Identification of the region required for maintaining pHW126 in its monomeric form

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Abstract

The pHW126-like plasmids are a recently discovered small group of cryptic plasmids replicating by the rolling circle mode. The replication origin of pHW126 consists of a conserved stretch, four perfect direct repeats and a so-called accessory region. The latter increases plasmid stability but is not absolutely necessary for replication. Here, we report that deletion of the accessory region causes rapid multimerization of pHW126. While the number of pHW126-units per cell remains constant, the number of physically independent plasmid molecules is reduced by approximately 40%, rendering random distribution to daughter cells less effective. A conserved inverted repeat within the accessory region could be identified as a sequence necessary for maintaining pHW126 in its monomeric form. A mutant version of pHW126 lacking this inverted repeat could be rescued by placing the single-strand initiation site (ssi) of pHW15 on the plus strand, while including the ssi in the opposite direction had no effect. Thus, our data provide evidence that multimer formation is, besides copy number reduction and ssDNA accumulation, an additional means how loss of a mechanism ensuring efficient lagging strand synthesis may cause destabilization of rolling circle plasmids.

Introduction

Plasmids of bacteria appear in a wide variety of sizes, have different copy numbers and may encode various functions. Accordingly, plasmids have evolved different strategies for their maintenance. Huge circular plasmids usually replicate by the theta mechanism, are frequently self-transmissible and have a low copy number of just a few molecules per cell. Consequently, these plasmids depend on systems mediating partitioning, multimer resolution and postsegregational killing to ensure distribution to daughter cells. Small plasmids may also use the theta mode, but many of them replicate by the rolling circle mechanism or by strand displacement (del Solar et al., 1998; Rawlings & Tietze, 2001; Khan, 2005). Small plasmids are nonself-transmissible but may possess systems mediating mobilization in the presence of a conjugal plasmid (Francia et al., 2004; Garellan-Barea et al., 2009). Owing to their high copy number, small plasmids can rely on random distribution. The only additional function they require is a system to ensure that multimers do not accumulate (Thomas, 2000), which would cause plasmid instability in a process known as the ‘dimer catastrophe’ (Summers et al., 1993). Even this ability does not seem to be essential for rolling circle plasmids, as their replication strategy disfavour accumulation of multimers (Thomas, 2000). Nevertheless, small plasmids may contain stabilization systems.

Recently, a novel class of rolling circle plasmids, the pHW126-like plasmids, was described (Rozhon et al., 2010). Currently, just four members of this group are known: pHW126, pIGRK and pGMS31 (Smorawinska et al., 2012), which were isolated from Enterobacteriaceae, and the distantly related pRAO1 (Ogata et al., 1999), which was found in Ruminobacter amylophilus. These plasmids are characterized by low G+C contents of 32–40% and small sizes of < 3 kb. They possess just two genes: one encodes a replication protein and the other one a putative mobilization protein. The replication proteins of both pHW126 (Rozhon et al., 2011) and pIGRK...
(Mazurkiewicz-Pisarek et al., 2009) have been shown to exhibit Mn$^{2+}$-dependent nicking activity on their cognate supercoiled plasmid DNA, thereby creating the 3‘-OH responsible for priming leading strand DNA synthesis. While the replication proteins of the pHW126-like plasmids are clearly related, their mobilization proteins belong to different classes.

So far, only the replication mechanism of pHW126 has been investigated in more detail (Rozhon et al., 2011). As revealed by deletion analysis, the replication origin of pHW126 can be divided into three parts: a conserved stretch and four perfect direct repeats, both are essential for replication, and a so-called ‘accessory region’. The latter is not absolute necessary for replication but its deletion increased the plasmid loss rate significantly. Here, we provide evidence that this can be attributed to rapid plasmid multimerization.

**Materials and methods**

**Media and growth conditions**

*Rahnella* and *Escherichia coli* strains were grown in MLB medium (10 g L$^{-1}$ peptone, 5 g L$^{-1}$ yeast extract, 5 g L$^{-1}$ NaCl, pH 7) at 30 and 37 °C, respectively. When necessary, ampicillin (100 mg L$^{-1}$), or kanamycin (30 mg L$^{-1}$) were added to the medium. The strain *Rahnella* genomospecies 3 DSM 30078 was used as a host for all experiments and *E. coli* XL1-blue was used for DNA manipulation.

**Cloning, transformation of bacterial strains and assay for replication**

Constructs were prepared by cloning restriction or PCR fragments of pHW126 (Supporting Information, Tables S1 and S2) into pBKanTII by standard techniques (Sambrook & Russell, 2001). The identity of the constructs was confirmed by restriction analysis and sequencing. Transformation of *E. coli* and *Rahnella* and the assay for autonomous replication were performed as described previously (Inoue et al., 1990; Rozhon et al., 2006, 2011).

**Assay for multimerization**

Bacteria were freshly transformed with the desired construct and plated on MLB-plates containing the appropriate antibiotics. Single colonies were used to inoculate overnight cultures. Plasmid DNA was isolated using the Xact Mini Prep Kit (Genxpress, Wiener Neudorf, Austria) and immediately loaded onto a 0.7% agarose gel and run in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) containing 200 µL L$^{-1}$ ethidium bromide.

**Partial digestion and chloroquine gel electrophoresis**

Plasmid DNA was isolated from a gel using the Xact DNA Gel Kit. For partial digestion, aliquots of the eluate were incubated with the indicated amount of PstI in a total volume of 20 µL at 37 °C for 5 min. The reaction was terminated by addition of 5 µL stop buffer (100 mM EDTA, 100 mM Tris–HCl pH 8.0, 40% glycerol, 0.05% bromophenol blue) and immediately analysed by agarose gel electrophoresis as described previously. For chloroquine electrophoresis, aliquots of the eluate and, as a control, linearized plasmid DNA were mixed with loading buffer (2 mM EDTA sodium salt pH 8.0, 40% glycerol, 0.05% bromophenol blue) and chloroquine was added to the same concentration as used for gel electrophoresis. The samples were loaded onto a 0.8% agarose gel and run in TAE buffer containing 0, 3 or 9 mg L$^{-1}$ chloroquine at 2.5 V cm$^{-1}$ and 4 °C for 15 h. Subsequently, the gel was washed five times with water, stained with ethidiumbromide (1 µg L$^{-1}$) and destained with water prior to photography.

**Secondary structure prediction**

The secondary structure of the DNA was predicted with MFOLD (http://mfold.rna.albany.edu; Zuker, 2003) using the settings for DNA and allowing a maximal distance between paired bases of 50 bp.

**Results**

**Deletion of the accessory region causes plasmid instability because of multimer formation**

As shown previously, deletion of the accessory region causes destabilization of pHW126. Determination of the plasmid copy number by qPCR revealed that all tested constructs had a similar copy number of approximately eight copies per genome, irrespective of whether the accessory region was included or deleted (Rozhon et al., 2011). Thus, the increased plasmid loss rate could not be attributed to a reduced copy number. To investigate the role of the accessory region in more detail, we analysed undigested DNA of different constructs by agarose gel electrophoresis to detect possible topological changes. All constructs containing the accessory region, pHW126InS, pHW126ΔHH, pHW126ΔHB2 and pHW126ΔHB1, were present predominantly as one distinct band of the expected size. In contrast, pHW126ΔHH2 and pHW126ΔStH2, the two constructs with a deletion of the accessory region, showed a multiple band pattern.
The smallest bands of pHW126ΔHH2 and pHW126ΔStH2 had the expected size, while the larger bands migrated at positions expected for plasmid dimers, trimers and tetramers (Fig. 1a). To exclude that a contamination of the original DNA preparation was responsible for the observed pattern, the bands corresponding to the monomer and the putative dimer of pHW126ΔHH2 were cut out of the gel and the DNA was isolated. Transformation of Rahnella genospecies 3 DSM 30078 with the monomeric plasmid pHW126ΔHH2 and subsequent analysis of plasmid DNA isolated from the bacteria yielded the same pattern as originally observed (Fig. 1b). DNA isolated from bacteria transformed with the putative plasmid dimer showed also a multiple band pattern except that the monomer band was present only in small amounts. This indicates that the other DNA species are rapidly formed from monomeric pHW126ΔHH2 in vivo, while the reverse process is slow.

To provide further evidence that the additional bands represent supercoiled plasmid multimers, the putative dimer was isolated from a gel and partially digested with PstI. As indicated in Fig. 2a, digestion of the putative dimer led, at low PstI concentrations, to formation of a linear fragment with twice the size of the completely digested plasmid. This fragment is expected if just one PstI site of the dimer is cleaved. Addition of more enzyme led to the formation of the same product as observed for the monomer. Moreover, the DNA topology was analysed by agarose gel electrophoresis in the presence of chloroquine. This intercalating agent differentially affects the electrophoretic mobility of DNA containing distinct numbers of supercoils resulting in characteristic ladders. In contrast, linear or nicked DNA molecules migrate in the presence of chloroquine also as single bands (Molloy et al., 2004). As expected, in the absence of chloroquine, the isolated monomer and dimer migrated, like linearized DNA, as single bands (Fig. 2b, left panel; the trace amounts of open circle and linearized
molecules present in the preparations of pHW126AHH2 monomer and dimer originate from shearing forces during DNA purification). The linearized fragment showed one single band in the presence of chloroquine, while the plasmid monomer and dimer displayed a multiple band pattern as expected for supercoiled DNA. A similar effect was also observed for the trimer (data not shown). These results confirm that deletion of the accessory region induces rapid formation of supercoiled plasmid multimers.

Quantification of the different forms revealed that the DNA isolated from cells freshly transformed with monomeric pHW126AHH2 consisted of approximately 34% monomers, 41% dimers, 16% trimers and 10% tetramers or higher multimers. As mentioned earlier, a copy number of approximately 8 has been reported for the constructs shown in Fig. 1a (Rozhon et al., 2011). However, qPCR measures only the number of pHW126-units per genome. Taking the multimerization of constructs lacking the accessory region into account, their number of physically independent plasmid molecules per cell is significantly lower, particularly < 5 per genome, providing an explanation for the increased plasmid loss rate.

Mapping the region required for maintaining pHW126 monomeric

To map the genetic elements necessary for maintaining pHW126 in its monomeric state, we prepared a number of truncated versions of pHW126. The constructs pHW126AHH2 to pHW126-80 could replicate autonomously, while plasmids with larger deletions (pHW126-81 to pHW126-84) were replication deficient (Fig. 3a and b). This is in good agreement with a previous study, which showed that the HpolI-SpeI fragment contains the origin of replication (Rozhon et al., 2011). However, the data presented here have an improved resolution and allow assigning the 5′ end of the origin of replication to base pair 1689 (previously: 1669). This is also supported by the high homology of the plasmids pHW126, pIGRK and pIGMS31 in this region (Fig. 3c).

The constructs capable of autonomous replication were assayed for multimerization by gel electrophoresis analysis. pHW126AHB1, pHW126-75 and pHW126-76 were present as monomers. The monomer band was also dominant for construct pHW126-77, but also small amounts of the dimer could be observed. The remaining three constructs, pHW126-78, pHW126AHB2 and pHW126-80 accumulated high levels of multimers (Fig. 3b).

An alignment of pHW126 with its closest homologues pIGRK and pIGMS31 revealed a small but highly conserved sequence in this region (Fig. 3c). The distance of the conserved part and the replication origin was variable in pHW126, pIGRK and pIGMS31. To investigate whether the distance between the conserved stretch and the origin of replication is important for the prevention of multimer accumulation, the spacing was increased to more than 1000 bp by inserting a kanamycin resistance cassette. Only a small fraction of the obtained plasmid pHW126InKan was present as dimers and higher multimers were below the detection limit (Fig. 3b), indicating that the distance between the accessory region and the replication origin has only a moderate effect on multimerization.

The accessory region contains a single-strand initiation site (ssi)

Secondary structure prediction of the pHW126 accessory region indicated the presence of two stem-loop structures (Fig. 3d). The second stem-loop structure is also present in pIGRK and pIGMS31, suggesting a functional relevance of this inverted repeat. Indeed, deletion of this stem-loop structure induced multimerization, while no effect was observed for construct pHW126-76, which lacks the first inverted repeat (Fig. 3a and b). Stem-loop structures are common in single-strand initiation sites (ssis) crucial for priming lagging strand synthesis (Bahk et al., 1988; Novick, 1989; Nomura et al., 1991; Honda et al., 1993; Jeong et al., 1997; Kramer et al., 1997). The ssis of plasmids are often not conserved in plasmids of the same family (Kramer et al., 1998; Khan, 2005), which allows the substitution of the ssi of a certain plasmid with an ssi of another unrelated plasmid or even a phage (Tanaka et al., 1994). However, priming of lagging strand synthesis at an ssi is generally dependent on host factors (del Solar et al., 1987; Gruss et al., 1987). Consequently, an ssi is usually only fully functional in its original host or closely related species (Kramer et al., 1995; Meijer et al., 1995). Thus, to provide experimental evidence that a functional ssi site is necessary to prevent multimer formation of pHW126, we replaced the conserved stretch upstream of the pHW126 minimal replicon with the ssi of pHW15, a CoEl1-like plasmid originally isolated from Rahnella genomospecies 2 (Rozhon et al., 2006). As ssis function in an orientation-dependent manner (Gruss et al., 1987) and the direction of leading strand synthesis in pHW126 is unknown, we placed the ssi of pHW15 in construct pHW126ssi(+) on the plus strand to direct synthesis of the antisense strand and in construct pHW126ssi(−) on the minus strand for priming of the sense strand. As shown in Fig. 3a and b, placing the ssi of pHW15 on the plus strand could fully substitute the deleted ssi of pHW126 as indicated by the absence of multimers. In sharp contrast, placing the ssi on the opposite strand could not prevent...
accumulation of plasmid dimers and higher mers. This result confirms that a functional ssi site directing synthesis of the antisense strand is necessary to prevent multimer formation of pHW126 and denotes an ssi function to the accessory region.

Discussion

Recently, we have shown that deletion of the so-called accessory region of pHW126 causes plasmid instability (Rozhon et al., 2011). Here, we demonstrate that this can
be addressed to rapid plasmid multimer formation. Although the number of pHW126-units per cell remains constant, multimerization decreases the number of physically independent plasmid molecules by about 40% presumably rendering random distribution to daughter cells less effective. A conserved sequence within the accessory region was identified to be crucial for keeping pHW126 derivatives lacking the palindromic region can be rescued by the ssi of pHW15, a plasmid unrelated to pHW126, clearly indicates that ssi activity rather than a potential physically linked function is crucial for keeping pHW126 in its monomeric form. Single-strand initiation sites function in an orientation-dependent manner (Gruss et al., 1987). Thus, it was expected that the ssi of pHW15 would rescue the multimerization phenotype of pHW126 deletion versions only if inserted in an appropriate direction. Indeed, we found that functional substitution of the ssi of pHW126 was only possible by inserting the ssi of pHW15 into the plus strand and thus directing priming of the antisense strand, while placing the pHW15 ssi in the opposite direction had no effect. This result suggests also that the origin of replication placed in the minimal replicon directs synthesis of the sense strand. Thus, the structural organization of the pHW126 backbone displays a pattern typical for rolling circle plasmids: the rep gene encoding the replication protein is located downstream of the replication origin and a region providing ssi function is placed upstream of the origin. The sequence with ssi activity is often referred to as sso for single-strand origin. However, rolling circle plasmids may contain more than one ssi signal, and thus, we hesitate to conclude that the ssi identified here represents also the sso.

The result that deletion of the ssi causes accumulation of multimers is surprising because ssi signal lacking derivatives of several other rolling circle plasmids including pTI181 (Gruss et al., 1987), pMV158 (Kramer et al., 1995) and pM4 (Yin et al., 2009) were shown to display remarkably decreased plasmid copy number and accumulation of single-stranded DNA, while formation of multimers was not reported. We aimed to investigate whether deletion of the ssi of pHW126, in addition to multimerization, also induces accumulation of ssDNA, but failed to detect this molecular species by Southern blot analysis (data not shown). However, it must be emphasized that the amounts of ssDNA formed by several rolling circle plasmids may be very low. For instance, pMV158 replicating in Streptococcus pneumoniae forms minute amounts (Kramer et al., 1995) and in the case of pMV158 replicating in Bacillus subtilis (Kramer et al., 1995) or pGT232 (Heng et al., 1999), the abundance is undetectably low. In Rathibella cells containing wild-type pHW126, the ssDNA is likely converted efficiently to dsDNA by the ssi. In constructs lacking the ssi lagging strand synthesis may be primed to some extend at other sites and remaining ssDNA molecules may undergo recombination with ds plasmids to form di- and multimers as single-stranded DNA is known to be highly recombinogenic (Persky & Lovett, 2008).

Rapid multimerization has been reported for different rolling circle plasmids with a failure in termination of replication caused either by specific mutations in the rep gene (Projan et al., 1987; Bidnenko et al., 1993) or by a deletion of a signal in the 5′ part of the replication origin (Yasukawa et al., 1998). Both reasons can be excluded for our pHW126 derivatives because: (1) sequencing confirmed the absence of any mutations within the rep gene, (2) increasing the distance between the replication origin and the accessory region to more than 1 kb had only minor effects [in case of pKYM insertion of even 27 bp induced massive multimerization (Yasukawa et al., 1998)] and (3) the multimerization phenotype could be rescued by including the functional ssi signal of pHW15. Furthermore, insertion of foreign DNA into rolling circle plasmids may cause formation of high-molecular weight plasmid multimers by an as yet unknown mechanism (Gruss & Ehrlich, 1988, 1989). This high-molecular weight DNA is believed to be composed of head-to-tail linear plasmid multimers (Gruss & Ehrlich, 1988). In contrast, the multimers of pHW126 derivatives lacking the accessory region are clearly supercoiled circular DNA molecules. While multimers were rapidly formed from plasmid monomers, the reverse process was less efficient. Monomerization of dimers of rolling circle plasmids may happen if replication is initiated at one origin and terminated at the second origin (Gruss & Ehrlich, 1989). This has also been shown for pHW126 (Rozhon et al., 2010). However, the rate of this process seems to be insufficient to keep constructs lacking the accessory region as monomers.

In summary, our results provide evidence that derivatives of certain rolling circle plasmids lacking a functional ssi signal may undergo multimerization, which lowers their number of physically independent plasmid molecules per cell. Thus, multimer formation seems to be an additional means, besides copy number reduction and ssDNA accumulation, by which loss of genetic elements ensuring efficient lagging strand synthesis may cause plasmid destabilization.
Acknowledgements

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Plasmds and constructs used in this study.

**Table S2.** Primers used in this study.

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