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Overproduction and localization of *Mycobacterium tuberculosis* ParA and ParB proteins

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SUMMARY

The ParA and ParB family proteins are required for accurate partitioning of replicated chromosomes. The *Mycobacterium tuberculosis* genome contains *parB*, *parA* and two *parA* homologs, Rv1708 and Rv3213c. It is unknown if *parA* and its homologs are functionally related. To understand the roles of ParA and ParB proteins in *M. tuberculosis* cell cycle, we have evaluated the consequences of their overproduction and visualized their localization patterns in *M. smegmatis*. We show that cells overproducing of ParA, Rv1708 and Rv3213c and ParB are filamentous and multinucleoid indicating defects in cell cycle progression. Visualization of green-fluorescent protein fusions of ParA and its homologues showed similar localization patterns with foci at poles, quarter-cell, midcell positions and spiral-like structures indicating that they are functionally related. On the other hand, the ParBGFP fusion protein localized only to the cell poles. The cyan and yellow fluorescent fusion proteins of ParA and ParB, respectively, colocalized at the cell poles indicating that these proteins interact and possibly associate with the chromosomal origin of replication. Collectively our results suggest that the *M. tuberculosis* Par proteins play important roles in cell cycle progression.

Keywords

Partitioning; segregation; nucleoid; septum; cell division

1. Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis, is responsible for 1.7 million deaths annually (WHO report 2008: Global tuberculosis control). The existence of multi-drug-resistant TB strains and the recent emergence of an extensively drug-resistant strain emphasize the need for novel drug therapies. The bacterial cell cycle is a complex process that includes chromosomal DNA replication, segregation of the newly replicated chromosomes into daughter cell compartments and cytokinesis. The division site is selected between the segregated chromosomes and the cell septum forms¹. Disruption of the chromosome segregation process can in turn disrupt cell division, causing aberrant septum formation and therefore, generation of anucleate cells². Latency, a hallmark of TB, describes a persistent state, in which *M. tuberculosis* possibly divide slowly but are restrained by the host immune system³. As *M. tuberculosis* can shift between active and restricted growth *in vivo*, the knowledge of chromosomal segregation with respect to the cell cycle may help to elucidate a mechanism for this transition.

Plasmid and chromosomal partitioning are mediated by orthologs of ParA and ParB proteins⁴⁻⁸. The *parA* gene encodes a dynamic protein that exhibits ATPase activity and the

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parB gene encodes a protein that binds DNA and forms a nucleoprotein complex at specific regions, called *parS* sequences, present on the plasmid or the chromosome near the origin of replication². *M. tuberculosis* genome encodes *parA* (Rv3918), *parB* (Rv3917), and two *parA*-like homologs, *Rv1708* and *Rv3213* (<http://genolist.pasteur.fr/TubercuList>). Himar-1 transposon mutagenesis indicates that both *parA* and *parB* are essential for the growth of *M. tuberculosis*⁹. Interestingly, the *M. smegmatis* *parB* counterpart is not essential and its overproduction has no phenotype¹⁰. ParB protein has been shown to bind to three origin-proximal *parS* sequences and in vitro this binding is enhanced by interaction with ParA¹⁰.

It is unknown if the *Rv1708* and *Rv3213* proteins are functionally related to ParA and how ParA, its homologs and ParB (collectively referred to as Par proteins) localize in actively growing cells. In this study, we have characterized the *M. tuberculosis* Par proteins with respect to their cellular localization and overexpression in *M. tuberculosis* and *M. smegmatis*. Our data indicate that *M. tuberculosis* ParA and its homologs show similar localization patterns and that ParA and ParB colocalize at cell poles suggesting that they interact in the partitioning complex *in vivo*.

2. Methods

2.1 Bacterial culture conditions

M. smegmatis and *M. tuberculosis* strains were grown in Middlebrook 7H9 broth (Becton-Dickenson) supplemented with oleic acid-albumin-dextrose-catalase (OADC) (Becton-Dickenson) and Tween 80 (Sigma). Kanamycin at 25 µg ml⁻¹ or hygromycin at 50 µg ml⁻¹ were added as needed. All plasmids were propagated in *E. coli* as described¹¹. Viability was measured by spreading appropriate dilutions of cultures grown with and without 0.2% acetamide for 6 hours on 7H10 plates with appropriate antibiotics and determining colony-forming units (CFU) as described¹¹.

2.2 Construction of Par and fluorescent fusion overproduction strains

parA, *parB*, *Rv1708* and *Rv3213* genes were amplified by polymerase chain-reaction from *M. tuberculosis* genomic DNA using gene-specific primers with appropriate restriction sites (Table 1) and cloned under the inducible *acetamidase* promoter in either pJAM2, a replicating shuttle vector, or pJFR19, an integrating vector¹¹. Expression was induced by the addition of acetamide to 0.2%. As needed, *gfp*, *cfp* or *yfp* was cloned into constructs containing the *par* genes (Table 2). The recombinant plasmids were electroporated into *M. smegmatis* and *M. tuberculosis* and transformants were selected on 7H10 agar plates containing appropriate antibiotics. To construct a strain producing ParA-CFP and ParB-YFP, we transformed *parA:cfp* into *M. smegmatis* bearing *parB:yfp* (Table 2).

2.3 Protein sequence alignments

Protein sequences for *M. tuberculosis* ParA (Rv3918), ParB (Rv3917), *Rv1708* and *Rv3213c* (<http://genolist.pasteur.fr/TubercuList/>) were aligned using the ClustalW2 alignment tool (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

2.4. Microscopy

Nucleoids were stained with a mixture of ethidium bromide (40 µg/ml) and mithramycin A (180 µg/ml) as described¹². For some experiments, actively growing *M. smegmatis* cells were incubated with the membrane dye, FM4-64 at a final concentration of 0.5 µg/ml for 1 to 1.5 hours. Next, cells were collected and stained with DAPI (Invitrogen) at 10 µg/ml for 5 to 10 min. The cells were visualized by brightfield and fluorescence microscopy using a Nikon Eclipse E600 microscope with a CoolSnap ES CCD camera (Photometrics) and a high-pressure mercury lamp (Nikon). A Nikon G-2E/C TRITC filter set (Ex528-553/

Em600-660) was used for ethidium bromide/mithramycin A and FM4-64 –stained cells and a Nikon B2A filter set (Ex450-490/Em515) was used for GFP strains. The CFP and YFP strains were imaged using a Nikon CFP filter (Ex490-510/Em520-550) and Nikon YFP filter (Ex426-446/Em460-500). DAPI-stained cells were imaged with a standard DAPI filter set (Ex325-375/Em435-485, Chroma Technology). Images were analyzed using MetaMorph 6.2 software (Universal Imaging Corporation). At least 100 cells from each set were scored for staining patterns and cell length measurements.

3. Results and Discussion

3.1. *M. tuberculosis* ParA and ParA homologs are potential ATPases

Multiple alignment of the *M. tuberculosis* ParA with Rv1708 and Rv3213c revealed high similarity among the three proteins. All three also have the conserved Walker A and B motifs, characteristic of several proteins that bind and hydrolyze ATP¹³ (Fig. 1). The *M. tuberculosis* and *M. smegmatis* ParA proteins show significant sequence similarity (not shown).

3.2. Consequences of overproduction of Par proteins

The recombinant *M. smegmatis* strains overexpressing *parA*, *parB*, Rv1708 and Rv3213 were stained with DAPI and visualized by fluorescence and brightfield microscopy (Fig. 2). In wild-type *M. smegmatis* MC²155, ~ 90% of cells had 1 to 4 nucleoids (as shown in Figs. 2 panels 1c and 5b) and only 10% showed >4 nucleoids/cell. The cells overproducing ParA were elongated and multinucleoidal with more than 4 nucleoids per filamentous cell (Fig. 2, panels 2c and 3c). Similar results were observed with Rv1708 and Rv3213c overproducing cells (data not shown). ParB overproduction resulted in 54% of nucleated cells with 5 to 8 nucleoids and 17% with more than 8 nucleoids/cell. However, in both cases nucleoids appeared more diffuse as compared to wild type suggesting defects in DNA segregation. This may, in turn, give appearance of multinucleoidal phenotype. About 1 to 3 % wild-type and the recombinant strains overproducing ParA and its homologous were anucleate. In contrast, 26% of ParB overproducing cells were short, i.e. ~ 2µm long and anucleate (Fig. 2 panels 6a and 6b). These latter results are similar to those reported for *P. aeruginosa* cells overproducing ParB¹⁴ and *B. subtilis* *spo0J* null mutants⁶.

Visualization of septa following staining with FM4-64 and nucleoids by DAPI revealed that the wildtype cells contained midcell septa with well-segregated nucleoids in both halves of the cell (Fig. 2 panels 1a-d). Some of the Par overproducing cells contained midcell septa with distinct nucleoids (not shown) while others were without distinct or developing septa at midcell position (Fig. 2, compare panels 1b with 2b, 3b and 4b). These results indicate that the cell division process is delayed under Par overproduction conditions. We also noted that approximately 5% ParB (Fig. 2, panel 4b), 1% ParA (Fig. 2, panel 2b) and 3.5% Rv1708 and Rv3213c (data not shown) cells contained septa at quarter-cell position (Fig. 2, see panel 2b, 4b). Thus, asymmetric septa presumably did not contribute much to the number of anucleate cells in cells overproducing ParB (Fig. 2-2a-d).

3.3. The ParB overproducing strain shows reduced viability

Our results showing accumulation of anucleate cells upon ParB overproduction suggest that these cells show reduced viability. Determination of viability by plating for CFU revealed that ParB overproduction led to a ~50% decrease in viability whereas that of the ParA, Rv3213c, or Rv1708 overproduction had little effect as compared to wild-type *M. smegmatis* (Fig. 3). Elevated levels of ParB cause growth inhibition in *P. aeruginosa*¹⁴. Presumably, an optimal ratio of ParB to ParA is required for optimal cell cycle progression in *M. smegmatis*, much like the scenario observed with the *B. subtilis* Soj (parA family) and

Spo0J (ParB family) proteins¹⁵. *M. smegmatis* $\Delta parB$ strain shows delayed growth and a ~10-fold increase in the number of anucleate cells¹⁰.

3.4. Mycobacterium tuberculosis Par protein localization suggests similar roles for ParA and ParA homologs in nucleoid segregation and possible interaction with ParB

To further examine the roles of *M. tuberculosis* ParA and ParB in DNA segregation, we tagged ParA (and its homologs) and ParB to GFP and visualized their cellular localizations in *M. tuberculosis* and *M. smegmatis* by fluorescence microscopy (Figs. 4-I and 4-II, respectively). As the various types of localizations in the two strains were similar, further observations were restricted to *M. smegmatis*. *M. tuberculosis* ParA:GFP and the ParA homolog-GFP fusions formed several similar types of localizations; these included polar foci (uni or bi-polar, Fig. 4-IIA'), foci at the quarter-cell (Fig. 4-IIB') and midcell positions (Fig. 4-IIC') and a spiral-like distribution (Fig. 4-IIE'). Presumably, ParA, like Soj protein of *B. subtilis*, is capable of dynamic pole-to-pole and internucleoid movement¹⁶. The Rv1708:GFP fusion localizations were similar to ParA, however, the spiral-like structures were observed more frequently (37% of cells, not shown). Finally, the Rv3213c:GFP showed a unique membrane localization (Fig. 4-IIF'). Since Rv3213c is predicted to be soluble, the membrane localization could be due to interactions with another membrane-bound protein. *M. tuberculosis* ParB:GFP primarily formed foci at one or both cell poles and only occasionally localized to the midcell (Fig. 4-II G', H', and K', respectively). Polar localization indicates possible association with the chromosome origin, which is oriented toward the cell poles during cell division.

To observe the localization of the Par proteins with respect to nucleoids, cells were stained with a mixture of ethidium bromide/mithramycin to visualize nucleoids (Fig. 5-I). ParA-GFP was found to be dispersed between or occasionally overlapping the condensed nucleoids (Fig. 5-Ia-d), which suggests possible colocalization with the chromosome. Similar results were noted with Rv1708-GFP and Rv3213c-GFP (not shown). *B. subtilis* Soj forms foci associated with the nucleoids and exhibits dynamic assembly and disassembly of these foci¹⁶. *M. tuberculosis* ParB was observed as distinct spots at the cell poles; however, a distinct association with condensed nucleoids was not apparent (Fig. 5-Ie-h). *B. subtilis* Spo0J is known to specifically associate with the region of the nucleoid proximal to the cell pole during both vegetative growth and sporulation^{17, 18}.

3.5. Mycobacterium tuberculosis ParA and ParB colocalize to cell poles

In order to explore the possibility that *M. tuberculosis* ParA and ParB colocalize, we created *M. smegmatis* strains producing ParA:CFP and ParB:YFP protein individually and together and visualized fluorescent fusion proteins. We routinely observed less ParA localization in the double strain when compared to strains in which only ParA-CFP was being overproduced (data not shown). Only 5% of cells expressed both the fusion proteins and ParA-CFP and ParB-YFP were colocalized at the cell pole in 74% of these cells (Figure 5-II). The double strain also grew much slower and exhibited extensive clumping in broth (not shown). Since ParB overproduction led to asymmetric septa formation, and since interactions between ParA and ParB are needed for segregation^{10, 14, 19}, our data indicate that the overproduction of both proteins presumably results in an enhanced interference with segregation, cell division and cell viability. Thus, our data also suggest that an optimal ratio of the two Par proteins is important for proper chromosome partitioning.

Collectively, our results suggest that *M. tuberculosis* ParA is a dynamic protein that possibly oscillates within the cell in a spiral-like formation and appears to colocalize with nucleoids and with ParB at one or both cell poles. Recently, Soj has been shown to dimerize and bind DNA in the presence of ATP²⁰. *M. tuberculosis* ParA appears to lack one of the two

arginines implicated in dimerization and DNA binding and therefore these properties remain to be tested. Considering the localization patterns, it is tempting to speculate that Rv1708 and Rv3213 are capable of similar dynamic oscillation and association with the nucleoids, cell poles or ParB.

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Rv1708      =====MPAGLPQQASVAVRLSCDVPPDARHHEPRP===== 30
Rv3213c
ParA      MSAPWGPVAAGPSALVRSQGASTIEPFQREMTPTPTPEAAHNPMTNVSR 50

Rv1708      GMTDHPDTGNGIGLTGRPPRAIPDPAPRSSHGPPRVIAMQNQKGGVGKTT 80
Rv3213c      =MTDT=====RVLAVANQKGGVAKTT 20
ParA      ETSSTFDTPIGAAAERAMRLVHTTHEPLQRPGRHRLVTIANQKGGVGKTT 100
           :                               Walker A motif  :*::: *****.***

Rv1708      STINLGAALGEYGRRLVLDMDPQGALSAGLGVPHYELDKTIHNVLVEPR 130
Rv3213c      TVASLGAAMVEKGRRLVLDLDPQGCLTFSLGQDPDKLPVSVHEVLG=E 69
ParA      TAVNIAAALAVQGLKTLVIDLDPQGNASTALGITDRQSGTSPSSYEMLIGE 150
           :. .:.*:  * :.*:::*****  :.*  :  .  :.  :

Rv1708      VSIDDLIHSR=VKNMDLVPSNIDLSAAEIQLVNEVGREQTLARALYPVL 179
Rv3213c      VEPNAVLTIT==MEGMTLLPANIDLAGEAAMLLMRAGREYALKRALAKFS 117
ParA      VSLHTALRRSPHSERLFCIPATIDLAGEIELVSMVARENRLRTALAALD 200
           *. . . *  :  :  : :*:::*****:  *  : .**  *  **  .

Rv1708      DR=YDYVLIDGQPSLGLTLVNLACTDGVIIPTCEFFSLRGLALLTDIV 228
Rv3213c      DR=FDVVIIDGPPSLGLTLNGLTAADKAIVPLQCEMLAHRGVGQFLRTV 166
ParA      NFDYDYVFDGPPSLGLLTINALVAAPVMIPIQCEYYALEGVSQIMRNI 250
           :  : *  :***  *****:*:*.*.:  ::*  :**  :.*.:  :.  :
           Walker B motif

Rv1708      DKVRDLNPKLDISGILITRYDPTVNSREVMAVVERFGDLVFDIVITR 278
Rv3212      ADVQQITNPNLRLLGALPTLYDSRTTHTRDVLVDVADRYDLQVLAPPPIR 216
ParA      EMVKAHLNPQLEVTTIVILTMYGRTKLADQVADEVRYFGSKVLRTVIR 300
           *  :  **:*  :  : *  *  *  :  : *  :  :.  *  : . * .

Rv1708      TVRFPETSVAGEPITTWAPKSAGALAYRALARELIDRFGM===== 318
Rv3213c      TVRFAEASAGSSVMAGR=KNKGAVAYRELAQALLKHWTGRPLPTFTVD 265
ParA      SVKVSEAPGYSMTIIDYDPSRGAMSYLDASRELAERDRPPSAKGRP==== 347
           :*:.*.:.  .  :.  .  :*****  :*  :.  :

Rv1708      =
Rv3213c      L 266
ParA      =

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Fig. 1.

Sequence alignment of *Mycobacterium tuberculosis* ParA and the ParA homologs Rv1708 and Rv3213c. Analysis revealed that the Walker A and Walker B motifs, associated with ATPase activity, are found in both ParA and the ParA homologs. * -amino acid residues that are identical in all sequences, : - conserved amino acid residues, and . - residues that are semi-conserved.

Fig. 2.

Septal and nucleoid staining of Par overproduction strains. *M. smegmatis* strains overproducing *M. tuberculosis* ParA, the ParA homologs Rv1708 and Rv3213, and ParB were stained during log phase growth with FM4-64 (FM) and DAPI to show cell septa and nucleoids, respectively. The grayscale images are brightfield (BF); black bars - 4 μ m. Magnification is 100x. Overlay panels were created by combining the fluorescent images. (1a-d) Wild-type *M. smegmatis* with mid-cell septum and well separated nucleoids. (5ab) Wild-type *M. smegmatis* stained with DAPI to show well separated nucleoids. (2a-d) ParA overproducing strain with asymmetric septum and nucleoids in both cell compartments. (3a-d) ParA overproducing strain without apparent septa formation; nucleoids are distributed throughout the cell. (4a-d) ParB overproducing strain with asymmetric septum; nucleoids are only seen in one cell compartment. (6a, b) Anucleate cell stained with DAPI (ParB overproducing strain).

Fig. 3.

Viability of strains overproducing Par proteins. The Par overproduction strains were grown in the presence of the 0.2% acetamide for 6 hours, then serially diluted and plated on Middlebrook 7H10 agar plates without acetamide; counts of viable colonies were used to calculate viability. Viability of the ParB overproduction strain was reduced by about 0.5 log compared with wild-type *M. smegmatis*. Overproducing ParA and the ParA homologs Rv1708 and Rv3213 did not lead to a significant loss of viability.

Fig. 4.

Localization of *M. tuberculosis* Par protein fusions with GFP in *M. tuberculosis* and *M. smegmatis*. *M. tuberculosis* strains overproducing the Par:GFP fusions were grown for 24 hours with 0.2% acetamide; *M. smegmatis* Par:GFP overproduction strains were grown with 0.2% acetamide for 3 hrs. Cells were imaged during log phase. Grayscale images are brightfield (BF) and green pseudocolor - Par:GFP fusions; black bars - 4 μm. Magnification is 100x. (IA,A') ParB:GFP localization in *M. tuberculosis* at cell poles. (IB,B') ParA:GFP localization at cell poles and midcell in *M. tuberculosis*. (IIA'-EE') Localization patterns of ParA:GFP in *M. smegmatis*. (IIF'-KK') Membrane-like distribution of Rv3213c:GFP in *M. smegmatis*. (IIG'-KK') Localization patterns of ParB:GFP in *M. smegmatis*.

Fig. 5.

Colocalization of *M.tb* Par:GFP fusions with nucleoids and ParA:CFP colocalization with ParB:YFP. For colocalization of Par:GFP fusions and nucleoids, *M. smegmatis* Par:GFP strains were grown with 0.2% acetamide for 3 hrs in log phase. Cells were stained with ethidium bromide and imaged. Grayscale images are brightfield (BF); green pseudocolor - Par:GFP fusions (GFP) and red pseudocolor - nucleoids stained with ethidium bromide (EtBr); black bars – 4 μ m. Magnification is 100x. An overlay panel was created from the fluorescence images. For colocalization of ParA:CFP and ParB:YFP, the *M. smegmatis* double overproduction strain was grown with 0.2% acetamide for 3hrs during logarithmic growth, then cells were imaged. Green pseudocolor - ParA:CFP and red pseudocolor - ParB:YFP; an overlay panel was created from the fluorescence images. (Ia-d) ParA:GFP shown with stained nucleoids in *M. smegmatis*. The arrow indicates colocalization. (Ie-h) ParB:GFP shown with stained nucleoids in *M. smegmatis*. (IIa-d and e-h) ParA:CFP and ParB:YFP foci in *M. smegmatis*. The arrow indicates colocalization.

Table 1

Primers used in this study

Primer	Gene	Sequence 5' to 3'
MVM 209	<i>Rv1708</i> forward	AGAGTACTTTGCCTGCGGGTCTCCCGGG ScaI
MVM 210	<i>Rv1708</i> reverse	AGTCTAGACATGCAAATCGTCGATCAAC XbaI
MVM 211	<i>Rv3213</i> forward	AGAGTACTATGACTGATACCCGGGTGCT ScaI
MVM 212	<i>Rv3213</i> reverse	AGTCTAGACAGGTCCACCGTGAAGGTGG XbaI
MVM 336	<i>parA(Rv3918)</i> forward	TCCAGTACTATGACCCAGCCGTCACGC ScaI
MVM 337	<i>parA(Rv3918)</i> reverse	GTGTCTAGACAGAGCGTCCCTGTGCAG XbaI
MVM 339	<i>parB(Rv3917)</i> reverse	GTGTCTAGATGGTTCGTCCTTCGCGGA XbaI
MVM 407	<i>parB(Rv3917)</i> forward	CGGGATCCCATATGAGTGCTCCGTGGGGCCCGGTG BamHI
MVM 418	<i>parB(Rv3917)</i> reverse	ACGGATCCTCTAGATCATGGTCGTCCTTCGCGGA XbaI
MVM 419	<i>Rv1708</i> forward	GCGAATCCATATGTTGCCTGCGGGTCTCCCGGGC NdeI
MVM 420	<i>Rv1708</i> reverse	CAGTTCTAGATC <u>CA</u> CATGCCAAATCGGTCGATCAACTC XbaI

Table 2

Plasmids used in the study

Plasmid	Gene Insert	Vector Used
pEM30	<i>parA</i>	pJAM2 (replicating vector)
pEM31	<i>Rv3213</i>	pJAM2
pEM32	<i>parA:gfp</i>	pJAM2
pEM33	<i>parB:gfp</i>	pJfr19 (integrating vector)
pEM34	<i>parB</i>	pJfr19
pEM35	<i>Rv1708</i>	pJfr19
pEM37	<i>Rv3213:gfp</i>	pJAM2
pEM38	<i>Rv1708:gfp</i>	pJfr19
pEM39	<i>Rv3213</i>	pMV306H (integrating vector)
pEM40	<i>parA</i>	pMV306H
pEM77	<i>parA:cfp</i>	pJfr19
pEM78	<i>parB:yfp</i>	pJAM2