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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Tn5Map, a Tn5 derivative containing the 18 bp I-SceI site, was delivered from a RP4-mobilizable, RK6-derived suicide vector to *Escherichia coli* HB101, *Brucella melitensis* and *Agrobacterium tumefaciens* C58, which all lack natural I-SceI sites in their genomes. Digestion of the DNA from Tn5Map-containing strains and analysis by pulsed-field gel electrophoresis (PFGE) revealed that these derivatives contained a single transposon insertion. These digests also gave direct and independent proof for the single circular chromosome of *E. coli*, and for the presence of two circular chromosomes in *B. melitensis* and of a circular and a linear chromosome in *A. tumefaciens* C58 (which also contains two large circular plasmids). This rapid and versatile technique is potentially applicable to the study of the genomic organization in all Gram-negative bacteria which support Tn5 transposition. Moreover, linearization of circular replicons could be the first step for a rapid method of physical mapping.

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

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).

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 Kan^R *A. tumefaciens* transconjugants was made on medium containing 200 µg kanamycin ml⁻¹.

Recombinant DNA techniques. These were as described by Sambrook *et al.* (1989). Restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase were from Boehringer Mannheim or New England Biolabs and were used as recommended by the manufacturers.

Delivery of Tn5Map. pGF2 was transferred from *E. coli* SM10λpir by standard filter mating techniques. SM10λpir(pGF2) was mixed with *E. coli* HB101, *B. melitensis* Ether^{SmR} 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 transferred to an LB plate and incubated overnight. Bacteria on the filter were 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 *Sma*I and *Pac*I were performed as previously described (Allardet-Servent *et al.*, 1993; Michaux *et al.*, 1993). Conditions of *I-Sce*I 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

Table 1. Strains and plasmids used in this study

Strain	Characteristics/genotype	Source/reference
<i>E. coli</i>		
SM10λpir	<i>thi thr leu tonA lacY supE</i> <i>recA::RP4-2Tc::Mu Km λpir</i>	Miller & Mekalanos (1988)
SY327λpir	<i>Δ(lac-pro) argE(Am) rif nalA</i> <i>recAλpir</i>	Miller & Mekalanos (1988)
HB101	<i>hsdS recA ara proA lacY galK rpsL xylL</i> <i>mtl supE</i>	Laboratory strain
HB101ω1	Tn5Map in the chromosome	This study
HB101ω2	Tn5Map in the chromosome	This study
<i>Brucella melitensis</i>		
Ether	Biovar 3	ATCC 23458
Ether ^{SmR}	Streptomycin resistant	This study
Etherω1 ^{SmR}	Tn5Map in the 2.2 Mb replicon, streptomycin resistant	This study
Etherω2 ^{SmR}	Tn5Map in the 1.1 Mb replicon, streptomycin resistant	This study
<i>Agrobacterium tumefaciens</i>		
C58	Streptomycin resistant	X. Nemes (Lyon, France)
C58ω1	Tn5Map in the 3.0 Mb replicon	This study
C58ω2	Tn5Map in the 2.1 Mb replicon	This study
C58ω3	Tn5Map in pAtC58 (550 kb)	This study
C58ω4	Tn5Map in pTi (200 kb)	This study
Plasmids		
ColE1::Tn5Map	Derivative of Tn5cos containing the 18 bp <i>I-Sce</i> I site	Hiller <i>et al.</i> (1994)
pGP704	π-dependent suicide vector	Miller & Mekalanos (1988)
pGF2	pGP704 containing Tn5Map	This study (Fig. 1)

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

ColE1::Tn5Map, a derivative of ColE1::Tn5cos (Zuber & Schumann, 1991) containing the 18 bp I-SceI 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 R6K π protein encoded by the *pir* gene, which can be provided *in trans*. A 7541 bp fragment containing Tn5Map was excised from ColE1::Tn5Map by first linearizing the plasmid with *Alw44I*, 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 *SmaI*, resulting in pGF2 (Fig. 1). The permissive host *E. coli* SY327 λ *pir* was transformed with the ligation mixture and ampicillin- and kanamycin-resistant transformants were selected. pGF2 was then transferred to *E. coli* SM10 λ *pir*, which provides the RP4 *tra* functions, for conjugation experiments.

Delivery of Tn5Map by conjugation

pGF2 was transferred by conjugation from *E. coli* SM10 λ *pir* into *E. coli* HB101, *B. melitensis* Ether^{SmR} 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 ColE1::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 pAtC58 (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 con-

Fig. 1. Structure of plasmid pGF2.

centration ($5 \times$) 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-SceI

Conditions for the I-SceI digestion of bacterial genomes in agarose plugs were optimized using *B. melitensis*; the presence of two circular replicons, one without a I-SceI 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-SceI 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-SceI 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-SceI and compared with non digested controls (Fig. 3). Wild-type HB101 was compared with two clones containing Tn5Map insertions (HB101 ω 1 and HB101 ω 2). Wild-type *B. melitensis* Ether was compared with Ether ω 1 (Tn5Map in the 2.2 Mb replicon) and Ether ω 2 (Tn5Map in the 1.1 Mb replicon). Digestion with I-SceI 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 $\times$ 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* Ether ω 2 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 ω 1 to ω 4 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 $\times$ DNA concentration were used. With this concentration only the 2.1 Mb replicon is visible in the PFGE gel. *I-SceI*-digested and undigested samples were again compared (Fig. 4). *I-SceI* digestion of C58 ω 1 intensified the 3 Mb band, consistent with linearization of a circular DNA molecule (Fig. 4a).

For the analysis of the 2.1 Mb chromosome and the plasmids, shorter pulse times were used. After digestion of C58 ω 3 and C58 ω 4 DNA with *I-SceI*, bands at 550 kb

Fig. 3. PFGE gel of I-SceI digests of *E. coli* (HB101) and *B. melitensis* Ether (*B. m.*) DNAs containing Tn5Map (see Table 1) and undigested controls. *S. cerevisiae* (*S. c.*) chromosomes were used as size markers. Migration conditions as for Fig. 2.

Fig. 4. I-SceI digestion of *A. tumefaciens* C58 DNA containing Tn5Map. (a) C58 ω 1 DNA digested by I-SceI and undigested control. PFGE migration conditions as for Fig. 1 (lanes marked *). (b) C58 ω 2, C58 ω 3, C58 ω 4 and wild-type DNAs digested by I-SceI and undigested controls. Migration conditions were: pulse ramp 60–130 s at 180 V in a 0.8% agarose gel for 24 h. *S. cerevisiae* (*S. c.*) chromosomes were used as size markers in both (a) and (b).

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).

C58 ω 2 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 C58 ω 2 DNA with I-SceI cleaved the 2.1 Mb

Fig. 5. Demonstration that the *A. tumefaciens* C58 ω 2 genome contains a single Tn5Map insert. (a) Ethidium-bromide-stained PFGE gel; comparison of *SwaI* and *SwaI/I-SceI* digestion. (b) Southern blot of the gel shown in (a) probed with labelled Tn5Map. (c) Ethidium-bromide-stained PFGE gel; *PacI/I-SceI*-digested C58 ω 2 compared to *PacI*-digested C58 ω 2. DNA was electrophoresed in a 1% agarose gel with two pulse ramps, 10–30 s for 15 h and 60–150 s for 30 h at 150 V. *S. cerevisiae* (*S. c.*) chromosomes were used as size markers in (a) and (c).

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 C58 ω 2 DNAs and also hybridized with the 1150 kb and 940 kb fragments, confirming their origin (data not shown).

***A. tumefaciens* C58 ω 2 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 C58 ω 2 was unique: (1) C58 ω 2 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* C58 ω 2. 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 C58 ω 2 into two fragments by I-SceI 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 *B. melitensis* and *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/endonuclease 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 (Strobel & Dervan, 1991), DNA-binding proteins ('Achilles' heel cleavage') (Koob & Szybalski, 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 using 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-SceI 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-SceI 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 *Brucella* species (unpublished results).

ACKNOWLEDGEMENTS

We thank Daniel Richard and Daisy Schneider for the photography, and Gisèle Bourg for excellent technical assistance.

David O'Callaghan was supported by 'L'Association Recherche et Partage'.

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Received 23 March 1995; revised 6 June 1995; accepted 12 June 1995.