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The segregation of *Escherichia coli* minichromosomes constructed *in vivo* by recombineering

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Abstract

Circularized regions of the chromosome containing the origin of replication, *oriC*, can be maintained as autonomous minichromosomes, *oriC* plasmids. We show that *oriC* plasmids containing precise, pre-determined segments of the chromosome can be generated by a simple *in vivo* recombineering technique. We generated two such plasmids carrying fluorescent markers. These were transferred to a recipient strain with a different fluorescent marker near the chromosomal copy of *oriC*. Thus the fates of the *oriC* plasmid and chromosomal origins could be followed independently in living cells by fluorescence microscopy. In contrast to a previous report, we show that there is a strong tendency of *oriC* plasmid copies to accumulate at the cell center as a single or double focus at the plane of cell division. This is not simply due to exclusion from the nucleoid space but rather appears to be a specific recognition and retention of the plasmid by some central-located cell site.

1. Introduction

E.coli minichromosomes are small autonomously replicating DNA molecules whose replication is driven by the chromosomal origin of replication *oriC*. They are often referred to as *oriC* plasmids. Isolation of F⁺ plasmids that replicate under *oriC* control has been reported by Hiraga (1976). Minichromosomes that replicate exclusively from *oriC* were isolated by *in vitro* ligation of chromosomal restriction fragments by Yasuda and Hirota (1977) and by *in vivo* recombination between specialized transducing phages by von Myenburg et al. (1979). Such *oriC* plasmids were found to be unstably inherited despite evidence that they were present at high average copy numbers. It was shown that they replicate under normal *oriC* control, but have no means of equitable segregation to daughter cells (Jensen et al., 1990; Leonard and Helmstetter, 1986). Thus, the copy number in some cells increases at the expense of others, and the overall copy number in the population depends on the strength of the antibiotic selection used to maintain them. In addition, multiple plasmid copies are grouped into a limited number of foci, further increasing segregational instability (Niki and Hiraga, 1999). Here we show that *oriC* plasmids containing precise, pre-determined regions of the chromosome can be generated by *in vivo*

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recombination. By following the fate of these plasmids by fluorescence microscopy in living cells, we study their properties and find that their spatial distribution differs radically from that previously described (Niki and Hiraga, 1999).

2. Methods

2.1 Bacterial Strains and Plasmids

All bacteria are derivatives of *Escherichia coli* K12. LT521 is MG1655 λ cl857 Δ (*cro-bioA*)] *pgl* Δ 8 *nadA::Tn10*. CC2373 is MG1655 Δ *lacI-lacA* containing the pMT1 *parS* site inserted at 84.3' min. with a linked chloramphenicol-resistance marker. The *parS* insertion was made as described (Nielsen et al., 2006b), using the primer pair:

5'GCAGGATGTTTGATTAAAAACATAACAGGAGAAAAATGCAGGAAGCGGA
ATTCCGGACC and
5'AATCGGTTACGGTTGAGTAATAAATGGATGCCCTGCGTAACTCGAGATGC
A GAAGACGCA to generate the PCR fragment. CC2535 was derived from CC2373 by deletion of the *recA* gene. CC4749 is MG1655 Δ *lacI-lacA* containing a P1 *parS* site inserted at 84.3' min. with a linked kanamycin-resistance marker (Nielsen et al., 2006a). The P1ParB-CFP and pMT1ParB-yGFP proteins were supplied from the pBR322-based plasmid pFHC2973 (Nielsen et al., 2006b), or, in the case of the cells in Fig. 6, from its derivative pALA2819, that has a modified -10 *plac* promoter sequence TATAA in place of CATTA in the *lacZ* promoter region upstream of the P1*parB-CFP* gene and a chromosomal *seqA* constitutive promoter sequence (AGCTTCTTTTTTCGCGTTGTCGGGTTAGGGTATTAT) inserted in the intergenic region between the P1*parB-CFP* and pMT1*parB-yGFP* genes.

2.2 Media

LB broth and AB minimal glucose media were as previously described (Nielsen et al., 2007). Kanamycin was used at 30 μ g/ml and Ampicillin was used at 30 μ g/ml where appropriate.

2.3 Primers and PCR conditions for recombineering

The following primer pairs were used to make the vector DNA to produce plasmids, *poriC-2* and *poriC-4*. The plain segments are homologous to the chromosome target sites, whereas, boldface sequence is homologous to the *kan/parS* cassette.

Primer pair for *poriC-2*

5' TGTTCCAGCGCTACGCTCAAAGGAGATATGAACCACTTCCATATCATG
CGATAAAAAGCCGAAGCCTTA
5'
TTTTATACGACACGCGGCATACCTCGAAGGGAGCAGGAGTGAAAAACGTG
CACAGCTTTAGAGCGTTTTGC

Primer pair for *poriC-4*

5' GTCGGTTTTTTGTTGCTTAATCATAAGCAACAGGACGCAGGAGTATAAAAA
CGATAAAAAGCCGAAGCCTTA
5'
TTTTATACGACACGCGGCATACCTCGAAGGGAGCAGGAGTGAAAAACGTG
CACAGCTTTAGAGCGTTTTGC

Platinum® *Taq* polymerase (Life Technologies, Carlsbad, CA) was used according to manufacturer protocol. One µl of an overnight culture of CC4749 was used for template. The PCR conditions were as follows: 94° - 2min, then 94° - 30s, 55° - 30s, 68° - 2min for 10 cycles followed by 94 - 30s, 55° - 30s, 68° - 2min + 5s/cycle for 30 cycles, and finally, 68° for 10min then hold at 4°. The ~1.6Kb fragment containing the kanamycin-resistance gene, P1 *parS*, and homologies to regions flanking the *E. coli oriC* (Fig. 1) was cleaned up using a Qiagen QIAquick® PCR purification kit (Qiagen, Valencia, CA).

2.4 Protocol for isolating *oriC* plasmids

The *oriC* plasmids were constructed using recombineering as follows. LT521 was made competent for Red-mediated recombination and electrotransformation by standard protocols (Sawitzke et al., 2007; Sharan et al., 2009). 1µl (~100ng) of the linear DNA PCR product containing the P1 *parS* site and a kanamycin-resistance gene (Fig. 2B) was electrotransformed into these cells using standard methods (Sawitzke et al., 2007; Sharan et al., 2009). After electroporation, 1ml of LB broth was added and cells allowed to recover at 32°C for 30 minutes after which a 0.1ml aliquot of culture and the remainder of the culture, spun down and resuspended in 0.1ml LB broth, were spread on sterile nitrocellulose filters (100mm) resting on an LB agar petri plate. These plates were incubated at 32°C for 4 hours after which the filters were shifted to LB + Kan plates and incubated at 32°C overnight (Yu et al., 2000). The next day candidate colonies were picked and single colony purified on LB + Kan plates and incubated at 32°C. An isolated colony was used to inoculate a 5ml overnight culture in LB broth and grown at 32°C to saturation. The *oriC* plasmid DNA was isolated from this culture using a Qiagen mini-prep kit following the manufacturers protocol (Qiagen, Valencia, CA). This purified DNA was used to transform electroMAX™ DH5α competent cells (Life Technologies, Carlsbad, CA) to kanamycin-resistance on LB + Kan plates. An isolated colony was used to inoculate a 100ml culture in LB + Kan which was grown to saturation and used to make a midi-prep (Qiagen, Valencia, CA). Midi-prep DNA was visualized on agarose gels (data not shown) and was used to transform CC2373 or CC2535.

2.5 Microscopy

Cultures were grown in AB minimal glucose casamino acids medium at 32°. Fluorescence microscopy was carried out as previously described (Nielsen et al., 2006b) and image data collected and analyzed automatically as described by Nielsen and Hansen (2010).

3. Results

3.1 Direct generation of *oriC* plasmids by recombineering

Recombineering is an efficient method of *in vivo* genetic engineering that exploits the properties of the phage γ Red recombination system (Yu et al., 2000). Among many applications is the ability to rescue precisely defined segments of the *E.coli* chromosome onto a plasmid vector, thus circumventing the need for the multiple steps required to clone chromosomal genes onto plasmid vectors by conventional genetic engineering (Court et al., 2002). This is achieved by recombination between a linearized plasmid DNA whose ends provide short regions of homology to the targeted chromosomal region in an “ends-in” configuration (Hastings et al., 1993) ; Fig. 1A.). Such substrates can be made readily by PCR amplification of the plasmid vector sequences using primers that incorporate the appropriate chromosomal homologies. This generates a linear DNA with the plasmid origin and selectable marker flanked by the chromosomal homologies (Fig. 1A). These are then introduced into a strain that supplies the γ Red proteins and the desired products can be recovered by simple plasmid preparation procedures (see Methods). We modified this procedure as illustrated in Fig. 1B. Here, the vector DNA contains a selectable gene, but no

replication origin. On rescuing a chromosomal segment, the resulting circular DNA has no means of replicating and cannot be maintained. However, if the rescued segment spans the chromosomal origin of replication *oriC*, an *oriC* plasmid is generated that is maintained as a plasmid under sole control of the included chromosomal origin (Fig. 1B.).

We carried out this procedure attempting to rescue two different overlapping sequences that include *oriC* and surrounding genes (see Methods and Fig. 2A). After the recombineering steps, plasmid DNA was isolated and used to transform strain DH5 α to kanamycin resistance. Transformants were obtained at a frequency of approximately one per hundred recipient cells for each isolate, suggesting that the targeted sequences had been rescued successfully and that the resulting plasmids could replicate. In the recipient DH5 α cells, the rescued region is also present on the host chromosome and thus the cells were merodiploid for the rescued sequences (data not shown). Consistent with the properties of conventionally constructed *oriC* plasmids, the plasmids were segregationally unstable and were progressively lost from the population in the absence of antibiotic selection (data not shown).

3.2. Localizing the *oriC* plasmids by fluorescence microscopy

Each of the *oriC* plasmid constructs included a P1*parS* site within the vector segment (Fig. 1B) designed to give fluorescent foci when the P1 ParB-CFP protein is supplied *in trans* (Fig. 2B). The P1ParB-CFP protein binds specifically to the P1*parS* site to form a core complex. Additional P1ParB-CFP proteins then bind to the complex and spread out on the surrounding DNA sequence to form a multi-subunit array which can readily be seen as a bright blue fluorescent focus using the fluorescence microscope (Nielsen et al., 2006b). Thus the fate of the *oriC* plasmids can be followed in living cells. Minichromosome DNA preparations from the DH5 α transformed strains were used to transform strain CC2535 by electrotransformation selecting for kanamycin resistance. The recipient strain has a pMT1*parS* marker at 84.3 min close to the chromosomal copy of the replication origin. The pMT1*parS* site binds the fluorescent pMT1 ParB-yGFP protein forming a yellow-green focus (Nielsen et al., 2006b). The purified isolates were then transformed with a plasmid that expresses both P1 ParB-CFP and pMT1 ParB-yGFP. Thus the fates of the *oriC* plasmid and chromosomal origins could be studied independently by fluorescence microscopy of the living cells (Nielsen et al., 2006b). Typical results are illustrated in Fig. 3. For clarity the *oriC* plasmid foci are false colored red, and the chromosomal origin foci are false colored green. In the majority of cases, the red and green foci are separated and differ in number or pattern consistent with the presence of free *oriC* plasmid copies. However, approximately 4% of the cells show coincidence of the two labels for each focus (figure 3). These may be examples of the ability of *oriC*-containing plasmids to integrate into the host chromosome in a recombination-deficient strain (Masters et al., 1979). Integration of the plasmid via its chromosomal homology would yield a composite chromosome with the two types of label relatively close together on the DNA.

3.3. Analysis of the numbers of fluorescent foci

With a few exceptions, the red and green foci were seen at different locations from each other in the cell. As is normal for this growth rate, where the *E. coli* chromosome typically has three pairs of active replication forks (Nielsen et al., 2007), most cells have two-to-four green chromosomal foci (Fig. 3,4). The average number of green foci in these cells was 2.6 per cell, consistent with the average number of origins in these cell populations (Nielsen et al., 2007). The average number of *oriC* plasmid foci was 1.8. However, Southern analysis of the total DNA extracted from the cells suggests that they have considerably more plasmid origins than chromosomal origins (ca. 4-fold, data not shown). Thus, it is likely that each plasmid focus contains more than one copy of the plasmid. The distribution of numbers of

foci and the presence of multiple copies of the plasmid in each focus are consistent with previous observations, where conventionally derived *oriC* plasmid foci were detected in fixed cells by fluorescence *in situ* hybridization (Niki and Hiraga, 1999).

3.4. Accumulation of *oriC* plasmids at the cell center

For cells labeled at the chromosomal origin there are generally two foci at cell birth and four prior to cell division under our growth conditions (Nielsen et al., 2007). Foci in the two foci cells are distributed along the long cell axis to form two well separated peaks at the cell quarter positions. Foci in the four foci cells are distributed in four separated peaks at the cell eighth positions (Fig. 5, (Nielsen et al., 2007)). Occasional cells have foci that have not yet duplicated their origins in an initial round of replication (Fig. 4) and have a single origin focus broadly distributed along the cell axis, (Nielsen et al., 2007). On average, there are fewer *oriC* plasmid foci than the chromosomal foci, and many cells lack a plasmid focus altogether (Fig. 3,4). The distribution of foci is generally less orderly than that of the chromosomal foci. Niki and Hiraga reported that many of these *oriC* plasmid foci were found in the cytoplasmic spaces near the cell poles (Niki and Hiraga, 1999). Fig. 5 shows an analysis of 2000 cells transformed with the *poriC-2* plasmid and measured automatically (see Methods). In sharp contrast to the Niki and Hiraga result, we found that the cell center is by far the most likely position to find an *oriC* plasmid focus. 34% of the cells had a focus within the central 10% of the cell length. Examples for *poriC-4* are shown in Fig. 6 and other examples can be seen for *poriC-2* in Fig. 3. Central foci consisted of a single focus or two foci side-by side on the plane of division. There was only a slight tendency for foci to accumulate at the cell poles. Only 7% of the cells had an *oriC* plasmid focus within the 10% of the cell length nearest to a pole. This is most readily seen in cells with two plasmid foci (Fig. 5). The presence of a single central focus in some cells at a late stage in cell division (Fig. 6) suggests that they may be about to divide to yield one plasmid free cell and one with a polar focus. However, as two foci are often present and multiple plasmids are present in each focus, we cannot rule out the possibility that such divisions yield a more equitable segregation that distributes plasmid copies to each of the daughter cells.

4. Discussion

We demonstrate that *E.coli* minichromosomes (*oriC* plasmids) can be generated in a single step by *in vivo* recombineering. The method should allow the generation of plasmids carrying the *oriC* locus and any defined chromosomal region that surrounds it, assuming that the resulting plasmid can be maintained. Generation of such plasmids with very large chromosomal regions should be possible, and may help to locate any sequences that might be involved in the organization or segregation of the chromosomal DNA. In choosing the sequences targeted in this paper, we deliberately avoided including the ATP synthase genes that are known to be toxic when present at higher than normal copy number (Hansen et al., 1981). It is possible that other toxic genes may be encountered in isolating large *oriC* plasmids. However, the flexibility of recombineering as a tool for generating defined deletions (Yu et al., 2000) may allow the generation of plasmids lacking toxic loci but retaining all other genes in a region.

Like minichromosomes observed by others, the new *oriC* plasmids are segregationally unstable. Analysis of the living cell population by fluorescence microscopy shows that plasmid-free cells accumulate in the population even under selection, emphasizing their unstable nature. The average plasmid-containing cell has greater than ten copies of the plasmid, but only 1 to 5 plasmid foci. This is fully consistent with results obtained with conventionally isolated *oriC* plasmids detected in fixed cells by fluorescence *in situ* hybridization (Niki and Hiraga, 1999). However, the distributions of the plasmid foci in the cells in our study differ radically from those previously published. Rather than finding the

majority of foci at the cell poles (Niki and Hiraga, 1999), we found that most foci were present at the cell division plane at the cell center. The source of this difference might be the method of focus detection, the use of living rather than fixed cells or to the growth conditions (our cells were grown in minimal glucose casamino-acids medium rather than minimal glucose medium). However, it is also possible that the difference is derived from the culture history of the cells. Division of cells with central foci should give rise to polar foci in the daughters. We see relatively few cells with polar foci in our newly established populations. However, as the plasmids lack an active segregation mechanism some may become trapped in the poles permanently. Such cells would accumulate in the population with time and thus polar foci would become prominent in older cultures.

Why do the *oriC* plasmids accumulate at the cell center? It is unlikely that this is due to a tendency to accumulate in the nucleoid-free cytoplasm (Niki and Hiraga, 1999). If cytoplasmic regions were favored as the primary position for plasmid localization, we would see more foci at the cell poles than at the center. The center only becomes nucleoid free at late stages of cell growth. The cell center is a site at which the chromosomal origin normally replicates (Koppes et al., 1999). Perhaps the origins of the *oriC* plasmids attach to the cellular replication machinery at the cell center, and being unable to segregate, they remain there. Alternatively, the plasmids may interact with the centrally located FtsK protein. This protein recognizes polarized sequences (KOPS) that are found asymmetrically arrayed around the chromosome, including the chromosomal regions present on the *oriC* plasmids. Interaction of KOPS sequences with FtsK normally directs the chromosome terminus to the cell center prior to cell division (Bigot et al., 2005). However, when acting on the relatively small plasmid, the equivalent interaction would likely constrain the whole plasmid to the cell center.

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Highlights

1. Defined *oriC* plasmids are constructed *in vivo* by recombination
2. *oriC* plasmids localize at the site of the cell division plane
3. This technology can be used to map chromosome organization and segregation functions.

Figure 1. Recombineering pick-up strategy for production of *oriC* plasmids

A. Pick up of a defined chromosomal segment by recombineering. A linear plasmid vector sequence containing the plasmid origin and a selectable marker is produced by PCR and has short primer sequences placed in an “ends in” configuration with respect to their homologies on the chromosome target. On introduction into a cell that expresses the γ Red proteins, recombination picks up the targeted sequence as a plasmid under vector control. B. The linear sequence has only a selectable marker and a *parS* site for a fluorescent tag. If the chromosomal sequence picked up contains the chromosomal origin *oriC*, an autonomous minichromosome (*oriC* plasmid) is produced that can replicate under *oriC* control and can be followed in living cells by its fluorescent tag.

Figure 2. Genetic maps of the components of *oriC* plasmids made by recombineering
 A. The chromosomal segments picked up in plasmid *poriC*-2 (upper), and *poriC*-4 (lower).
 B. The cassette sequences present in the two plasmid constructs. Recombineering (Fig. 1) joins the chromosomal and cassette sequences to form a circular plasmid in the relative orientations shown here.

Figure 3. Double-labeled cells containing the plasmid *poriC-2*

The cells were derived from strain CC2535 and the fluorescent ParB proteins supplied from plasmid pFC2973. Cells are labeled near the origin of replication of the chromosome (green) and on the *oriC* plasmid *poriC-2* (red). Note that some cells appear to lack the plasmid, and the plasmid is present as one or two foci at the cell center in many others. Only a minority of cells has plasmid foci at the cell poles.

Figure 4. Distributions of numbers of foci per cell

Histogram of the number of cells containing a given number of foci for the chromosomal origin marker (black bars) and the *porC-2* plasmid (grey bars) in the cell population shown in Fig. 3.

Figure 5. Histograms of numbers of cells with foci at a given relative position on the long cell axis
 The histograms were generated from data collected automatically from the population shown in Fig. 3. Numbers are normalized such that the area under each graph is equal to one. Panels at left show chromosomal foci and at right, plasmid foci. Upper panels: cells with one focus. Center panels: cells with two foci. Lower panels: cells with four foci. a). Chromosomal foci (green) show typical chromosome origin distributions with one broadly distributed focus, two foci at the cell quarter positions, or four foci at the cell eighth positions. Plasmid *poriC-2* foci are mostly at the cell center (cells with one or two foci). The very few cells with four foci appear to show a more random pattern of distribution, but the numbers of cells are too low to reveal more than a general trend.

Figure 6. Double labeled cells containing the plasmid *porC-4*

The cells were derived from strain CC2535. The fluorescent ParB proteins were supplied from plasmid pALA2819. Selected cells from a single field are shown. Note the frequent cells with one or two central *oriC* plasmid foci. The arrow indicates a cell that is in a late stage of cytokinesis with a single plasmid focus. This may give rise to a cell with no foci and one with a polar focus.