Rapid Mapping of Cloned DNA Fragments on the Salmonella Chromosome

Genetic analysis in bacteria often involves mapping novel mutations. Traditionally, mapping has been performed using time-consuming methods such as conjugation and transductional analysis. However, an alternative strategy is to use physical mapping using restriction enzymes, which cleave bacterial chromosomal DNA at relatively few sites, generating DNA fragments that can be resolved by pulsed-field gel electrophoresis (PFGE) (6). For example, the Salmonella typhimurium LT2 chromosome can be cut into 11, 23 and 7 fragments using the restriction endonucleases BlnI, XbaI and CeuI, respectively (3,5,11).

We and others have used transposons, such as TnphoA (7) and polymerase chain reaction (PCR) with degenerate primers, to identify novel regions of S. typhimurium DNA, for example those required for virulence. As a step towards the full characterization of novel loci, the influence of individual genes within the loci and any polar effects should be fully assessed. To achieve this, it is usually necessary to map the region and construct a series of defined mutations. Here we report a simple technique, particularly applicable to Salmonella, that allows the simultaneous construction of defined mutations and rapid physical mapping of the novel loci within the genome.

S. typhimurium C5 TnphoA::440 is a novel, unmapped TnphoA insertion mutant of S. typhimurium C5. Salmonella strains harboring TnphoA::440 are attenuated for virulence in mice (7). The salmonella genomic sequences bordering the TnphoA::440 transposon insertion site of TnphoA::440 were
Benchmarks

cloned, sequenced and shown to be from a previously unidentified region of the genome that has some homology to bacteriophage tail fiber encoding sequences and invertible DNA sequences (T. Ali, unpublished results).

The scheme for mapping the mutation in TnphoA::440 and simultaneously constructing a defined mutation is shown in Figure 1. To facilitate physical mapping, BlnI and XbaI restriction enzyme sites were introduced within a DNA cassette encoding a kanamycin-resistance gene. For this purpose, the kanamycin cassette in plasmid pUC4K (Pharmacia Biotech, Milton Keynes, England, UK) was used as a template for PCR (conditions for the PCR amplification were initial denaturing at 95°C for 5 min, 30 cycles of 94°C for 40 s, 55°C for 1 min, 72°C for 1 min and a final extension of 72°C for 6 min). Primers were designed to incorporate an EcoRI site upstream and XbaI, BlnI and EcoRI sites downstream of the kanamycin-resistance coding region upon amplification (Km-XB, Figure 1). The PCR product encoding Km-XB was then digested with EcoRI and cloned into the EcoRI site of pBluescript®(+) (Stratagene, Cambrige, England, UK) to produce plasmid pEBXKAN. Approximately 635 bp upstream of the point equivalent to TnphoA integration in S. typhimurium C5, TnphoA::440 was amplified from wild-type S. typhimurium C5 using primers with PstI and EcoRI restriction sites designed into the 5′ and 3′ ends, respectively. The PCR product was then subjected to digestion with the appropriate restriction enzymes and cloned into the PstI and EcoRI restriction sites of pBluescript(+) to produce pTPE1. A 470-bp region downstream of the TnphoA insertion site was also amplified using PCR but using primers designed to incorporate EcoRI and SalI, and the EcoRI-, EcoRII, SalI-cleaved fragment, was cloned into the EcoRI and SalI sites of pTPE1 to give pTPES. The plasmid pEBXKAN was cleaved with EcoRI, and the released Km-XB cassette was cloned into the EcoRI site of pTPES to give pTPKS. This plasmid was then cleaved with SacI and SalI, and the released insert was cloned into the SacI and SalI sites of the suicide vector pGP704 (8) to give plasmid pGPKS (Figure 1).

pGPKS was transformed into E. coli SM10, and the plasmid was then transferred to S. typhimurium LB5010 (NalR) by conjugation. For conjugation, 100 µL of both SM10 and LB5010 grown to late logarithmic phase were mixed onto an L agar plate and incubated at room temperature for 30 min before replating out onto L agar containing kanamycin and nalidixic acid. These antibiotics select for S. typhimurium LB5010 transconjugants acquiring the Km-XB cassette. The resulting colonies were then assessed for nalidixic acid resistance, kanamycin resistance and ampicillin sensitivity as such colonies could be S. typhimurium LB5010 with the Km-XB cassette inserted into the chromosome by homologous recombination. Bacteriophage P22 transduction was then used to transfer the modified region into S. typhimurium LT2 and C5. Southern blot and PCR analysis showed that the defined mutation had been transferred into the same locus of these recipient strains (results not shown).

A modification of a previously described method (5) was used to prepare the genomic DNA. Briefly an overnight culture was diluted by a factor of ten into fresh LB broth and grown at 37°C with vigorous shaking for 3 h. The bacteria were harvested and resuspended in cell suspension solution (10 mM Tris- HCl [pH 7.2], 20 mM NaCl, 100 mM EDTA) to a concentration of 5 × 10^8 cells/mL. The resuspended bacteria were then mixed with an equal volume of 1.6% low-melting-point agarose (Life Technologies, Paisley, Scotland, UK), poured into molds and allowed to solidify. The resulting blocks of embedded bacteria were then lysed (1 mg/mL lysozyme) at 37°C for 1 h and treated with protease K (1 mg/mL) at 55°C for 18 h. Proteinase K was inactivated with phenylmethylsulfonyl fluoride (PMSF; 1 mM) for 1 h at room temperature. The treated blocks were washed twice with wash solution (20 mM Tris- HCl [pH 8.0], 50 mM EDTA) and then once with storage solution (2 mM Tris- HCl [pH 8.0], 5 mM EDTA). They were then stored at 4°C in storage solution.

The agarose blocks were sliced into 3-mm fragments and incubated with 1×
restriction buffer for 15 min at room temperature. This was repeated again with fresh buffer. The buffer was then replaced with fresh buffer containing either XbaI or BlnI restriction enzyme at a final concentration of 0.2 U/µL and incubated overnight at 37°C.

The restriction enzyme-digested DNA fragments were separated using a CHEF-DR® II pulsed-field apparatus (Bio-Rad, Hemel Hempstead, England, UK) in a 1% agarose gel containing 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). Pulse times were ramped from 120–180 s for 6 h, 6–12 s for 6 h, 24–36 s for 6 h and 70–90 s for 6 h, all at a constant 6 V/cm (1). The gel was then stained in ethidium bromide and photographed.

The DNA from the gels was transferred to a nylon membrane (Amer sham International, Little Chalfont, Bucks, England, UK) using standard procedures as described before (9). Probes were made using the random prime method (Amersham International) and hybridized overnight at 65°C. After washing, the membrane was autoradiographed.

The PFGE patterns of C5 and LT2 show a number of common fragments as expected since they are both S. typhimurium strains. It can be seen from the PFGE that the 830-kb BlnI fragment (fragment C) of S. typhimurium LT2-defined mutant has been reduced in size to approximately 800 kb, and there is a new fragment of 33 kb (Figure 2A). This suggests that the sequence of interest maps at either 40.9 centisomes or at 56.5 centisomes on the chromosome map [1 centisome equals 48.0 kb (4)]. Since the 457-kb fragment (fragment D) is missing from the XbaI digestion (Figure 2B), it can be concluded that the sequence maps are at 40.9 centisomes. Due to the uncertainty of knowing where the products of fragment D are in the XbaI digest, a Southern blot analysis of an equivalent gel was performed (Figure 2C). Probing the membrane with the PCR product of primers TP10 and TR12 that were used in the construction of the plasmid pTPES allowed the localization of the positions of the missing fragments and, hence, confirmed the initial results as well as allowing the orientation of the sequence to be determined in respect to the origin of replication. By selecting for one of the orientations of the Km-XB during the initial cloning, in particular the production of transcripts in the same direction as the surrounding ORF, we are able to study the resulting phenotype produced by the defined lesion.

In conclusion, we have developed a simple and complete scheme for simultaneously mapping and mutating novel loci within the salmonella genome. Although the introduction of rare restriction sites into the S. typhimurium and other genomes for mapping purposes has been demonstrated before (2, 5, 10–12), we have introduced a single-step PCR procedure to insert the rare restriction sites into the kanamycin cassette. This modified kanamycin cassette simultaneously simplifies the construction of a defined mutant; a

![Figure 2. Agarose blocks containing chromosomal DNA of S. typhimurium strains C5 - defined mutant, C5 TnphoA::440, C5, LT2 - defined mutant and LT2 digested with either restriction enzyme BlnI or XbaI and then separated using PFGE. λ concatemers were used to determine the fragment sizes. (A) In the chromosomal DNA digested with BlnI, the 830-kb fragment of LT2 has been reduced in size to 800 kb and 33 kb in the defined mutant, whereas chromosomal DNA digested with XbaI (B) shows that a 457-kb fragment is missing from the defined mutant. (C) Southern blot of the gel probed with the PCR product of primers TP10 and TR12 allowed the localization of one of the missing fragments in (B).]
necessary step in further characterization of the gene or locus of interest. This scheme will provide a simplified and generally applicable approach to characterize the salmonella genome.

REFERENCES


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