Identification of C-terminal hydrophobic residues important for dimerization and all known functions of ParB of *Pseudomonas aeruginosa*

J. Mierzejewska,1† A. A. Bartosik,1 M. Macioszek,1 D. Plochocka,1 C. M. Thomas2 and G. Jagura-Burdzy1

1The Institute of Biochemistry and Biophysics, PAS, Pawinskiego 5A, 02-106 Warsaw, Poland
2School of Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

The ParB protein of *Pseudomonas aeruginosa* is important for growth, cell division, nucleoid segregation and different types of motility. To further understand its function we have demonstrated a vital role of the hydrophobic residues in the C terminus of ParB<sub>P.a.</sub>. By *in silico* modelling of the C-terminal domain (amino acids 242–290) the hydrophobic residues L282, V285 and I289 (but not L286) are engaged in leucine-zipper-like structure formation, whereas the charged residues R290 and Q266 are implicated in forming a salt bridge involved in protein stabilization. Five parB mutant alleles were constructed and their functionality was defined *in vivo* and *in vitro*. In agreement with model predictions, the substitution L286A had no effect on mutant protein activities. Two ParBs with single substitutions L282A or V285A and deletions of two or seven C-terminal amino acids were impaired in both dimerization and DNA binding and were not able to silence genes adjacent to parS, suggesting that dimerization through the C terminus is a prerequisite for spreading on DNA. The defect in dimerization also correlated with loss of ability to interact with partner protein ParA. Reverse genetics demonstrated that a parB mutant producing ParB lacking the two C-terminal amino acids as well as mutants producing ParB with single substitution L282A or V285A had defects similar to those of a parB null mutant. Thus so far all the properties of ParB seem to depend on dimerization.

INTRODUCTION

The essential role of *par* loci in plasmid partitioning has long been appreciated (Williams & Thomas, 1992), while the function of chromosomally encoded *par* loci in the segregation of bacterial chromosomes is less clear. The chromosomally encoded *par* loci are highly conserved and belong to type I partitioning systems (Gerdes et al., 2000). Besides the high level of identity in amino acid sequences of the chromosomal homologues of ParA and ParB, the parS sequences are also extremely well conserved at least between so called primary chromosomes. The localization of the parAB genes, as well as the majority of parS sites, in close vicinity to the oriC region could indicate a role of *par* systems in replication/segregation of chromosomes, as genes known to be crucial for these processes are situated within the oriC domain (20% of the chromosome around oriC). Moreover, the chromosomal *par* systems are able to promote active segregation and stabilization of otherwise unstable replicons even in heterologous host cells (Bartosik et al., 2004; Godfrin-Estevenon et al., 2002; Lin & Grossman, 1998; Yamaichi & Niki, 2000). Although these features of chromosomally encoded *par* loci suggest that they should play a similar biological function in chromosome segregation to that which plasmid *par* systems play for plasmid DNA, studies on *par* mutants in different bacteria have revealed a more complex picture. With the exception of *Caulobacter crescentus*, the chromosomal *parA* and parB genes are not essential for cell viability (Mohl & Gober, 1997). However, mutations in the *parA* (soj) and *parB* (spo0f) genes lead to defects in the sporulation of *Bacillus subtilis* (Cervin et al., 1998; Quisel et al., 1999; Quisel & Grossman, 2000) and *Streptomyces coelicolor* (Jakimowicz et al., 2002, 2006, 2007; Kim et al., 2000) and in vegetative chromosome partitioning of *B. subtilis* (Ireten et al., 1994), *Pseudomonas aeruginosa* (Lasocki et al., 2007; Bartosik et al., 2009) and *Pseudomonas putida* (Lewis et al., 2002; Godfrin-Estevenon et al., 2002). Several studies have indicated a role for *par* genes in origin localization and segregation (Bowman et al., 2008; Ebersbach et al., 2008;
The increased frequency of chromosome loss observed in et al. (Bartosik culture growth. The other phenotypic defects (motility slower growth rate and perturbations in colony formation increase in the number of anucleate cells, longer cells, cells. Their lack causes visible phenotypic defects (more P. aeruginosa system of P. aeruginosa sequences are located in the (Bartosik locus has been identified ~8 kb from (Bartosik ParB to dimerize and also on an intact N terminus a putative helix–turn–helix (H–T–H) motif, the ability of parS to spread on DNA and silence genes adjacent to the centromere-like sequences (parS, respectively. It demonstrates the ability to interact with ParA and to dimerize and to bind centromere-like sequences (parS). ParBparBparB mutants (Godfrin-Estevon et al., 2002; Lewis et al., 2002), but only in the transient and stationary phase of culture growth. The other phenotypic defects (motility defects, changed colony morphology, increase in cell size) caused by the lack of ParBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparBparB
**‘Silencing’ assay.** *E. coli* DH5α(pAB811parS233) and DH5α(pGB2) cells were transformed with the appropriate pGBT30taep-parB derivatives. Undiluted and 10- and 100-fold dilutions of the initial transformation mixture were plated to select for incoming plasmid (Luria agar supplemented with penicillin) or for both incoming and resident plasmid (Luria agar with penicillin and streptomycin) with and without 0.5 mM IPTG to induce ParB production. After 24 h incubation at 37 °C the colonies were counted and the number of different class transfectants in the original transformation mixture was estimated.

**Purification of His₆-tagged proteins.** *E. coli* strain BL21(DE3) was transformed with pET28mod derivatives encoding histidine-tagged (MGSHHHHHHSSGLVPRGSHSEF) ParB derivatives and protein overexpression and purification was carried out as described previously (Bartosik et al., 2004).

**Cross-linking with glutaraldehyde.** His₆-tagged polypeptides purified on Ni²⁺-agarose columns (at 0.1 mg ml⁻¹) were cross-linked by use of glutaraldehyde (Jagura-Burdzy & Thomas, 1995) and separated on 10 % (w/v) SDS-PAGE gels. The proteins were transferred onto nitrocellulose membranes and Western blot analysis was performed with anti-ParB antibodies, as described previously (Bartosik et al., 2004).

**Analysis of protein–DNA interactions by electrophoretic mobility shift assay (EMSA).** To check the ability of mutated ParBₚₐ derivatives to bind parS DNA in vitro, a nonradioactive EMSA (Leonard et al., 2004) was performed. A 16 bp dsDNA fragment (annealed oligonucleotides, #17 and #18, Table S1) containing the parS233 (Bartosik et al., 2004) was used in EMSA. Samples (5.6 pmol) of parS233 oligonucleotides without or with increased concentration of purified His₆-tagged ParB and its derivatives were incubated under conditions described previously (Kusiat et al., 2011). Negative control of the binding reaction was provided by use of unrelated dsDNA with palindromic sequence (annealed primers #19 and #20, Table S1) and the same amounts of ParB in the incubation mixture. The samples were analysed on 10 % (w/v) non-denaturing polyacrylamide gel in TBE buffer (Sambrook et al., 1989). DNA bands were stained with 0.5 µg ethidium bromide ml⁻¹ and visualized on a UV transilluminator.

**Growth experiments and sample preparation for Western blotting.** The growth of bacteria was monitored by measuring OD₆₀₀; the cultures were diluted and plated on Luria agar to establish c.f.u. ml⁻¹. Bacteria were harvested, resuspended in sonication buffer (50 mM phosphate buffer, pH 8.0, and 300 mM NaCl) and disrupted by sonication. Crude extracts from the same number of cells were analysed by SDS-PAGE followed by Western blotting performed as described previously (Bartosik et al., 2004).

**Bacterial adenylate cyclase two-hybrid system (BACTH system).** The interactions between ParB mutant derivatives and either wild-type ParB or ParA were analysed by using the bacterial two-hybrid system BACTH (Karimova et al., 1998). The C-terminal parB mutant alleles have been cloned as EcoRI–HincII fragments from pET28mod derivatives into pLKB4 (derivative of pUT18C) to create translational fusions with the T18 catalytic domain of Bordetella pertussis adenylate cyclase, CryT18–ParB. The wild-type parA and wild-type parB alleles have been cloned into pLKB2 (modified pKT25) to produce translational fusions: CryT25–ParA and CryA25–ParB. *E. coli* BTH101 cya strain was co-transformed with both pLKB4 and pLKB2 derivatives and plated on indicator MacConkey base medium supplemented with 1 % maltose (as the only carbon source), penicillin, kanamycin and 0.5 mM IPTG. The plates were incubated for 48 h at 27 °C.

**Motility assays.** The swimming, swarming and twitching assays were performed according to the method of Rashid & Kornberg (2000) with modifications described previously (Lasocki et al., 2007). All sets of plates were standardized by using the same volume of medium.

**DAPI staining and immunofluorescence microscopy.** The DAPI staining procedure and immunofluorescence microscopy were carried out as previously described (Bartosik et al., 2004; Bignell et al., 1999). Cells were examined using an Eclipse E800 light fluorescence microscope (Nikon) fitted with an ORCA ER CCD camera (Hamamatsu). Images were captured and manipulated on PC Windows XP Professional PL with the Lucia General 5.0 (Laboratory Imaging).

**In silico ParBₚₐ dimer modelling.** Amino acids sequences of ParB of *P. aeruginosa*, ParB of *P. putida*, KorB of RK2/RP4 (IncP-1α) and R751 (IncP-1β) were aligned using MAFT (Katoh & Toh, 2008), CLUSTAL W (Larkin et al., 2007) and T-Coffee (Notredame et al., 2000) servers, and manually adjusted. A structural model of the monomeric C terminus of *P. aeruginosa* ParB was obtained using Sybyl-x1.1 package (TRIPOS) on the basis of ParBₚₐ and KorBₚₐ alignment (Fig. S1) and KorBₚₐ crystal structure (Delbrück et al., 2002). The structure of the C-terminal dimer of ParBₚₐ (superposition on KorB dimer) was subjected to energy minimization (100 steps) using the AmberFF99 force field as implemented in Sybyl-x1.1.

**RESULTS**

**Predicting amino acids essential for ParB dimerization**

A comparison of ParBₚₐ (290 amino acids) with other chromosomal homologues revealed highly conserved segments designated BoxI (S66–R79) and BoxII (Y86–A97) (Yamaichi & Niki, 2000), a H–T–H motif and regions 1 to 4 (R6–L16, L123–A138, V211–L224, G270–I289, respectively) (Bartosik et al., 2004) (Fig. S2). Previous studies on ParBₚₐ using in vivo and in vitro methods (Bartosik et al., 2004) identified a C-terminal fragment of 56 amino acids (ParB235–290) as the dimerization domain for ParBₚₐ and indicated that deletion of the last seven residues from this domain (yielding ParB235–283) abolished its dimerization.

The 3D structure of ParBₚₐ has not yet been solved. The sequence of the C terminus of ParBₚₐ aligns well with the C-terminal part of the ParB homologue – KorB of RK2 (IncP-1α) (Fig. S1) and moreover the two domains are functionally interchangeable. Replacement of 61 amino acids from the C terminus of ParB by the C-terminal 100 amino acids of KorB (ParB1–229–KorR258–358) restores its ability to dimerize and bind parS with high affinity in vitro as well as to transcriptionally silence genes near a parS site in vivo (Bartosik et al., 2004). Therefore, on the basis of crystallographic analysis of the C-terminal part of KorB of RK2/RP4 (Delbrück et al., 2002) a model of the C-terminal domain of ParBₚₐ was built in silico (Fig. 1). According to this model the hydrophobic residues L282, V285 and I289 are engaged in a leucine-zipper-like structure, whereas the charged R290 and Q266 are implicated in forming a salt bridge involved in stabilization of the ParB dimer. To verify this model, two alleles of parB with C-terminal deletions of either seven (parB1–283) or two...
| Plasmid          | Relevant features                                                                                   | Reference or source                  |
|------------------|-----------------------------------------------------------------------------------------------------|--------------------------------------|
| pABB811          | pGB2 with parS2/3 sequence                                                                          | Bartosik et al., (2004)              |
| pAKE600          | oriMB1, oriTB2, ApR, sacB                                                                             | El-Sayed et al., (2001)              |
| pET28mod         | oriMB1, KmR, T7p, lacO, His tag, no BamHI site, T7 tag deleted                                       | G. Jagura-Burdzy, Warsaw, Poland     |
| pBBR1MCS         | IncA/C broad-host-range cloning vector, lacZa-MCS, mob, T7p, T3p, CmR                              | Kovach et al. (1995)                 |
| pGB2             | oriG, SmR, repA gene downstream of MCS                                                               | Churchward et al. (1984)             |
| pGEM-T Easy      | oriMB1, ApR, lacI, tacp, expression vector                                                          | Jagura-Burdzy et al. (1991)          |
| pJMB500          | pBBR1MCS with lacT tacp-parB                                                                         | Lasocki et al. (2007)                |
| pKLB2            | pGBT30 with tacp-parB                                                                               | Bartosik et al. (2004)               |
| pKLB2.8          | pET28mod with T7p-parB                                                                              | Bartosik et al. (2004)               |
| pKT25            | oriMB1, KmR, lac-cyaT25                                                                             | Karimova et al. (1998)               |
| pKT25-zip        | Derivative of pKT25 in which the leucine zipper of GCN4 is translationally fused with cyaT25 fragment | Karimova et al. (1998)               |
| pLKB2            | pKT25 modified with MCS                                                                             | L. Kusiak, Warsaw, Poland            |
| pLKB220          | pLKB2 with translationally fused cyaT25-parA                                                        | L. Kusiak, Warsaw, Poland            |
| pLKB233          | pLKB2 with translationally fused cyaT25-parB                                                         | L. Kusiak, Warsaw, Poland            |
| pLKB4            | pUT18C modified with MCS                                                                             | L. Kusiak, Warsaw, Poland            |
| pLKB433          | pLKB4 with translationally fused cyaT18-parB                                                         | L. Kusiak, Warsaw, Poland            |
| pUT18C           | oriMB1, ApR, lac-cyaT18                                                                             | Karimova et al. (1998)               |
| pUT18C-zip       | Derivative of pUT18C in which the leucine zipper of GCN4 is translationally fused with cyaT18 fragment | Karimova et al. (1998)               |
| pJMB26           | parB1–288 allele PCR amplified using #1 and #4 primers                                               | This study                           |
| pJMB27           | parB1–283 allele PCR amplified using #1 and #5 primers                                               | This study                           |
| pJMB28           | 379 bp fragment PCR amplified using #11 and #12 primers                                               | This study                           |
| **pET28mod derivatives** |                                                                                                       |                                      |
| pJMB100          | pKLB2.8 derivative T7-parB282 (site-directed mutagenesis with pair of primers #21 and #22 to introduce substitution L282A into ParB) | This study                           |
| pJMB101.1        | pKLB2.8 derivative T7-parB285 (site-directed mutagenesis with pair of primers #23 and #24 to introduce substitution V285A into ParB) | This study                           |
| pJMB102          | pKLB2.8 derivative T7-parB286 (site-directed mutagenesis with pair of primers #25 and #26 to introduce substitution L286A into ParB) | This study                           |
| pMMB5.2          | pKLB2.8 derivative T7-parB1–288 (inserted EcoRI–SalI fragment of pJMB26)                           | This study                           |
| pMMB6.2          | pKLB2.8 derivative T7-parB1–283 (inserted EcoRI–SalI fragment of pJMB27)                           | This study                           |
| **pAKE600 derivatives (suicide vector for gene exchange)** |                                                                                                       |                                      |
| pJMB400          | EcoRI–SalI fragment of pMMB5.2 carrying parB1–288                                                    | This study                           |
| pJMB401          | EcoRI–SalI fragment of pMMB6.2 carrying parB1–283                                                    | This study                           |
| pJMB402          | 379 bp SalI–BamHI fragment of pJMB28 inserted into pJMB400                                          | This study                           |
| pJMB403          | 379 bp SalI–BamHI fragment of pJMB28 inserted into pJMB401                                          | This study                           |
| pJMB404          | EcoRI–SalI fragment of pMJB100 carrying parB282                                                      | This study                           |
| pJMB405          | EcoRI–SalI fragment of pMJB101.1 carrying parB285                                                   | This study                           |
| pJMB406          | 379 bp SalI–BamHI fragment of pJMB28 inserted into pJMB404                                          | This study                           |
| pJMB407          | 379 bp SalI–BamHI fragment of pJMB28 inserted into pJMB405                                          | This study                           |
| **pBBR1MCS derivatives** |                                                                                                       |                                      |
| pJMB501          | BamHI–SalI fragment of pJMB604 carrying lacT and tacp-parB1–283 transcriptional fusion              | This study                           |
| pJMB502          | BamHI–SalI fragment of pJMB603 carrying lacT and tacp-parB1–288 transcriptional fusion              | This study                           |
| pJMB503          | BamHI–SalI fragment of pJMB600 carrying lacT and tacp-parB282 transcriptional fusion                | This study                           |
amino acids, I289 and R290, (parB1–288) were amplified by PCR and three alleles coding for ParBs each with a single amino acid substitution – L282A, V285A and L286A – were constructed by applying PCR site-directed mutagenesis. These alleles were introduced into appropriate vectors and their products were tested for the ability to dimerize, bind DNA \textit{in vitro}, spread on DNA and interact with ParA \textit{in vivo}.

### Spreading on DNA \textit{in vivo} – ‘silencing test’ in \textit{E. coli}

The ParB\textsubscript{P.a.} protein recognizes the parS sequence as a dimer then self-associates and spreads on DNA causing transcriptional silencing of genes adjacent to parS (Bartosik \textit{et al.}, 2004). The plasmid pGB2 (Churchward \textit{et al.}, 1984) used for the ‘silencing test’ is an Sm\textsuperscript{R} stable replicon based on pSC101 in which a multiple cloning site (MCS) is inserted approximately 200 bp upstream of the promoter for the initiator gene repA. The presence of parS close to the repA promoter in pABB811 does not influence plasmid stability unless wild-type ParB is produced in trans from pKLB2 (tacp-parB) (Bartosik \textit{et al.}, 2004). The transformation frequency of \textit{E. coli} DH5\textalpha{} (pABB811) with pKLB2 in the absence of IPTG with selection for incoming and resident plasmid was two- to threefold lower than the transformation frequency when only the incoming plasmid is selected. Addition of 0.5 mM IPTG to transformation plates with double selection (conditions of ParB over-production) decreases the number of transformants more than 100-fold (Table 2) in comparison with the number of transformants grown on double selection plates without IPTG.

### Table 1. cont.

| Plasmid | Relevant features | Reference or source |
|---------|-----------------|---------------------|
| pJMB504 | BamHI–SalI fragment of pJMB601.1 carrying lacI\textsuperscript{q} and tacp–parB285 transcriptional fusion | This study |
| pGBT30 derivatives | | |
| pJMB600 | EcoRI–SalI fragment of pJMB100 to form a tac–parB282 transcriptional fusion | This study |
| pJMB601.1 | pKLB2 derivative tacp–parB285 (PCR site-directed mutagenesis with pair of primers \#23 and \#24 to introduce V285A substitution into ParB) | This study |
| pJMB602 | EcoRI–SalI fragment of pJMB102 to form a tacp–parB286 transcriptional fusion | This study |
| pJMB603 | EcoRI–SalI fragment of pJMB26 to form a tacp–parB1–288 transcriptional fusion | This study |
| pJMB604 | EcoRI–SalI fragment of pJMB27 to form a tacp–parB1–283 transcriptional fusion | This study |
| BACTH system plasmids (pUT18C derivatives) | | |
| pJMB700 | EcoRI–HincII fragment of pJMB100 inserted into pLKB4 between restriction sites EcoRI and Smal to create a cyaT18–parB282 translational fusion | This study |
| pJMB701.1 | EcoRI–HincII fragment of pJMB101.1 inserted into pLKB4 between restriction sites EcoRI and Smal to create a cyaT18–parB285 translational fusion | This study |
| pJMB702 | EcoRI–HincII fragment of pJMB102 inserted into pLKB4 between restriction sites EcoRI and Smal to create a cyaT18–parB286 translational fusion | This study |
| pJMB703 | EcoRI–HincII fragment of pMMB5.2 inserted into pLKB4 between restriction sites EcoRI and Smal to create a cyaT18–parB1–288 translational fusion | This study |
| pJMB704 | EcoRI–HincII fragment of pMMB6.2 inserted into pLKB4 between restriction sites EcoRI and Smal to create a cyaT18–parB1–283 translational fusion | This study |

Fig. 1. Model of a dimer of C termini of ParB\textsubscript{P.a.} (amino acids 242–290). The mutagenized residues are shown as sticks in the red subunit (labelled according to their position in the ParB\textsubscript{P.a.} sequence). The indicated residues L282 and V285 in the grey subunit are possibly involved in a leucine zipper formation. The distance between R290 of one monomer and Q266 of another facilitates the electrostatic interactions (magnification at the left).
The silencing test was repeated to establish the effect of overproducing the modified ParB proteins on the stability of pABB811 in E. coli DH5α(pABB811). The numbers of transformants with selection for either incoming plasmid (Pn) or both incoming and the resident plasmids (Pn Sm) with and without IPTG present are shown in Table 2. Only ParBL286A (pJM602) caused significant instability of pABB811 and a loss of streptomycin resistance of the recipient strain when ParB was overproduced during growth with IPTG (more than 100-fold decrease in the number of transformants on Pn Sm IPTG plates, effect observed for wild-type ParB delivered from pKLB2). The other plasmids tested had very little impact on stability of pABB811 (two- to threefold decrease in the number of double transformants grown in the presence of inducer in comparison with the number of transformants selected for incoming plasmid). The deletions of seven (pJM604) or two amino acids I289 and R290 from the C terminus (pJM603) as well as the single amino acid substitutions V285A or L282A impaired significantly the ability of monomers as deletion of seven (ParB1–283) or even two (ParB1–288) amino acids impaired significantly the ability of monomers to interact.

**ParBPₐ DNA binding in vitro (EMSA)**

A previous study (Bartosik et al., 2004) indicated that self association of ParB is important for efficient DNA binding (deletion of the C terminus in ParB1–229 significantly decreased the DNA binding ability but did not completely stop it from binding parS). All purified ParBPₐ derivatives were tested for binding to parSₐ using a standard EMSA (Fig. 2b). Non-radioactive EMSA was performed on parS (annealed oligonucleotides #17 and #18) and an unrelated palindrome motif (annealed oligonucleotides #19 and #20) as a control. Wild-type ParB did not bind the control oligonucleotides at tested concentrations. ParBL286A showed affinity towards parS approximately twofold higher than wild-type ParB whereas ParBV285A and ParBL282A bound parS but with twofold lower affinity. ParB1–288 and ParB1–283 hardly shifted the double-stranded parS oligonucleotides at tested concentrations. Therefore the ability to bind parS seems to correlate with the degree of dimerization proficiency as illustrated by comparing Fig. 2(a) and (b).

**ParBPₐ self association and interaction with ParApₐ in vivo**

To check the interactions of mutated ParB proteins with wild-type ParB and ParA in vivo, the bacterial adenylate...
cyclase two-hybrid system in *E. coli* (Karimova et al., 1998, 2000) was applied. Mutated ParB derivatives were translationally fused to CyaT18 fragment (pUT18C derivatives), whereas wild-type ParB and ParA were fused to CyaT25 fragment (pKT25 derivatives). *E. coli* BTH101, an adenylate-cyclase-deficient strain (*cya*), was co-transformed with a mixture of appropriate pairs of BACTH system plasmids and plated on MacConkey base medium supplemented with 1 % (w/v) maltose, 0.5 mM IPTG and selective antibiotics. The results of *in vivo* BACTH analysis confirmed the conclusions from the *in vitro* dimerization studies presented above. The two short deletion mutants ParB1–283 and ParB1–288 were unable to associate with wild-type ParB whereas interactions between ParBL286A and wild-type ParB were similar to self-association of wild-type ParB as demonstrated by BTH101 (pLKB702)(pLKB233) transformants (Fig. 3). Interactions of ParBL282A and ParBV285A with wild-type ParB were weaker than control interactions between pLKB433 and pLKB233 but still very clear. The analysis of interactions of mutant ParB derivatives and ParA showed a correlation between the efficiency of ParB dimerization and the ability to interact with ParA in the BACTH system. ParBL286A demonstrated interactions with

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**Fig. 2.** ParB<sub>P.a</sub> self-association and DNA binding *in vitro*. (a) Cross-linking with glutaraldehyde (GA) of ParB variants. Purified His<sub>6</sub>-tagged proteins (0.1 mg ml<sup>−1</sup>) were incubated at room temperature for 20 min without (0) or with increasing concentrations (1×, 2×, 5× and 10×10<sup>−3</sup> %) of glutaraldehyde. The samples were separated by SDS-PAGE on 12 % gels and analysed by Western blotting with anti-ParB antibodies. Monomeric, dimeric and higher forms are indicated by m, d and h, respectively. (b) DNA binding affinity of ParB derivatives (EMSA). Purified His<sub>6</sub>-tagged proteins (10, 20, 30 and 50 pmol) were incubated with 5.6 pmol double-stranded *parS<sub>P.a</sub>* oligonucleotide at 37 °C for 15 min. As a control, double-stranded oligonucleotides with an unrelated palindromic motif were used under the same conditions.
ParA similar to those for wild-type ParB. Visibly weaker interactions between ParA and ParBL282A or ParBV285A and no interactions between ParA and ParB1–283 or ParB1–288 were detected. This strongly suggests that the dimer form of ParB is required for interactions with ParA.

**Introduction of mutant parB alleles into the P. aeruginosa chromosome**

The C-terminal modifications of ParB (ParB1–283 and ParB1–288 as well as ParBL282A and ParBV285A) cause defects in dimerization and in turn defects in DNA binding, transcriptional silencing and interaction with ParA. To determine the phenotypic effect of these mutations all four alleles were introduced into the P. aeruginosa chromosome using suicide vector pAKE600 and allele exchange via homologous recombination (El-Sayed et al., 2001). The new P. aeruginosa PAO1161 RifR parB null strain (Bartosik et al., 2009). Neither of the new tested mutants was disturbed in twitching (data not shown) but all four were strongly impaired in swarming and slightly affected in swimming (Fig. 4). The parB282 and parB285 mutants demonstrated lesser defects in swimming when compared with parB null, parB1–288 and parB1–283 mutants.

To look at the effects of parB mutations on P. aeruginosa PAO1161 RifR growth, time-course experiments in both rich (Luria broth) and minimal (M9) medium were conducted. Each growth experiment was performed using cells freshly taken from a deep-frozen stock to reduce the possibility of accumulation of secondary mutations. The new PAO1161 RifR parB mutants (short deletions and single amino acid substitutions) demonstrated changes in the growth rate similar to the parB null strain. They showed ~10% longer mean generation time (mgt) in comparison with wild-type PAO1161 RifR when grown in Luria broth or minimal medium (M9) at 37 °C, and ~20% longer mgt when grown in Luria broth at 30 °C (Table 3).

The parB mutant strains were also examined for the frequency of anucleate cell formation. Bacterial cells were collected from cultures at late exponential growth phase...
The cells were fixed and DAPI-stained to visualize chromosomes. The number of cells without chromosomes and the mean cell length were estimated using fluorescence microscopy combined with appropriate software. The frequency of anucleate cell formation for the ParB null mutant was more than 100-fold higher than for the wild-type strain under the same growth conditions on the sample of at least 1000 cells (Table 3). The short C-terminal deletion mutants PAO1161 RifR, parB1–283, parB1–288 as well as the substitution mutants parB282 and parB285 produced anucleate cells at similar frequencies to those observed for the PAO1161 RifR ParB null mutant. Measurements of cell length showed that all ParB mutants produce cells up to 10% longer on average than those of the wild-type similarly to the PAO1161 RifR ParB null mutant (Table 3).

In order to check the intracellular concentration of the mutated ParB s, equal numbers of cells of the PAO1161 RifR strain and ParB mutants from the same growth phases were collected and analysed by Western blotting with anti-ParB antibodies. The amount of ParB truncated at the C terminus was approximately five- to sixfold lower than the mean cell length of wild-type ParB in actively dividing cells of the PAO1161 RifR strain, which is a key representative of the large family of ParB proteins encoded by both plasmids and chromosomes. Our earlier work described in this paper adds important details to our understanding of ParB from *P. aeruginosa*, which is a key representative of the large family of ParB proteins encoded by both plasmids and chromosomes.

### DISCUSSION

The work described in this paper adds important details to our understanding of ParB from *P. aeruginosa*, which is a key representative of the large family of ParB proteins encoded by both plasmids and chromosomes.
in vivo studies revealed that overproduced ParB of P. aeruginosa is able to silence the expression of genes adjacent to the parS site (Bartosik et al., 2004) and that spreading activity relies on dimer formation by the C terminus, DNA binding and N-terminal polymerization domain (Bartosik et al., 2004; Kusiak et al., 2011). Accumulating evidence has shown that this activity is a common feature of ParB family members of type IA (Bingle et al., 2005; Rodionov et al., 1999; Schumacher et al., 2007, 2010), also including the chromosomal homologues (Bartosik et al., 2004; Breier & Grossman, 2007). This silencing is thought to be a consequence of spreading on DNA due to ParB–ParB interactions through the N-terminal polymerization domain (Kusiak et al., 2011). However, the physiological role of both plasmidic (Rodionov & Yarmolinsky, 2004) and chromosomal ParBs spreading on DNA is unclear. The recent studies on Spo0J of B. subtilis showed that Spo0J spreads around each parS site on chromosomal DNA over dozens of kilobases (Breier & Grossman, 2007) but under the conditions tested, this process did not significantly affect expression of the majority of genes near parS, with the exception of some sporulation genes. The crystallographic studies on ParB homologues of type IA (Delbrück et al., 2002; Khare et al., 2004; Leonard et al., 2004; Schumacher et al., 2007) combined with further experimental verification should help to elucidate the exact role of ParB spreading on DNA and whether this role is universal for all ParB homologues. The discoveries of interactions of chromosomal ParB homologues with DnaA (control of initiation of replication), different proteins involved in chromosome organization and ori domain localization (SMC, PopZ, TipN) and cytokinesis (FtsZ, MipZ) suggest an important biological role of Par proteins in a wide spectrum of processes, some of them possibly species-specific (Bowman et al., 2008; Donovan et al., 2010; Ebersbach et al., 2008; Gruber & Errington, 2009; Kadoya et al., 2011; Murray & Errington, 2008; Ptacin et al., 2010; Schofield et al., 2010; Scholefield et al., 2011; Sullivan et al., 2009; Thanbichler & Shapiro, 2006; Toro et al., 2008). In P. aeruginosa ParB seems to be involved not only in the chromosome segregation but also in the control of growth rate, cell motilities and colony morphology (Bartosik et al., 2009). Its role in some but not all of these processes depends on interactions with its cognate ParA counterpart (M. Kusiak and G. Jagura-Burdzy, unpublished data). It was unclear whether a ParB dimer is required for interactions with ParA and other
putative partners. To correlate the structural information with the physiological role of ParB we looked closely at the C-terminal domain which we had previously established to be the dimerization domain of ParB.

Although ParB<sub>P,a</sub> has not been crystallized yet, the putative 3D structure for the C-terminal (242–290 amino acids) domain of ParB<sub>P,a</sub> has been predicted, based on crystallographic data for its homologue, the KorB protein of plasmid RK2/RP4 (Delbrück et al., 2002) (Fig. 1). We constructed five parB mutant alleles to define the functionality of proteins modified in the C-terminal region. Both in vitro and in vivo tests on the ability of ParB derivatives to dimerize indicated that the last two amino acids at the C terminus within the conserved region 4 are essential for the ability of ParB<sub>P,a</sub> to self-associate. Removal of I289 and R290 rendered ParB inactive in dimer formation. In agreement with the structural prediction in silico two hydrophobic residues L282 and V285 have been confirmed to play a vital role in ParB dimerization. The alanine substitution derivatives ParBL282A and ParBV285A showed detectable changes in self-association in vitro and in association with wild-type ParB in vivo. On the other hand, L286, which should be directed outwards from the putative dimer (Fig. 1), has been confirmed experimentally not to be involved in self-associations. The alanine substitution derivative ParBL286A behaved like wild-type ParB in all tests with the exception of EMSA when it seemed to bind parS with even higher affinity than wild-type ParB.

The four ParB mutant derivatives impaired to various extents in dimerization were also impaired to similar extents in parS binding, strongly implying that ParB binds to parS as a dimer. None of these mutants was also able to silence genes within the boundaries of the nucleoid. The four ParB mutant derivatives impaired to various extents in parS binding, strongly implying that ParB binds to parS as a dimer. None of these mutants was also able to silence genes within the boundaries of the nucleoid.

When these four parB alleles were introduced into the P. aeruginosa chromosome by allele exchange they caused defects in growth rate and motilities (swarming and swimming) and more than 100-fold increase in the frequency of anucleate cell formation. Immunofluorescence microscopy showed that in contrast with wild-type ParB, which is organized into one–four regularly distributed foci, the modified ParBs formed multiple smaller foci dispersed within the boundaries of the nucleoid.

It has been observed that all modified ParBs are present in lower quantities per cell and are more prone to degradation than wild-type ParB, probably due to their inability to be protected by ParA (Lasocki et al., 2007; Bartosik et al., 2009). The elevation of mutant ParB production to the level observed for wild-type ParB did not suppress the parB mutant phenotypes in the constructed merodiploid strains, suggesting that the decreased level of protein is not the main factor responsible for the visible deficiencies of the mutants.

Despite the fact that ParBs with single amino acid substitution (ParB282 and ParB285) seem to be significantly less impaired in dimerization, DNA binding or interactions with ParA than the truncated derivatives ParB1–283 and ParB1–288, the phenotypes of four new parB mutants were almost identical (with slight difference between the deletion and point mutants in swimming defects) and they resembled the phenotype of the parB null mutant (Bartosik et al., 2009). The data presented suggest that even small changes in the dimerization ability of ParB may translate into lower affinity of parS binding and in turn result in inability to spread on DNA (silencing test). The spreading on DNA has been shown to determine the biological function of ParB in P. aeruginosa (Kusiak et al., 2011).

In conclusion, an in silico model of the ParB<sub>P,a</sub> C-terminal dimerization domain has identified the hydrophobic residues L282 and V285 and charged residue R290 as vital for dimerization. Substitution of hydrophobic residues by alanine or removal of the two last amino acids I289 and R290 impairs ParB<sub>P,a</sub> in dimerization, parS binding and ParA interaction and renders it inactive in spreading on DNA (transcriptional silencing). Since such truncation of ParB as well as alanine substitution of two hydrophobic residues led to the same deficiencies in growth, genome segregation and motilities as a complete lack of ParB in P. aeruginosa, it is clear that dimerization is a vital prerequisite for the function of ParB in the cells.

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