Plasmid 68 (2012) 159–169

Contents lists available at SciVerse ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

rctB mutations that increase copy number of *Vibrio cholerae oriCII* in *Escherichia coli*

Birgit Koch , Xiaofang Ma, Anders Løbner-Olesen *

Department of Science, Systems and Models, Roskilde University, Universitetsvej 1, 4000 Roskilde, Denmark

ARTICLE INFO

Article history:
Received 22 February 2012
Accepted 25 March 2012
Available online 3 April 2012
Communicated by Dr. Dhruba K. Chattoraj

Keywords:
Vibrio cholerae
Oricii
DNA replication
Initiation
Origin

ABSTRACT

RctB serves as the initiator protein for replication from *oriCII*, the origin of replication of *Vibrio cholerae* chromosome II. RctB is conserved between members of *Vibrionaceae* but shows no homology to known replication initiator proteins and has no recognizable sequence motifs. We used an *oriCII* based minichromosome to isolate copy-up mutants in *Escherichia coli*. Three point mutations $rctB_{R269H}$, $rctB_{L439H}$ and $rctB_{Y381N}$ and one IS*10* insertion in the 3′-end of the *rctB* gene were obtained. We determined the maximal C-terminal deletion that still gave rise to a functional RctB protein to be 165 amino acids. All *rctB* mutations led to decreased RctB–RctB interaction indicating that the monomer is the active form of the initiator protein. All mutations also showed various defects in *rctB* autoregulation. Loss of the C-terminal part of RctB led to overinitiation by reducing binding of RctB to both *rctA* and *inc* regions that normally serve to limit initiation from *oriCII*. Overproduction of $RctB_{R269H}$ and $RctB_{L439H}$ led to a rapid increase in *oriCII* copy number. This suggests that the initiator function of the two mutant proteins is increased relative to the wild-type.

1. Introduction

The genomes of *Vibrio cholerae* and other members of *Vibrionaceae* are distributed between two circular chromosomes (Okada et al., 2005). Characterization of the replication origins of *V. cholerae*, *oriCI* and *oriCII* has indicated that *oriCI* is similar to the origin of replication of the *Escherichia coli* chromosome, *oriC*, whereas *oriCII* is more similar to iteron-bearing plasmid origins (Egan and Waldor, 2003). In these plasmids the gene encoding the replication initiator protein is found adjacent to the origin which carries an array of replication initiator binding sites (iterons) required for initiation of replication (Chattoraj, 2000; Paulsson and Chattoraj, 2006; del Solar et al., 1998). Iterons with regulatory functions are often found outside the minimal origin region (Chattoraj, 2000; Paulsson and Chattoraj, 2006; del Solar et al., 1998). *oriCII* is situated in the right site of the *rctA*/*rctB* intergenic region (Egan and Waldor, 2003; Fig. 1A). The *rctB* gene encodes the protein RctB which function as the *oriCII* initiator protein (Egan and Waldor, 2003; Duigou et al., 2006).

Regulation of initiation of replication from *oriCII* is complex. Binding of RctB to iterons and to a 39-mer in the left side of the *rctA*/*rctB* intergenic region (*inc*) as well as binding to the transcribed ORF *rctA* can negatively regulate initiation of replication by RctB titration (Venkova-Canova et al., 2006; Venkova-Canova and Chattoraj, 2011). In iteron bearing plasmids negative control of initiation of replication is exerted by a process called handcuffing, which is coupling of iterons via initiator bridges (Chattoraj, 2000; Paulsson and Chattoraj, 2006). The 39-mer of the *inc* region promotes *oriCII* iteron handcuffing, presumably via initiator remodeling (Venkova-Canova and Chattoraj, 2011). A 39-mer and a 29-mer with sequence similarity to the 39-mer in the *inc* region and with the ability to bind RctB are found in *rctA* and the *rctB* promoter region, respectively (Venkova-Canova and Chattoraj, 2011; Fig. 1A). As many replication initiators RctB functions as an autorepressor (Egan et al., 2006; Pal et al., 2005) by binding to the

* Corresponding author. Fax: +45 4674 3011.
E-mail address: lobner@ruc.dk (A. Løbner-Olesen).

0147-619X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.plasmid.2012.03.003

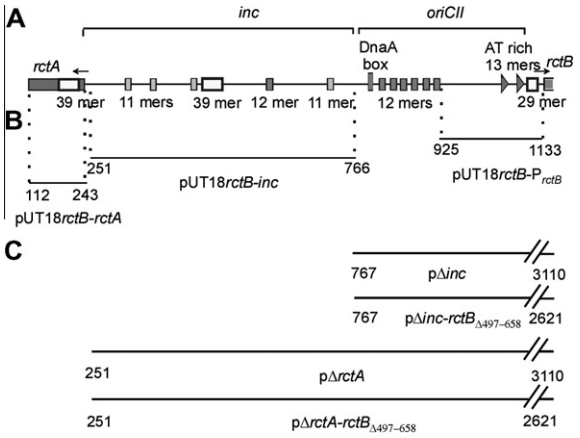

Fig. 1. Map of the *V. cholerae oriCII* region. A. The minimal region needed for autonomous replication in the presence of RctB in *E. coli* is designated *oriCII*, whereas *inc* relates to the region that controls *oriCII* activity. The region is flanked by the two genes *rctA* and *rctB*. The position of the AT rich 13-mers, the DnaA-box and the 11-mer and 12-mer iterons are based on data published by Egan and Waldor (Egan and Waldor, 2003). The 39-mer and 29-mer RctB binding sites in *rctA*, *inc* and the *rctB* promoter respectively (Venkova-Canova and Chattoraj, 2011) are also indicated. B. Shows the regions cloned into pUT18*rctB*. The numbers below the lines are *V. cholerae* N16961 coordinates of fragment ends obtained from GenBank: AE003853.1. C. Shows the *oriCII* region cloned in combination with *rctB* or $rctB_{\Delta 497-658}$ to give the minichromosome p*inc*, p*inc*-$rctB_{\Delta 497-658}$, p*rctA* and p*rctA*-$rctB_{\Delta 497-658}$ respectively. The numbers below the lines are *V. cholerae* N16961 coordinates of fragment ends obtained from GenBank: AE003853.1.

29-mer in the *rctB* promoter region (Venkova-Canova et al., 2012). Further there is a link between replication and segregation of chromosome II (chrII) through the partitioning protein ParB2 which influence initiation of chrII replication by binding to *rctA*, while binding of RctB to *rctA* activates *parAB2* expression (Yamaichi et al., 2011).

The sequence of the 658 amino acid RctB protein shows no homology to known initiator proteins and there are no recognizable sequence motifs (Duigou et al., 2008). A specific residue R269 has been demonstrated to be involved in ATP binding (Duigou et al., 2008). The ATP-bound form of RctB is inactive for *oriCII* replication and mutations in this residue resulted in a protein insensitive to ATP inhibition of binding to *oriCII* (Duigou et al., 2008). In contrast the ATP-bound form of the replication initiator protein DnaA is required for initiation of replication from *oriC* of *E. coli* and from *oriCI* (Ozaki and Katayama, 2009; Koch et al., 2010).

E. coli expressing RctB supports replication of plasmids with *oriCII* as their sole origin (Egan and Waldor, 2003). *oriCII* minichromosomes carrying *rctA* are unstable in *E. coli* unless *rctB* is overexpressed (Duigou et al., 2008; Venkova-Canova et al., 2006; Venkova-Canova and Chattoraj, 2011; Yamaichi et al., 2011). The C-terminal 159 amino acids of RctB was found to be dispensable for initiator function but loss of this region altered RctB activity in such a way that it was still able to bind to *oriCII*, but unable to bind to *rctA*, hence C-terminal deletions in RctB led to increased copy number and stability of *oriCII* minichromosomes in *E. coli* (Yamaichi et al., 2011).

In this study we have isolated and characterized *rctB* mutations that result in an elevated copy number of an *oriCII* based minichromosome in the surrogate host *E. coli*. We have characterized the mutations alone or in combinations with deletions of *rctA* and *inc* regions. We have also assessed the ability of resulting mutant proteins to autorepress *rctB* transcription, and to dimerize (oligomerize).

2. Methods

2.1. Bacterial strains, plasmids, primers and growth conditions

All bacterial strains and plasmids are listed in Table 1. Primer sequences are listed in Supplementary data Table S1. The structure of all plasmids was confirmed by DNA sequencing. Cells were grown in LB medium (Bertani, 1951) or AB minimal medium (Clark and Maaløe, 1967) supplemented with 0.2% glucose (Glu), 0.5% casamino acids (CAA) and 10 μg/ml thiamine (ABTG-casa medium) at 30 or 37 °C. Antibiotics were used at the following concentrations: ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), and kanamycin (50 μg/ml).

2.2. Construction of minichromosomes and plasmids

For construction of *oriCII* minichromosomes, the *oriCII* regions equipped with NotI and BamHI restriction sites were PCR amplified from *V. cholerae* Bah-2. The NotI/BamHI digested PCR products were cloned into NotI/BamHI digested pSW29TSacB. The following primers were used: for p*oriCII* primer pair 4/1, for pΔ*rctA* primer pair 4/2, for pΔ*inc* primer pair 4/3, for p*oriCIIrctB*$_{\Delta 497-658}$ primer pair 5/1, for p*oriCIIrctB*$_{\Delta 495-658}$ primer pair 6/1, for p*oriCIIrctB*$_{\Delta 494-658}$ primer pair 7/1, for p*oriCIIrctB*$_{\Delta 493-658}$ primer pair 8/1, for p*oriCIIrctB*$_{\Delta 492-658}$ primer pair 9/1, for pΔ*rctA-rctB*$_{\Delta 497-658}$ primer pair 2/5 and for pΔ*inc-rctB*$_{\Delta 497-658}$ primer pair 3/5. p*oriCII-gfp* was constructed by cloning a BclI fragment containing a P_{BAD}–*gfp* cassette from pALO249 in the BamHI site of p*oriCII*. To enable a precise comparison of the copy number effects of the pointmutations in p*oriCIIrctB*$_{Y381N}$-*gfp* and p*oriCIIrctB*$_{L439H}$-*gfp* with the other mutations in *rctB* the *rctA-oriCII-rctB* region was PCR amplified from p*oriCIIrctB*$_{Y381N}$-*gfp* and p*oriCIIrctB*$_{L439H}$-*gfp*, respectively with the primer pair 1/4 and cloned into NotI/BamHI digested pSW29TSacB. For construction of an *oriCII* minichromosome without *rctB*, the region was PCR amplified from *V. cholerae* Bah-2 using the primer pair 1/28. The NotI/BamHI digested PCR products was subsequently cloned into NotI/BamHI digested pSW23T. DNA encoding RctB, $RctB_{L439H}$, $RctB_{Y381N}$ and $RctB_{R269H}$ was amplified from minichromosomes carrying no or the indicated mutations using primers 19/20. DNA encoding $RctB_{\Delta 498-658}$ was amplified from p*oriCII* using primer pair 19/21. The PCR products were cut with NdeI/HindIII and cloned into NdeI/HindIII digested pET26b.

2.3. Genomic blots

To determine the copy numbers of p*oriCII* derived minichromosomes DNA was isolated from cells growing exponentially at 37°C in ABTG-casa medium with kanamycin.