Non-canonical structure of the *repABC* module identified in the chromid of *Allorhizobium ampelinum* S4

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INTRODUCTION

The secondary chromosome (**chromid**) of *Allorhizobium ampelinum* S4 belongs to the ***repABC* replicon family**, widely distributed among ***Alphaproteobacteria***. The *repABC* operons contain three genes: (i-ii) ***repAB*** – encoding proteins involved in the **active partitioning** of newly duplicated replicon copies into daughter cells, and (iii) ***repC*** – encoding **replication initiator**[1] and containing **origin of replication** within its coding sequence[2] **(Fig. 1)**. The proper functioning of the modules also depends on the presence of several required *cis*-acting sequences. The partitioning process relies on short ***parS*** motifs, primarily located within the *repABC* modules[2]. After replication, the *parS* sites are bound by the RepB protein, and the resulting **RepB-*parS*** complexes are actively partitioned to opposite cell poles by the RepA protein, thereby positioning the origin regions in both daughter cells **(Fig. 2)**.

Interestingly, the *repABC* module of *A. ampelinum* S4 chromid does not contain any predicted ***parS***s[3]. However, several motifs matching the ***parS* consensus** sequence have been identified within two intergenic (*igs*) regions **far downstream** of ***repC*** – one *parS* site approximately **18 kb** downstream of *repC* and additional two *parS*s about **35 kb** downstream of *repC*[3].

For some extrachromosomal replicons (but not of those of the *repABC* family), it has been shown that binding of the chromosomal **replication initiator DnaA** can enhance the efficiency of the replication initiation[4]. Notably, the *repABC* module of *A. ampelinum* chromid (unlike typical *repABC* replicons) contains two predicted DnaA binding sites (**DnaA-boxes**) within an unusually long *igs* **between *repB* and *repC*** genes (about 950 bp-long whereas in other *repABC* replicons it ranges from 150 to 200 bp[3]).

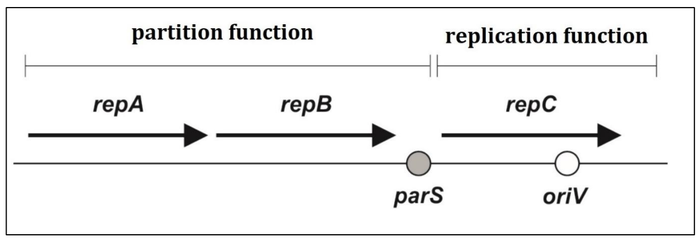

Fig. 1. Genetic organization of *repABC* module

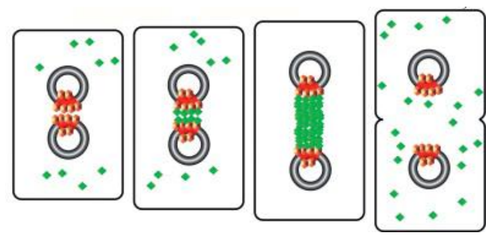

Fig. 2. Plasmid DNA segregation into dividing cells mediated by the partition system

RESULTS

Chip-Seq analysis of RepB, RepC and DnaA interactions with *A. ampelinum* S4 chromid DNA

This project aimed to explore the maintenance systems of the *A. ampelinum* S4 chromid using chromatin immunoprecipitation-sequencing (**ChIP-seq**), a method for genome-wide identification of protein–DNA interactions. As a first step, we mapped binding motifs for **RepB**, **RepC** and **DnaA** proteins both within the *repABC* module and the entire *A. ampelinum* chromid.

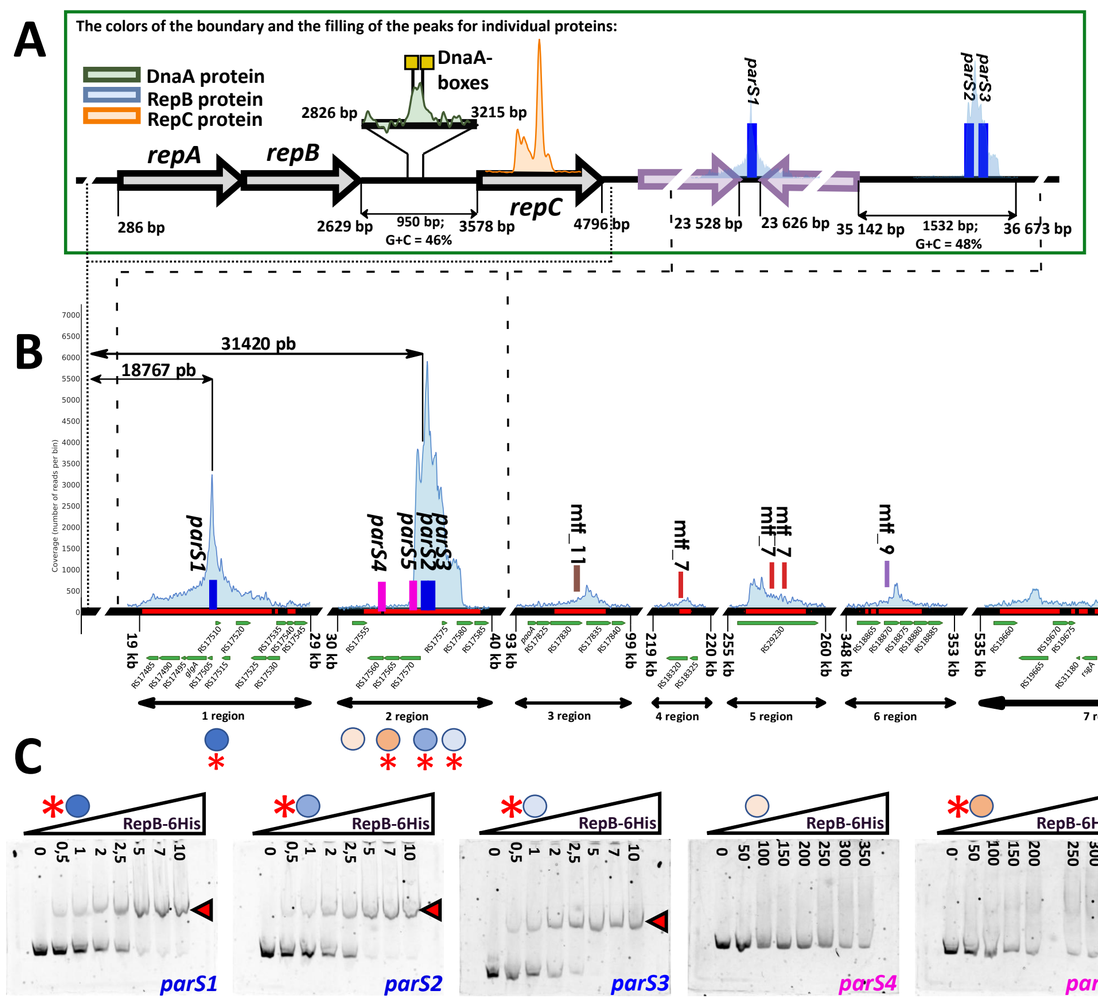

Figure 3. (A) The genetic organisation of the *A. ampelinum* S4 chromid *repABC* module with marked interactions of DnaA, RepB and RepC proteins detected by ChiP-seq analysis. (B) Strong RepB binding to 12 chromid regions containing consensus and degenerate *parS* sites. (C) Experimental verification of RepB-6His interactions with *parS* sequences using EMSA assays.

The analysis revealed that:

- **RepB binds strongly to *parS* sites matching the canonical *repABC* consensus**, confirming their role in chromid segregation. **RepC binds with high affinity within *repC***, indicating the replication origin. **DnaA shows weak binding in the *repB–repC* intergenic region (Fig. 3)**.
- **RepB also binds degenerate *parS*** with a conserved 8-nt half-motif, a feature not seen in other RepB proteins, potentially enhancing replicon segregation **(Fig. 3BC)**.
- **EMSA** confirmed **strong RepB-6His binding to *parS1–parS3***, supporting their role in chromid maintenance. Just 5 pmol of protein fully bound 1 pmol of these sites. Under **higher concentration**, RepB-6His also bound degenerate *parS* motifs with a conserved second half **(Fig. 3C)**.

Chip-Seq analysis of RepB, RepC and DnaA interactions with *A. ampelinum* S4 chromosome DNA

In addition to the above observations, ChIP-seq analysis also revealed interesting data on the interactions of RepC, RepB, and DnaA with the *A. ampelinum* chromosome.

The analysis revealed that:

- **A strong RepC binding signal was detected** in the chromosome within an ORF encoding a PAS domain-containing protein, around position 2787 kb **(Fig. 4A)**.
- Marker frequency analysis (MFA) indicated that the chromid replicates unidirectionally. Its replication initiates later than that of the primary chromosome, ensuring **synchronized termination of replication** of both replicons within the cell cycle **(Fig. 4B)**.
- Based on these findings, we hypothesize that the identified **RepC binding site plays a key role in synchronizing replication termination**. DNA replication through this checkpoint site enables RepC to dissociate and subsequently bind to the chromid origin, triggering its replication **(Fig. 4C)**.

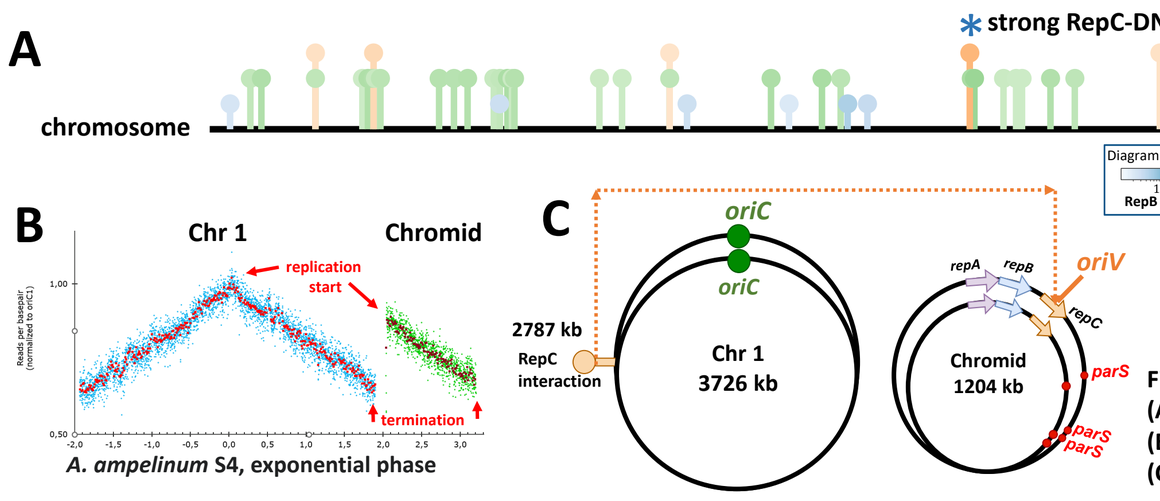

Figure 4.
(A) Genome-wide map showing binding sites of RepB, RepC, and DnaA proteins across the primary chromosome.
(B) MFA results indicating the replication direction of the main chromosome and chromid during exponential growth.
(C) Proposed model of replication termination synchronization between the chromosome and chromid.

CONCLUSIONS

- Our findings demonstrate that the maintenance systems of *repABC* replicons extend beyond the canonical *repABC* module. We identified additional elements, located distantly from the module, that may play important roles in the segregation of the *A. ampelinum* chromid.
- The RepB protein was shown to bind both consensus and degenerate *parS* sequences, particularly those retaining second half of the consensus motif. These interactions, previously not described for RepB proteins, may enhance segregation efficiency and/or serve regulatory role under conditions of RepB overproduction.
- We present the first ChIP-seq analysis of the RepC protein of a RepABC-type replicon. Our results indicate that DNA replication of the chromid and *A. ampelinum* chromosome is synchronized. The chromid RepC protein may play a key role in this synchronization process.

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