNA was purified from strain J2315 as described previously for *P. putida* (10), except that digestion of the lysate with sodium dodecyl sulfate and protease K at 50°C was prolonged to 15 h and phenol extraction of the digest was repeated four times. Plasmids and DNA fragments were purified using QIAGEN kits. In vitro DNA methodology and electrotransformation were performed according to standard procedures.

Plasmid stabilization test. Cultures of DH10B derivatives carrying a ParAB producer plasmid and a *parS*-bearing mini-F were started by 400-fold dilution from overnight cultures into M9-CSA with gentamicin and Cm (selective for pBBR1mcs5 and mini-F, respectively) and incubated at 37°C for five generations to an optical density at 600 nm of ~0.2. Cells were then diluted in M9-CSA without Cm to allow growth of mini-F-free segregants, maintained in the exponential growth phase for 25 to 30 generations by sequential dilution, and assayed at intervals for maintenance of mini-F (Cm resistance) as follows. Cells were spread onto an initial layer of L agar, covered with a second layer, and incubated at 37°C overnight. The colonies were counted and then overlaid with a third layer, this one containing Cm, and again incubated overnight. Cm^r colonies continued to grow and were counted as large colonies the next day. The number of generations was calculated from optical density measurements.

Silencing assay. Cells of MC1061 derivatives lysogenic for λRS88 *parS*-P*ldc*::*lacZYA* were diluted from cultures grown overnight and were grown exponentially in LB plus kanamycin to an optical density at 600 nm of ~0.25. Cultures were sampled and assayed for β-galactosidase as previously described (27).

RESULTS

Origin-proximal location of *parAB* loci. The *B. cenocepacia* J2315 genome comprises chromosomes of 3.87, 3.22, and 0.88 Mbp (c1, c2, and c3, respectively) and a 92.7-kbp plasmid (p1). It has been sequenced by the Sanger Centre. Using the BlastP program (46) with Soj and other ParA sequences as probes, we found several *parA* homologues but only four that are situated immediately upstream of open reading frames for ParB-like proteins, one in each replicon (Fig. 1). These *parAB* loci are close to the cumulative GC-skew minima in c1, c2, and c3, consistent with proximity to origins of bidirectionally replicated chromosomes (35). The GC skew in p1 changes direction only locally, possibly indicating overall unidirectional replication of the plasmid; *parAB* is situated at the most pronounced local minimum. The likelihood that the *parAB* loci do indeed lie in the origin regions is increased by their proximity to DnaA boxes; c1 and c2 have additional boxes (not shown) close to their GC-skew minima.

The environment of the *parAB* loci reveals two types of replication origin domains (Fig. 1). In c1, it includes the only *gidAB*, *dnaA*, *rpmH*, *rnpA*, and *gyrB* genes in the genome, all characteristic of chromosomal origins; *gidAB* and *parAB* actually appear to belong to the same operon. In c2, c3, and p1, on the other hand, *parAB* is adjacent to elements typical of low-copy-number plasmids: a gene (*repA*/*trfA*) for a plasmid-like replication control/initiator protein and a cluster of directly repeated sequences similar to iterons (not shown). Thus, although the size, rRNA gene content, and GC skew qualify c2 and c3 as chromosomes, the replication control regions in which their *parAB* loci are embedded are typical of low-copy-number plasmids.

Diversity of *parAB* loci. The ParA homologues of J2315, including that of the plasmid, resemble those of other bacterial chromosomes in sharing the A, A′, and B components of Walker box ATPase active sites and in lacking the extended N-terminal domain that regulates *par* operon transcription in many low-copy-number plasmids. However, alignment of their peptide sequences (Fig. 2A) showed that only the c1 ParA is closely related to known chromosomal ParAs. Those of c2, c3, and p1 differ chiefly in that they lack two peptide stretches that flank a predicted (46) helical region (centered on position 80 in

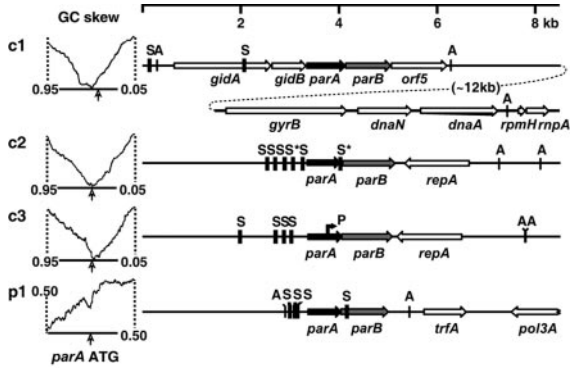

FIG. 1. Location of *parAB* loci and *parS* sites. At left, arrows indicate the position of each *parA* homologue start codon relative to the GC skew of the 10th (0.95 to 0.05) of the replicon centered on the GC-skew minimum (c1 to c3) or of the whole replicon (p1). Genetic map sketches are aligned at *parA* homologue start codons and show only genes characteristic of chromosome origin regions or relevant to plasmid maintenance. orf5 is a gene of unknown function, named for its position in the *gid-par* operon. *repA* (c2 and c3) signifies generic resemblance to plasmid replication control genes; *trfA* indicates homology with the corresponding RK2 gene. S, *parS* sites (c1) or *parS*-like palindromes (c2, c3, and p1); S*, degenerate *parS*-like sequences; P, predicted promoter internal to *parA* of c3; A, predicted DnaA box motifs based on the consensus 5′-TTATCCAC.

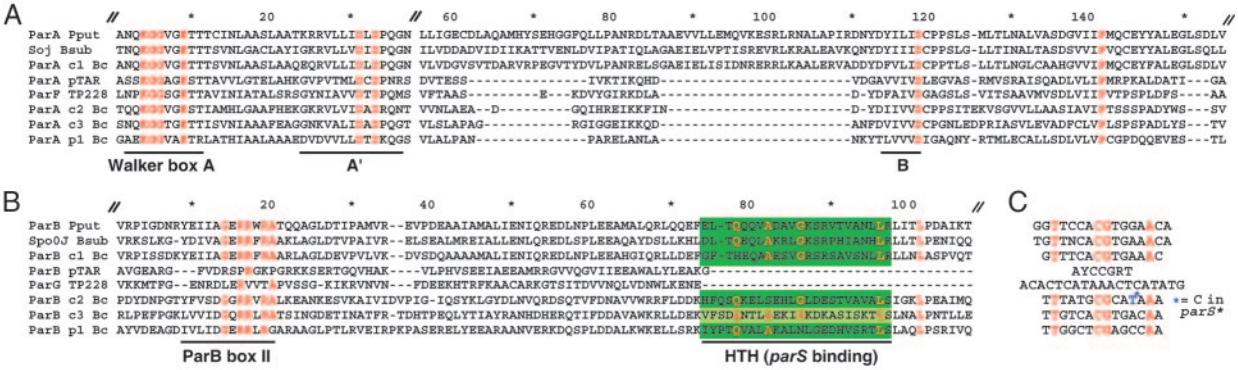

FIG. 2. Parts of the aligned ParA (A) and ParB (B) amino acid sequences that distinguish subgroups. Residues that are identical (A) or nearly so (B) in all examples shown are in red. Numbers above show distances, not coordinates, and double hatch marks denote sequences not shown (18 residues just after the A box in A). The Walker A (nucleotide binding), A′ (catalytic), and B (Mg binding) motifs constitute the ATPase active site. ParBII is a motif identified previously (52). The helix-turn-helix (HTH) motifs are shaded green. The HTH of c3 is weakly predicted relative to the others. Note the short A′-B-box interval in the c2, c3, and p1 ParAs, very similar to the pTAR and TP228 homologues, and the absence of all HTH region homology in the latter ParBs. (C) Aligned *parS* and *parS*-like sequences with fully conserved bases in red. Note the dissimilarity of the pTAR and TP228 *par* region repeats.

Fig. 2A), which in c1 ParA is predicted to be relatively unstructured, indicating a taxonomic, and possibly functional, distinction. The ParAs of c2, c3, and p1 show notable similarity to ParA of the plasmid pTAR (23) and ParF of TP228 (1).

The *B. cenocepacia* ParB-like sequences (Fig. 2B) are more divergent than the ParAs but contain the motifs typical of the ParB group (52). The relationship with the pTAR and TP228 homologues shown by the ParAs is not maintained by the ParBs, particularly with respect to the DNA-binding motif, which is predicted to be a helix-turn-helix in the *B. cenocepacia* homologues but in TP228 is of a different type, termed ribbon-helix-helix, located at the C terminus (11). More general relationships within the ParB group came to light upon an examination of the phylogeny of ParAs and ParBs in bacteria known to have multipartite genomes (Fig. 3). This analysis showed first that the phylogeny of ParB proteins broadly follows that of their ParA partners, presumably reflecting their coevolution. Second, and more strikingly, it shows that the differentiation of the largest chromosome, c1, from the others is characteristic of all multipartite genomes: the ParABs of these chromosomes

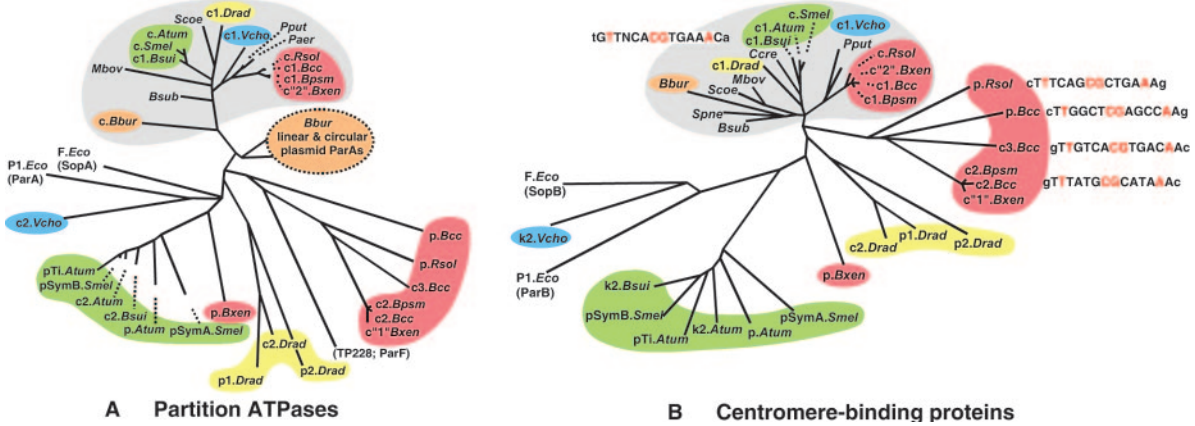

FIG. 3. Phylogeny of partition proteins of bacteria with multipartite genomes. Predicted amino acid sequences of ParA homologues (A) and ParB homologues (B) were aligned and phylogenetically compared using ClustalX. Large blocks of heterology, such as N-terminal extensions present in some ParAs but absent from others, were discarded from the analysis. The option "tree-correct for multiple substitutions" was used. Phylogenetic trees were viewed using Treeview. Homologues of individual multipartite genomes or closely related groups are indicated in color, and others are single-chromosome species included as markers. All branches are of the correct length, but some are extended by dotted lines for clarity. Abbreviations for full species names are as follows: *Atum*, *Agrobacterium tumefaciens*; *Bbur*, *Borrelia burgdorferi*; *Bcc*, *Burkholderia cenocepacia*; *Bpsm*, *Burkholderia pseudomallei*; *Bsub*, *Bacillus subtilis*; *Bsui*, *Brucella suis*; *Bxen*, *Burkholderia xenovorans*; *Ccre*, *Caulobacter crescentus*; *Drad*, *Deinococcus radiodurans*; *Eco*, *Escherichia coli*; *Mbov*, *Mycobacterium bovis*; *Paer*, *Pseudomonas aeruginosa*; *Pput*, *Pseudomonas putida*; *Rsol*, *Ralstonia solenacearum*; *Scoe*, *Streptomyces coelicolor*; *Smel*, *Sinorhizobium meliloti*; *Spne*, *Streptococcus pneumoniae*; *Vcho*, *Vibrio cholerae*. The group comprising the largest chromosome of every species, denoted as c or c1, forms a distinct cluster, as shown by the shaded area. Secondary replicons are shown as c2 and c3 or in the case of megaplasmids by their given names or p1 and p2. Large and small replicons of *B. xenovorans* were named chromosomes 2 and 1, respectively (Joint Genome Institute) and are thus shown in quote marks here. The *Borrelia burgdorferi* secondary replicon ParBs are less well defined than the ParA group and are not shown. In B, the canonical *parS* sequence is shown next to the large chromosome group and the *parS*-like palindromes of *B. cenocepacia* and *R. solanacearum* are shown beside their replicons, with conserved bases in red.

are more similar to each other and to those of monochromosomal bacteria than to their smaller chromosome or megaplasmid cohabitants. In contrast, the secondary replicon ParABs of each species and its close relatives tend strongly to form distinct clusters that correspond to host phyla (Fig. 3), indicating host selection at some level for compatibility or performance of these ParA-ParB pairs.

Replicon specificity of candidate *parS* sequences. To identify *parS* sites, we first searched the genome for examples of the "universal" centromere sequence (34). Only two were found, both upstream of the *parAB* locus in c1 (Fig. 1). However, a search of the *parAB* regions for inverted repeat sequences of any kind revealed novel but related palindromes in c2, c3, and p1 (Fig. 2B and 3C). These putative *parS* sites share with the "universal" *parS* an inverted repeat of 7 bp with T and C at positions 2 and 7, respectively. Among themselves, they share a singular arrangement distinct from the c1 *parS* sites: on all three replicons, the candidate *parS* sites are clustered at similar positions with respect to their *parAB* loci (Fig. 1), and the sequences of the sites on a given replicon are identical and specific to that replicon (Fig. 2). The only exceptions are on c2, whose putative *parS* cluster and *parA-parB* intergenic region each include a single imperfect *parS*-like palindrome (Fig. 1 [denoted S*] and 2C), and p1, which carries an additional site in its *parB* N terminus.

The location of the *parS*-like sites strongly suggested that they are linked functionally to the putative *parAB* loci.

Partition activity of *parABS* systems. To assess partition function, we examined the ability of *parAB* expressed from p*lac* on a multicopy vector to stabilize mini-F plasmids carrying single *parS* or *parS*-like sites in *E. coli*. This foreign-host assay has been used previously to demonstrate the stabilization capacity of the *B. subtilis soj-spo0J-parS* and the *P. putida parABS* systems (10, 52). As an initial approach, it is preferable to one employing *B. cenocepacia* itself in that it avoids the complications of incompatibility with the resident replicons and of possible pleiotropy of *parAB* mutants (which we have not yet isolated). Figure 4A to D shows the effects of producing ParA and ParB proteins without IPTG (isopropyl-β-D-thiogalactopyranoside) induction on the loss of mini-F-*parS* plasmids during ~25 generations of exponential growth. In each case, mini-F-*parS* responded only to its own *parAB* module, with noncognate *parS* plasmids being lost at rates not significantly different from that of pDAG203, the original mini-F vector (~9% per generation). The *parABS* systems are thus specific.

The nature of the stability change varied, however: *parAB* of c1 conferred complete stability on mini-F carrying c1 *parS*, *parAB* of c2 and p1 increased stability of their mini-Fs only modestly, and *parAB* of c3 strongly destabilized the mini-F carrying its *parS*-like site. Suspecting that this destabilization resulted from an oversupply of the Par proteins, we repeated the tests using an expression vector with a ~4-fold-lower copy number (Fig. 4E, shaded bars). Reducing the c3 *parAB* gene dosage eliminated destabilization and actually improved the stability of its mini-F-*parS* about threefold relative to that of pDAG203 (Fig 4E, bar 10). Thus, all the *parAB* loci can increase the stability of their cognate *parS* plasmids. The palindromes found in c2, c3, and p1 can be considered true *parS* sites, and their *parABS* ensembles can be considered genuine partition systems.

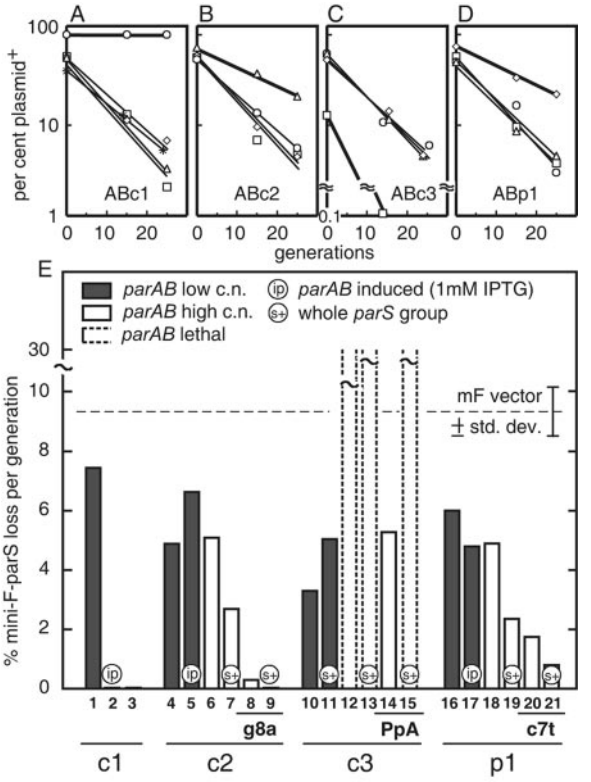

FIG. 4. Partition activity of *B. cenocepacia parABS* elements in *E. coli*. Shown is the effect of pBBRmcs5 carrying *parAB* of (A) c1, (B) c2, (C) c3, or (D) p1 on the loss rates of mini-F vector pDAG203 carrying a single *parS*-like unit of c1 (○), c2 (△), c3 (□), p1 (◇), or none (* [shown only in A]). Slight differences in slope, e.g., *parS* of c2 and p1 in A, are not significant. The *y* axis in C is broken to accommodate the accelerated loss of the c3 *parS* plasmid caused by its own ParAB. (E) Effects of modifying *parAB* and *parS* elements on mini-F stabilization. Empty and shaded bars show loss rates for p*lac-parAB* at high (pBBRmcs5) and low (pAM238) copy numbers (c.n.), respectively; dotted-line bars indicate that growth with selection for the mini-F was so perturbed and variable that loss rates could not be reproducibly measured; the horizontal dotted line shows the spontaneous loss rate of the mini-F vector; below the bar numbers, mutations in *parS** (g8a), *parB* promoter in *parA* (PpA), and *parS* (c7t) are shown. Variability of loss rate was ≤18% (standard deviation). Cognate ParAB-*parS* interactions shown by thicker lines in A to D are represented by columns 3, 6, 12, and 18, respectively.

The other replicons showed various responses to lowered ParAB levels. Stability of the c1 mini-F-*parS* (Fig. 4E, bar 3) was almost completely lost at a low *parAB* copy number (bar1) but was restored upon induction of *parAB* expression with IPTG (bar 2), presumably because the *B. cenocepacia* sequence upstream of *parA* in the ParAB producer plasmid does not contain an *E. coli* promoter. In contrast, the modest stabilizing effect of the c2 and p1 proteins (bars 6 and 18) was essentially unaffected by the induction of their *parAB*s or the reduction their copy number (bars 4 and 5 and 16 and 17, respectively), reflecting the presence of promoters in the upstream *B. cenocepacia* sequences; some factor other than *parAB* copy number or ParAB concentration appears to limit stabilization in these cases.

Inefficiency of a single *parS* site relative to its cluster was one

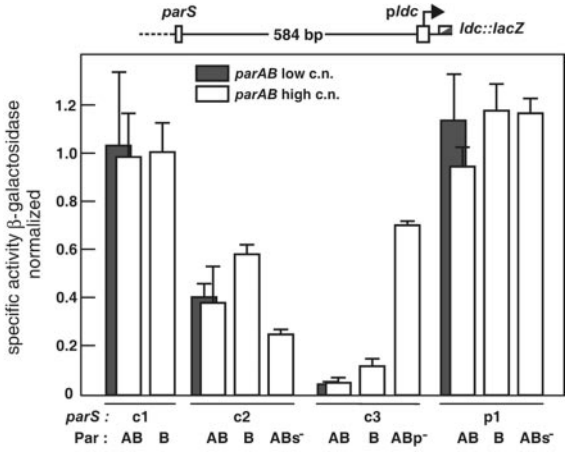

FIG. 5. *parABS*-mediated silencing. Exponential-phase cultures of strains carrying *parAB* on a plasmid and a *parS*-p*ldc*::*lacZ* module in an integrated λ prophage were assayed for β-galactosidase, and specific activities were normalized to those of the same strains without *parAB* (~250 Miller units). Error bars are standard deviations. ABs$^-$ and ABp$^-$ carry the mutations shown in Fig. 4.

such factor. Repeating the tests with mini-F plasmids carrying entire *parS* clusters resulted in a twofold reduction in the loss rate for c2 and p1 (Fig. 4E, bars 6 and 7 and 18 and 19, respectively). Again, the c3 system was different; its *parS* cluster aggravated destabilization (bars 11 and 13). Functional motifs internal to the *parAB* operons, the *parS*-like sites near the 5′ end of c2 and p1 *parB* and a predicted promoter in the c3 *parA* gene (Fig. 1), also appeared to be potential influences on stabilization capacity. Silent mutations in the conserved central positions of the extra *parS* sites improved stabilization by the resulting *parAB* loci (Fig. 4E, bars 8 and 20) and improved it still further when coupled with mini-Fs carrying full *parS* clusters (bars 9 and 21), providing essentially complete stability in the case of c2. The mutated c2 *parAB* continued to stabilize after reinsertion of the normal *parS** at an ectopic site (data not shown), indicating that centromere-based incompatibility had not been responsible for the limited stabilization. Mutation of the promoter in c3 *parA* abolished the destabilization of the single *parS* mini-F (bar 14) but was insufficient to stabilize the *parS* cluster plasmid (bar 15). No effect on noncognate *parS* plasmid stability was observed in tests with the more efficient mutant *parAB* loci (data not shown).

The beneficial effects of these mutations on partition activity suggested that the motifs in question might normally be used to regulate partition through the modulation of *parAB* expression. One way in which they might do this is through ParB-mediated silencing: partition complexes of several plasmids are known to be able to further recruit molecules of their centromere-binding protein and so spread along neighboring DNA, silencing it (53).

Silencing by the Par proteins. To assess the silencing capacity of the *B. cenocepacia* Par proteins, we inserted each of the *parS* sites upstream of an *E. coli ldc* promoter-*lacZ* fusion integrated at *att*λ and measured the effect of ParB, with and without ParA, on β-galactosidase synthesis (Fig. 5). The c2 and c3 ParB proteins reduced *lacZ* expression about 1.7- and 8-fold, respectively, and reduced it slightly more in the presence of ParA. No silencing was seen in strains carrying noncognate *parS* sites (data not shown). The lowered expression did not alter significantly following a ~4-fold decrease in *parAB* copy number (Fig. 5, shaded bars), implying that variation in silencing among the *parABS* systems results less from limitations in protein supply than from differences in the properties of ParB proteins. Nevertheless, the moderate increase in silencing that occurred upon mutating the *parS* site in c2 *parAB* could reflect an increased availability of ParB once a competing site has gone, and the reduction in silencing by c3 *parAB* mutated at the internal promoter presumably results from the decreased synthesis of c3 ParB protein. Although these interpretations must remain provisional pending a direct measurement of protein concentrations, the results suggest that silencing by ParB could play an autoregulatory role in the functioning of the c2 and c3 partition systems.

DISCUSSION

Species of the genus *Burkholderia* are notable for the breadth of their physiology and habitat (36). It is reasonable to assume, in view of their relatively large genomes, that they have achieved this versatility through the addition of new genetic material to the basic genome needed for a free-living existence. While imported DNA could in principle be accommodated by integration into the chromosome, it is clear that *Burkholderia* spp. have opted for keeping it on separate replicons. This choice can be expected to place extra demands on the mechanism that ensures orderly segregation, especially since three of the replicons are large enough to qualify as chromosomes. Our results show that each *B. cenocepacia* replicon harbors a single *parAB* locus and a group of *parS* centromere sites, that each *parABS* set can partition an unstable plasmid vector, and that each *parAB* functions only with the *parS* of its own replicon. These *parABS* systems thus have the potential to confer specificity and direction to the partition process in their mother organism. How this potential is realized should become evident from future studies based on *parAB* mutants of *B. cenocepacia*.

Of the three strategies for managing chromosome segregation that we considered, *B. cenocepacia* appears to have adopted independent action of ParAB proteins at individualized *parS* clusters in preference to collective partition by a single master *parAB* locus. Coordinated partition by cross-reaction among *parABS* systems is not entirely ruled out, because we have not yet measured the ability of *B. cenocepacia* ParA proteins to stabilize through the interaction with noncognate ParBs. However, in other ParB family proteins, it is the amino acids of a relatively unstructured N terminus that determine the specificity of interaction with ParAs (32, 44, 45, 49). If the same is true for *B. cenocepacia* ParBs, the dissimilarity of their N-terminal primary sequences (not shown) suggests that noncognate ParA-ParB interactions are unlikely to occur. A possibility of another type, which we have not investigated here, is the prevention of *ori*/*par* region interference by localization at distinct and specific intracellular sites. The origins of the two chromosomes of *Vibrio cholerae* were clearly seen to occupy distinct locations (6), whereas the separation of the *Sinorhizobium meliloti* chromosome and megaplasmid origins appeared less clear cut (22). The involvement of *parABS*

systems in the positioning of these origins has not been reported.

Our conclusions are based on the use of *E. coli* to test the function of exotic partition systems. This raises the issue of their relevance to behavior in their natural *Burkholderia* host. The c1 and modified c2 *parABS* systems stabilize mini-F as effectively as the vector's own system (*sop*). This observation directly demonstrates their independence of host-specific partition factors, thus removing the doubt on this issue which previous reports of partial stabilization (2, 10, 52) had allowed to persist. Participation of the host must either be at a general level, via the membrane or the nucleoid, for example, or involve a structurally conserved factor such as the bacterial actin MreB (9, 25, 37). The weak partition activity of the unmodified c2, c3, and p1 systems may be partly a consequence of intrinsic inefficiency, sensed even in *Burkholderia*, whereas the efficient c1 *parAB* is normally supplemented with only two isolated *parS* sites, and those of c2, c3, and p1 are accompanied by three to four nearby *parS* sites in a clustered arrangement which may serve to compensate for a lower effectiveness of its protein partners. On the other hand, the finding that mutations expected to alter Par protein supply enhanced stabilization by the c2, c3, and p1 systems suggests that imbalances in Par protein levels interfere with partition in *E. coli*, as previously observed for plasmid systems (7, 19, 26), and points to the existence of host-specific regulatory mechanisms. Regulation of chromosomal *parABS* systems, at any level, has not been described. The presence of *parS* sites or promoters internal to the *B. cenocepacia parAB* operons (Fig. 1) and the ability of c2 and c3 ParB proteins to silence a nearby promoter suggest that there are mechanisms for autoregulating Par protein production that are distinct from the classic promoter repression mode of autoregulation described so far for *parAB* operons. Such possibilities will be best explored in *B. cenocepacia* itself, where the full complement of relevant regulators is present.

A characteristic that has united all genomes with *parABS* systems described to date is the virtual identity of the *parS* sites found on their chromosomes (34). Moreover, these sites had not been seen in smaller chromosomes and plasmids. It was therefore surprising to find that the secondary replicons of *B. cenocepacia* contain *parS* elements whose sequence is distinct from but strongly resembles that of the principal *parS*, a 14- to 16-bp palindrome with a 5′-CG-3′ center and no spacer. These *parS* sites exhibit some notable features. They are present, and function better, as clusters rather than as dispersed copies typical of the arrangement of the canonical *parS*. The c2 *parS* is also found in the secondary chromosomes of other *Burkholderia* species (Fig. 2), but we found no examples of these *parS*s in other multipartite genomes outside the *Burkholderia* group (the secondary replicon of the closely related *Ralstonia solenacearum* carries its own, similar *parS*). Also striking is that the p1 replicon, which in other respects is a typical conjugative plasmid, carries a *parS* whose uninterrupted palindrome structure and general sequence relate it more closely to the canonical chromosome *parS* than to any known plasmid centromere. Any type of plasmid centromere should have sufficed if replicon compatibility were the only issue. It is possible that certain types of *parS* sites, or *parS*-ParB partners, function better than others in *Burkholderia* species and are therefore preferentially established upon entry into the cell. The replicons carrying this kind of *parS* would then, by accretion of DNA from the chromosome or from further imports and by suitable reorganization, become secondary chromosomes. The p1 plasmid might be in the initial stages of this process, as witnessed by the possible beginnings of a GC skew (Fig. 1). The smaller of the two chromosomes in the other *Burkholderia* genomes characterized, *Burkholderia pseudomallei* (16) and *Burkholderia mallei* (40), possesses *parABS* systems very similar to that of *B. cenocepacia* c2, including an identical *parS* (Fig. 3), suggesting that these species are following the evolutionary track already traveled by *B. cenocepacia*. The phylogenetic distinction between c1 ParA and ParB proteins and those of the other replicons support this view. The second chromosome of *Vibrio cholerae* also betrays a plasmid ancestor through the phylogeny of its ParA (48) as well as by the nature of its replication origin (5). In examining partition in *B. cenocepacia* itself, we not only might come to understand the role of the *parABS* systems in the mechanism but might also gain insight into the evolution of the multichromosome state.

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