Study of the organization of the genomes of Escherichia coli, Brucella melitensis and Agrobacterium tumefaciens by insertion of a unique restriction site

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INTRODUCTION

The bacterial chromosome has long been considered to be a unique circular molecule. In the last 15 years, a few reports proved that this is not always the case: several bacterial species have a linear chromosome (for a review see Hinnebush & Tilly, 1993), and others have multiple chromosomes (for a review see Cole & Saint Girons, 1994).

The experimental determination of genome topology makes use of pulsed-field gel electrophoresis (PFGE), which allows separation of linear genome-sized DNA molecules, but not of circular DNA molecules larger than 15 kb, which are excluded by the agarose gel. Usually a small proportion of circular DNA molecules suffer a random double-strand break during preparation and are thus linearized, producing a diagnostic faint band (Sobral et al., 1991). A much better distinction between circular and linear genomes could be made if these had an unique restriction site for specific and quantitative linearization of circular genomes or cutting linear genomes into two parts. In this paper we describe how this can be achieved by delivering a Tn5 derivative carrying the I-SceI restriction site. I-SceI is an endonuclease encoded by a group I intron of the Saccharomyces cerevisiae mitochondrial 21S rRNA gene (Monteilhet et al., 1990; Perrin et al., 1993). I-SceI recognizes, with high specificity, a non-symmetrical double-stranded sequence extending over 18 bp. In optimum conditions, no cleavage sites were found in the entire yeast nuclear genome, or in a range of bacterial genomes.

A technique combining transposon delivery using a wide-host-range, conjugative, suicide plasmid vector, I-SceI digestion of genomic DNA, and PFGE, was tested on three bacterial species with known genome topology: Escherichia coli, which contains a single circular chromosome, Brucella melitensis, which possesses two circular chromosomes (Michaux et al., 1993), and Agrobacterium tumefaciens C58, which is believed to contain both a 3 Mb circular and a 2.1 Mb linear chromosome together with two circular plasmids of 550 and 200 kb (Allardet-Servent et al., 1993).

Keywords: bacterial chromosomes, unique restriction site, genome organization, physical mapping
METHODS

Strains and growth conditions. Bacterial strains and plasmids are listed in Table 1. E. coli and A. tumefaciens were grown in LB medium at 37 or 28°C respectively. B. melitensis was grown at 37°C on Brucella Agar or in TSB (Gibco). When required, media were supplemented with ampicillin (100 µg ml⁻¹), streptomycin (100 µg ml⁻¹) and kanamycin (25 µg ml⁻¹). A spontaneous streptomycin-resistant mutant of B. melitensis Ether was selected by plating on Brucella Agar containing streptomycin. Its agglutination, phage sensitivity, and restriction profile in PFGE were identical to those of the parental strain. Plating A. tumefaciens C58 on medium with 25 µg kanamycin ml⁻¹ gave rise to a high frequency of resistant mutants. To avoid this problem, selection of KanR was performed by standard filter mating techniques. SM10pir(pGF2) was mixed with E. coli HB101, B. melitensis EtherSM1R or A. tumefaciens C58 (donor: recipient ratio 1:5) in a 5 ml syringe and filtered onto a 0.45 µm filter. The filter was then recovered in LB and plated onto media containing streptomycin and kanamycin to select transposition events. Colonies were visible after overnight incubation (E. coli) or 3 d (Brucella and Agrobacterium).

Endonuclease digestion of high-molecular-mass genomic DNA. Intact genomic DNA was prepared in agarose plugs as described by Schwartz et al. (1983). The cells were harvested in exponential growth. The usual DNA concentration (1 ×) was about 2 µg per plug but 5 × concentrated plugs were used when indicated. Digestions with Sall and PciI were performed as previously described (Allardet-Servent et al., 1993; Michaux et al., 1993). Conditions of I-SceI digestion, defined by the manufacturer (Boehringer Mannheim), were further optimized, first following previously described indications (Thierry et al., 1991) and then as follows: the plugs were preincubated at 4°C with the enzyme in the complete buffer for 100 min, in order to allow the enzyme to diffuse, and then transferred at 37°C for 15 min for DNA digestion. Double digestions were done sequentially. The first enzyme was eliminated by overnight incubation of the plug at 37°C in 0.5 M EDTA (pH 8), 1 mg proteinase K ml⁻¹ and 1 % (w/v) N-lauroyl sarcosine. Plugs were then washed twice in TE-PMSF (10 mM Tris pH 8, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and three times in TE (10 mM Tris pH 8, 0.1 mM EDTA) before digestion with the second enzyme.

PFGE of intact and digested DNA. PFGE was performed in a contour-clamped homogeneous electric field apparatus (Chu et al., 1986) CHEF-DRII (Bio-Rad) in 0.5 × TBE (Sambrook et al., 1989). S. cerevisiae chromosomes (BioRad) served as size standards.

Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics/genotype</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td>E. coli S100pir</td>
<td>thi thr leu tonA lacY supE recA: RP4-2Tc::Mu Km ppir</td>
<td>Miller &amp; Mekalanos (1988)</td>
</tr>
<tr>
<td>SY327pir</td>
<td>Δ(lac-pro) argE(Am) rif nalA recA ppir</td>
<td>Miller &amp; Mekalanos (1988)</td>
</tr>
<tr>
<td>HB101</td>
<td>hsdS recA ara proA lacY galK rpsL xylL mtl supE</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>HB101a1</td>
<td>Tn5Map in the chromosome</td>
<td>This study</td>
</tr>
<tr>
<td>HB101a2</td>
<td>Tn5Map in the chromosome</td>
<td>This study</td>
</tr>
<tr>
<td>Brucella melitensis Ether</td>
<td>Biovar 3</td>
<td>ATCC 23458</td>
</tr>
<tr>
<td>EtherSM1R</td>
<td>Streptomycin resistant</td>
<td>This study</td>
</tr>
<tr>
<td>EtherSM18R</td>
<td>Tn5Map in the 2.2 Mb replicon, streptomycin resistant</td>
<td>This study</td>
</tr>
<tr>
<td>EtherSM28R</td>
<td>Tn5Map in the 1.1 Mb replicon, streptomycin resistant</td>
<td>This study</td>
</tr>
<tr>
<td>Agrobacterium tumefaciens C58</td>
<td>Streptomycin resistant</td>
<td>X. Nemes (Lyon, France)</td>
</tr>
<tr>
<td>C58o1</td>
<td>Tn5Map in the 3.0 Mb replicon</td>
<td>This study</td>
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<tr>
<td>C58o2</td>
<td>Tn5Map in the 2.1 Mb replicon</td>
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<td>C58o3</td>
<td>Tn5Map in pArtC58 (550 kb)</td>
<td>This study</td>
</tr>
<tr>
<td>C58o4</td>
<td>Tn5Map in pTi (200 kb)</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids CoEl::Tn5Map</td>
<td>Derivative of Tn5o containing the 18 bp I-SceI site</td>
<td>Hiller et al. (1994)</td>
</tr>
<tr>
<td>pGF2</td>
<td>pGP704 containing Tn5Map</td>
<td>This study (Fig. 1)</td>
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markers. Details of migration conditions are given in the figure legends.

Southern blots and hybridization. Southern blots of PFGE agarose gels were prepared using nylon membranes (Boehringer Mannheim) by vacuum blotting using a VacuGene XL Blotting System (Pharmacia LKB) with 0.4 M NaOH as the eluant. Probes were labelled by a non-isotopic method (Digoxigenin DNA labelling kit, Boehringer Mannheim). Non-digested 2.1 Mb replicon probe was purified by Gene Clean II (Bio-101) after separation in low-melting-temperature agarose (Seaplaque) and then labelled by the same method.

RESULTS

Construction of pGF2

ColEl::Tn5Map, a derivative of ColEl::TnSeq (Zuber & Schumann, 1991) containing the 18 bp I-Scel recognition site inserted into the unique BamHI site, was available (Hiller et al., 1994). To facilitate the transfer of Tn5Map to a wide range of bacterial species, it was first subcloned into the suicide vector pGP704 (Miller & Mekalanos, 1988). This plasmid has the R6K origin of replication and the oriT sequence from RP4. The R6K origin requires for its function the R6Kγ protein encoded by the pir gene, which can be provided in trans. A 7541 bp fragment containing Tn5Map was excised from ColEl::Tn5Map by first linearizing the plasmid with AluI, filling in the overhangs with Klenow enzyme, and then recutting with EcoRI. The 7541 bp fragment was ligated into pGP704, which had been digested with EcoRI and Smal, resulting in pGF2 (Fig. 1). The permissive host E. coli SY327pir was transformed with the ligation mixture and ampicillin- and kanamycin-resistant transformants were selected. pGF2 was then transferred to E. coli SM10pir, which provides the RP4 tra functions, for conjugation experiments.

Delivery of Tn5Map by conjugation

Fig. 1. Structure of plasmid pGF2.

pGF2 was transferred by conjugation from E. coli SM10pir into E. coli HB101, B. melitensis EtherRsmRK and A. tumefaciens C58. Kanamycin- and streptomycin-resistant exconjugants were selected. Undigested DNA from individual clones was separated by PFGE, and tested for the presence of Tn5Map by hybridization using labelled ColEl::Tn5Map as probe (Fig. 2). E. coli HB101 and B. melitensis possess, respectively, one and two circular chromosomes which migrate as faint bands in PFGE. Hybridization showed the presence of Tn5Map in the transconjugants. Eight B. melitensis transconjugant clones were studied: two carried the Tn5Map on the 1.1 Mb chromosomes, while the others carried it on the 2.2 Mb chromosome (data not shown). None of the clones had Tn5 simultaneously in both chromosomes. Separation of the two large replicons of A. tumefaciens requires long pulse times: the faint band corresponded to the 3 Mb circular chromosome and the intense band to the 2.1 Mb linear chromosome. Two faint bands, corresponding to the cryptic circular plasmids pAtGC58 (550 kb) and the tumour-inducing plasmid pTi (200 kb), were separated using shorter pulses. The three circular replicons were visible only when plugs containing a high DNA concentration (5X) were used. At this concentration, the 2.1 Mb linear chromosome migrated as a thick and intense band of DNA. Eleven A. tumefaciens transconjugant clones were studied: one of them carried the Tn5 on the 2.1 Mb chromosome, one on the 550 kb plasmid, one on the 200 kb plasmid and eight on the 3.0 Mb chromosome. Four transconjugants with Tn5Map integrated in each of the four replicons were kept for further study.

Digestion of genomic DNA with I-Scel

Conditions for the I-Scel digestion of bacterial genomes in agarose plugs were optimized using B. melitensis; the presence of two circular replicons, one without a I-Scel site, allowed the assessment of the levels of non-specific activity. The conditions described in Methods gave optimal results. Longer digestion times increased the levels of non-specific degradation of the DNA. This non-specific degradation was caused either by the I-Scel enzyme or by contaminants in the enzyme preparation. No non-specific degradation was seen when plugs were incubated in TE buffer or reaction buffer without the enzyme (data not shown). Since no discrete new fragments appeared on prolonged incubation, it is unlikely that cutting occurred at secondary I-Scel sites, which may occur if suboptimal magnesium concentrations are used (Thierry et al., 1991).

Plugs containing genomic DNA from E. coli HB101 and B. melitensis were digested with I-Scel and compared with non digested controls (Fig. 3). Wild-type HB101 was compared with two clones containing Tn5Map insertions (HB101ω1 and HB101ω2). Wild-type B. melitensis Ether was compared with Ethero1 (Tn5Map in the 2.2 Mb replicon) and Ethero2 (Tn5Map in the 1.1 Mb replicon). Digestion with I-Scel specifically increased the intensity of the bands corresponding to the replicons containing Tn5Map, consistent with linearization of circular molecules. Despite optimization of reaction conditions, the increase in the intensity of the bands after digestion was always relatively faint, suggesting partial digestion. To
Fig. 2. Tn5Map insertion in E. coli HB101 (HB101), B. melitensis Ether (B. m.) and A. tumefaciens C58 (A. t.) genomes. ω, strains with Tn5Map insertions (see Table 1); wt, wild-type strains. (a) Ethidium-bromide-stained PFGE gel of 5× concentrated undigested DNA samples; S. cerevisiae (S. c.) chromosomes were used as size markers. (b) Southern transfers of the gels shown in (a) probed with labelled Tn5Map DNA. Migration conditions for E. coli and B. melitensis DNAs: pulse ramp 60-130 s at 180 V in a 0.8% agarose gel for 24 h; and for A. tumefaciens DNA: pulse ramp 90-300 s at 150 V in a 0.8% agarose gel for 45 h (lanes marked *) or a fixed pulse of 40 s for 20 h at 200 V in a 1% agarose gel (lanes marked **).

test this hypothesis a B. melitensis Ethero2 DNA preparation was first digested by I-SceI, which was then eliminated by proteinase K, and then digested by SpeI. The SpeI fragment containing the I-SceI site totally disappeared, giving rise to two new fragments, indicating that the Tn5Map insertion is unique in the B. melitensis genome and that the I-SceI digestion was complete (data not shown).

Analysis of the genome of A. tumefaciens C58

We used digestion by I-SceI to study the genome of A. tumefaciens C58. The experiments presented in Fig. 2 identified C58 derivatives ω1 to ω4 containing Tn5Map inserted in each of the four replicons. Fivefold-concentrated plugs were used to visualize the four replicons on ethidium-bromide-stained gels. For the experiments described below, plugs containing 1× DNA concentration were used. With this concentration only the 21 Mb replicon is visible in the PFGE gel. I-SceI-digested and undigested samples were again compared (Fig. 4). I-SceI digestion of C58ω1 intensified the 3 Mb band, consistent with linearization of a circular DNA molecule (Fig. 4a).

For the analysis of the 21 Mb chromosome and the plasmids, shorter pulse times were used. After digestion of C58ω3 and C58ω4 DNA with I-SceI, bands at 550 kb
and 200 kb, respectively, appeared, consistent with the linearization of the circular molecules corresponding to the large cryptic plasmid pAtC58 and the tumour-inducing plasmid pTi (Fig. 4b).

C58ω2 carries Tn5Map in the 2·1 Mb replicon. Digestion of wild-type C58 DNA with I-SceI did not alter the intensity or the apparent size of the 2·1 Mb replicon. Digestion of C58ω2 DNA with I-SceI cleaved the 2·1 Mb
band and produced new fragments of 1150 kb and 940 kb (Fig. 4b), consistent with the presence of a single I-SceI site in a linear molecule of 2.1 Mb. A probe prepared from gel-purified 2.1 Mb replicon hybridized to the 2.1 Mb bands from both wild-type and C58co2 DNAs and also hybridized with the 1150 kb and 940 kb fragments, confirming their origin (data not shown).

A. tumefaciens C58co2 contains a unique Tn5Map insertion in the 2.1 Mb replicon

Three experiments were performed to confirm that the insertion in the 2.1 Mb replicon of C58co2 was unique: (1) C58co2 DNA was digested by a rare-cutting endonuclease, SwaI, which does not cleave Tn5Map. The 14 restriction fragments were separated by PFGE, and then probed with Tn5Map (Fig. 5a, b). Only the 770 kb SwaI-C fragment hybridized. (2) SwaI/I-SceI double digestion cleaved SwaI-C giving two new fragments of 530 kb and 240 kb, both of which hybridized to Tn5Map (Fig. 5a, b). (3) PacI digestion located Tn5Map in the 965 kb PacI-B band while PacI/I-SceI double digestion produced two new bands of 600 kb and 375 kb (the 375 kb band comigrated with the PacI-E band) (Fig. 5c). All three experiments are consistent with the insertion of a single Tn5Map in the 2.1 Mb replicon of A. tumefaciens C58co2. Further, a tandem insertion would produce a fragment of at least 6.6 kb, which was not observed. This replicon must therefore be a linear molecule.

DISCUSSION

The results of this study show that the delivery of a unique I-SceI site to a bacterial genome using a transposon provides a useful new tool for the analysis of genome topology. To test this application we chose three bacterial species with different genomic organizations. Previously, the linearity of the 2.1 Mb chromosome of A. tumefaciens C58 was proposed mostly on the basis of indirect arguments by comparison with the PFGE behaviour of the supposedly circular 3.0 Mb chromosome. This view
was further supported by data from premigration experiments which removed linear DNA from the plug, and by the behaviour of the replicons in PFGE after X-ray irradiation (Allardet-Servent et al., 1993). The cutting of the 2-1 Mb replicon of C8802 into two fragments by I-SreI in the present study now provides direct proof of its linearity. Furthermore, in the physical mapping experiments using PFGE, the restriction fragments generated by rare-cutting enzymes are put together to form a circle. Most of these experiments still do not rule out a linear chromosome with fixed ends and terminal repeats (which may cause erroneous interpretation of the hybridization results). These arguments emphasize the need to analyse the intact chromosome, or the chromosome cut only once, as we propose. Furthermore, the ability to introduce a unique restriction site in each replicon of \textit{B. melitensis} and \textit{A. tumefaciens} demonstrates directly the existence of several replicons in the genomes of these bacteria.

Several techniques have been described to reduce the number of cleavable sites in eukaryotic and bacterial genomes. Methyltransferase/endoribonuclease combinations readily give a reduction of the number of cleavable sites, but not necessarily to one (Nelson & McClelland, 1992; Weil & McClelland, 1989). Triple helices containing a modified oligonucleotide creating a site for chemical ('artificial nuclease') cleavage (François et al., 1989), protection of a site from methylation using triple helices (Strobe1 & McClelland, 1989), DNA-binding proteins ('Achilles' heel cleavage') (Koob & Szybalinski, 1990) or RecA/oligonucleotide/chromosome complex (RecA-assisted restriction endonuclease cleavage) (Ferrin & Camerini-Otero, 1991) have also been used with success. These methods, however, can be applied without modification only to well-studied organisms since they depend upon either nucleotide sequence data and/or the availability of DNA-binding proteins. Similarly, insertion of a site by targeted recombination requires cloned DNA sequences. We chose to insert a unique restriction site into a modified transposon. This approach has already been used by Hanish & McClelland (1991) and by Wong & McClelland (1992) to introduce rare but not unique sites. Insertion of a unique I-SreI restriction site by a transposon such as Tn5 is an attractive procedure since it is rapid, efficient and versatile.

The combination of Tn5Map and I-SreI is a new tool for the study of bacterial replicon topology, since we have been able to introduce this site into several Gram-negative species. This will be a good test for rapidly identifying linear chromosomes and for estimating the number of replicons in the bacterial genomes. Moreover, the insertion of a unique restriction site, with defined flanking sequences, will be useful for the construction of physical maps of circular bacterial genomes by partial digestion. This method has allowed us to rapidly construct restriction maps of the genome of four \textit{Brucella} species (unpublished results).

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**REFERENCES**


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