Rapid Mapping of *Escherichia coli*::Tn5 Insertion Mutations by REP-Tn5 PCR

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We describe a novel method to map chromosomal *Escherichia coli*::Tn5 insertion mutations rapidly. This method utilizes the ends of Tn5 and the *E. coli* REP sequence as primer binding sites for the polymerase chain reaction (PCR). The unique *E. coli* chromosomal sequence located between these primer binding sites is amplified by PCR and used as a probe to identify the recombinant clones from the Kohara phage ordered *E. coli* mini-set bank that contains the Tn5 mutated loci. We used this approach to map two Tn5 insertion mutations previously identified by their effect on glycerol metabolism. The insertion mutations mapped to *glpD*, the aerobic sn-glycerol-3-phosphate dehydrogenase gene. Phenotypic analysis of the mutant strains revealed one with partial GlpD activity, suggesting transposon-mediated alteration of promoter activity. This mapping method should be applicable to the rapid physical mapping of any insertion mutation in the *E. coli* chromosome.

Transposon Tn5 mutagenesis is a powerful tool for generating random mutations in the *Escherichia coli* chromosome. The desired mutant colonies can be recovered readily by employing an appropriate genetic screen^(1,2); however, physical mapping of the transposon insertion mutation can be laborious, particularly if mutations in a number of genes are capable of generating the same phenotype. In a previous study,⁽³⁾ we isolated 21 independent Tn5 insertion mutations into the *E. coli* K-12 strain HB101⁽⁴⁾ chromosome which diminished the cell's ability to ferment glycerol, as evidenced by an altered colony color phenotype on MacConkey-glycerol plates. Although 16 of the 21 mutations mapped to the *glpFK* operon, the remaining five did not. Two of these five, Tn5-5 and Tn5-14, showed normal GlpK enzymatic activity but were nonviable when transformed with a plasmid containing the *glpK* gene. A lethal effect caused by an increased copy number of the *glpK* gene was hypothesized to explain this observation, suggesting that Tn5 insertions in a gene or genes downstream in the biochemical pathway resulted in the buildup of a toxic intermediate.^(5,6)

To map these two interesting insertion mutations rapidly, we developed a strategy, based on the polymerase chain reaction (PCR),⁽⁷⁾ that does not require prior knowledge of host chromosomal DNA sequences at the insertion site. This method is similar to a technique developed in eukaryotes that uses primers to human *Alu* repeat elements to amplify human specific se-

quences in rodent-human somatic cell hybrids (*Alu*-PCR),⁽⁸⁾ and methods recently developed in prokaryotes to amplify unique *E. coli* chromosomal DNA sequences between REP (repetitive extragenic palindrome) elements, called REP-PCR, and ERIC (enterobacterial repetitive intergenic consensus) sequences, called ERIC-PCR.⁽⁹⁾ The REP element has been predicted to comprise up to 1% of the genome of *E. coli*.⁽¹⁰⁾ This ~35-bp palindromic sequence has been identified in the intergenic regions of numerous operons, and its putative stem-loop structure has been proposed to decrease expression of downstream genes⁽¹¹⁾ while stabilizing transcripts of upstream sequences.⁽¹²⁾ It has also been proposed to play a role in determining chromosome structure and to bind DNA gyrase and the histone-like protein HU.^(13,14)

When an outwardly facing primer corresponding to either half of the REP palindrome and a Tn5-specific primer are used with chromosomal DNA from a Tn5 insertion mutant strain as the template, PCR amplification products contain unique sequences that lie between the transposon and adjacent REP site(s) as well as between adjacent REP sequences themselves. Using PCR conditions to favor amplification from Tn5 to REP, and by comparison to an amplification using REP primers alone, the resulting Tn5-REP amplification products can be identified readily. These DNA fragments are then used as probes for the mutated locus by screening an ordered *E. coli* genomic library.⁽¹⁵⁾ In this manner, the insertion can be mapped quickly to a specific

region of the chromosome. Standard genetic mapping techniques using conjugation and transduction⁽¹⁶⁾ can be time consuming and are accurate to about 1 min, or 47 kb,⁽¹⁷⁾ although this can be improved with refined genetic mapping methods. In contrast, this PCR-based mapping strategy allows positive assignment of the mutation to ± 50 bp (the limit of resolution on an agarose gel), when used in conjunction with the Kohara⁽¹⁸⁾ physical map. In addition, the method provides physical material of a manageable size, in the form of the PCR product, for additional characterization of the insertion mutation site.

METHODS

Phenotypic Analysis

Strains HB101::Tn5-5 and HB101::Tn5-14 were grown overnight in rich media (LB) containing 40 μ g/ml kanamycin, pelleted by centrifugation, and resuspended in M9 media containing no carbon source. The OD₆₀₀ of each culture was measured, and 1×10^6 cells were inoculated into 5 ml of M9 media containing 10 mg/liter Leu, Pro (the HB101 auxotrophic markers), and thiamine in addition to one of the following carbon source(s): 10 mM glucose, 10 mM glucose/25 mM glycerol, 25 mM glycerol, 40 mM succinate, or 40 mM succinate/25 mM glycerol.⁽¹⁹⁾ Cultures were incubated at 37°C with shaking, and the OD₆₀₀ of each was measured at specific time intervals. Strain HB101 was cultured under similar conditions with the omission of kanamycin during initial growth.

REP-Tn5 PCR

PCR amplification primers were prepared with an Applied Biosystems oligonucleotide synthesizer and DNA sequence information from the published sequence of Tn5,⁽³⁾ and the REP consensus sequence.⁽¹⁰⁾ The Tn5 primer 5'-CTGGAAAACGGGAAAGGTTCCG-3', primer E,⁽³⁾ matches a sequence 30 bp from the end of the transposon; the REP1R-I primer 5'-IIICGICGICATCIGGC-3'⁽⁹⁾ (I = inosine, where I is a degenerate position in the sequence) is the reverse complement of the left side of the stem of the REP consensus palin-drome,⁽¹⁰⁾ and the REP2-I primer 5'-ICGICTTATCIGGCCTAC-3' corresponds to the right side of the stem of

the REP palindrom.⁽¹⁰⁾ Amplification reactions were performed in 50- μ l reaction volumes using buffer and reagent concentrations (primers, deoxyribonucleotide triphosphates, template DNA, enzyme) as recommended by Perkin-Elmer Cetus. Amplification was conducted in a Perkin-Elmer Cetus first-generation thermal cycler as follows: denaturation at 94°C for 4 min, 25 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 1.5 min, and polymerase extension at 72°C for 2.5 min. PCR products were analyzed by electrophoresis through a 1% agarose gel and visualized with ethidium bromide staining. Products were purified using the Gene-clean II kit (Bio101), radiolabeled with [α -³²P]dCTP using a random hexanucleotide priming kit (Pharmacia), and hybridized to a commercially available membrane (Takara Shuzo Co., Ltd.) containing the Kohara et al.⁽¹⁸⁾ ordered miniset λ phage library of the *E. coli* chromosome using published methods.⁽¹⁵⁾ The background λ phage clones were visualized by addition of 0.1 ng of ³²P-labeled *Hind*III-digested λ DNA to the hybridization solution. The blots were exposed to X-ray film for 24–72 hr (with an intensifying screen) at –70°C.

Tn5-*glpD* PCR

The *glpD* primer 5'-AACTTCTGGTGC-GCGTCGGC-3', which corresponds to nucleotides 272–291 of the sense strand,⁽²⁰⁾ was used with the Tn5 primer. Fifty nanograms of template DNA were added to the PCR reaction tubes containing 10% DMSO.⁽²¹⁾ Reaction mixtures contained 50 pmoles of each primer, 62.5 nmoles of each of four dNTPs, and 2 units of AmpliTaq (Cetus) DNA polymerase. The amplification conditions consisted of an ini-

tial denaturation of 94°C (6 min); amplification proceeded with 30 cycles of denaturation at 90°C for 1 min, annealing at 62°C for 1 min, and polymerase extension at 70°C for 5 min. Products were analyzed by electrophoresis through a 0.8% agarose gel and visualized with ethidium bromide staining.

RESULTS

Phenotypic Characterization of Mutants

Isolation of the Tn5 insertion mutants affecting glycerol metabolism has been described previously.⁽³⁾ The glycolytic defect of the two insertion mutations, Tn5-5 and Tn5-14, was assessed by examining growth in defined media prepared from minimal media supplemented with specific carbon sources.⁽¹⁹⁾ A *glpD* mutation will allow normal growth on substrates other than glycerol. In addition, normal growth will be seen if glycerol is given with a carbon source that represses glycerol metabolism (i.e., glucose). However, if glycerol is present alone or with a noninhibitory substance (i.e., succinate), abortive glycerol metabolism will lead to growth stasis.⁽²²⁾ This effect has been attributed to the accumulation of L- α -glycerophosphate within the cell.⁽⁵⁾ When cultured in these defined media, strain HB101::Tn5-5 displayed a classic *glpD* phenotype: It was unable to grow in glycerol and grew very poorly on succinate with glycerol, but if glucose was present, growth was normal. However, strain HB101::Tn5-14 differed phenotypically: It evidenced poor growth on glycerol alone, but on succinate with glycerol, growth approached that of strain HB101 (Table 1).

TABLE 1 Growth of Tn5 Insertion Mutants on Various Carbon Sources

Bacterial strain	Sugar(s) added to M9 minimal broth				
	Glucose	Glycerol	Glucose and glycerol	Succinate and glycerol	Succinate
HB101::Tn5-5	+++	–	+++	±	++
HB101::Tn5-14	+++	+	+++	++	++
HB101	+++	++	+++	++	++

(–) No growth; (±) OD₆₀₀ $\leq$ 0.05; (+) 0.05 $\leq$ OD₆₀₀ $\leq$ 0.1; (++) 0.3 $\leq$ OD₆₀₀ $\leq$ 0.4; (+++) OD₆₀₀ $\geq$ 0.5.

A. Southern blot of DNA from *Salmonella enteritidis* strain 1040/90. Lanes from left to right: 1 kb ladder, HB101, Tn5-5, Tn5-14, neg. control, 1 kb ladder. Molecular weight markers are indicated on the left: 3054 bp, 2036 bp, 1636 bp, 1018 bp, 506, 517 bp. Bands for Tn5-5 and Tn5-14 are visible at approximately 1018 bp.

B. Southern blot of DNA from *Salmonella enteritidis* strain 1040/90 after EcoRI digestion. Lanes from left to right: 1 kb ladder, HB101, Tn5-5, Tn5-5 Eco RI, Tn5-14, Tn5-14 Eco RI, neg. control, 1 kb ladder. Molecular weight markers are indicated on the right: 3054 bp, 2036 bp, 1636 bp, 1018 bp, 506, 517 bp. Bands for Tn5-5 Eco RI and Tn5-14 Eco RI are visible at approximately 1018 bp.

C. Schematic diagram of the *glpD* gene region. The *glpD* gene is shown as a horizontal line with a right-pointing arrow. The *glpD* primer binding site is indicated by a shaded box at position -225. The *EcoRI* site is indicated by a vertical line at position +1. The Tn5-14 and Tn5-5 transposon insertion sites are indicated by hatched boxes. The REP (Resistance Element) is shown as a box at position +1692. Distances are indicated: 1700 bp from the *glpD* primer binding site to the Tn5-5 site, 390 bp from the Tn5-5 site to the REP, 1100 bp from the *glpD* primer binding site to the Tn5-14 site, and 890 bp from the Tn5-14 site to the REP.

1100-bp product (Fig. 1B). To establish further the identity of these products, the presence of an *Eco*RI site, located 415 bp from the 5' end of the *glpD* primer,⁽²⁰⁾ was assayed. When digested with *Eco*RI, the length of both PCR products was indeed reduced by approximately 400 bp (Fig. 1B). Thus, the mutations Tn5-5 and Tn5-14 were localized to the *glpD* locus with the Tn5-5 insertion 1420 ± 50 bp and the Tn5-14 insertion 820 ± 50 bp downstream of the translation start site.⁽²⁰⁾ Nucleotide sequence data for the *glpD* locus⁽²³⁾ became available after com-

FIGURE 2 Correlation of the genetic and physical maps. (A) Autoradiogram of a filter containing the Kohara phage ordered miniset library in a dot blot array. The fragment obtained from amplification of HB101::Tn5-5 DNA was labeled and used as the probe. Exposure time was 72 hr at -70°C . The two extraneous nonspecific signals do not align with phages. (B) Autoradiogram of the same filter using the labeled fragment from the amplification of Tn5-14 DNA. Exposure time was 24 hr at -70°C . (C) Alignment of the Kohara⁽¹⁸⁾ physical map and the Bachmann⁽¹⁷⁾ genetic map. The hatched box indicates the region of overlap of all three phages identified from the autoradiograms.

pletion of this work. The authors reported two REP sequences beginning 60 bp downstream of the putative translational stop site, lying in opposite orientations. The REP2-I primer was localized to nucleotides 1676–1692 with identity of all bases other than inosine to the antisense strand. Calculation of the position of the two Tn5 insertions using the known REP location yielded results consistent with those derived from the data presented here.

DISCUSSION

We have described a physical mapping technique that takes advantage of the presence of repetitive sequence elements in prokaryotic genomes. The method enables one to map insertion mutations quickly, by using the insertion element as one primer binding site and the REP sequence as the other genomically anchored, primer binding site for PCR amplification. The unique sequence spanned by the amplification

product can be used as a probe to identify genomic regions corresponding to the insertion site. The application of the REP-Tn5 PCR-based physical mapping technique was demonstrated by rapidly identifying the location within *glpD* of two Tn5 insertion mutations affecting glycerol metabolism.

The locations of these insertions within the *glpD* locus suggest a functional explanation for the phenotypic differences observed between the two mutants. Tn5-14 appears to retain some *glpD* function; although it is unable to grow on glycerol alone, it can tolerate its presence in the media. Tn5 IS50 sequences have been shown to possess an outwardly directed promoter activity.^(24–26) A partial *glpD* transcript initiated from within the transposon could be synthesized and translated to yield a functional, though incomplete, gene product lacking the amino-terminal region of GlpD. Such a mechanism does not appear to apply to the Tn5-5 insertion mutation,

which is located more distally in the *glpD* coding sequence. REP sequences have not been found in coding regions,⁽¹⁰⁾ and the proximity of the Tn5-5 insertion to the REP sequence suggests that it interrupts the distal coding sequence of the *glpD* gene, leading to insertional inactivation. In this position, outwardly directed promoter activity from Tn5 into *glpD* would be quite unlikely to lead to gene function. Partial activity of a truncated GlpD protein cannot solely account for these phenotypes since the insertion at the 3' end of *glpD*, Tn5-5, completely abolished GlpD function. Because the mutant with an insertion in the 5' half of *glpD*, Tn5-14, contains partial activity, an outwardly directed promoter activity from the end of the transposon is likely.

Furthermore, these results suggest regions of GlpD that are critical for its activity. Assuming transposon-mediated promoter activity, a partial Tn5-14 *glpD* transcript would contain only the latter half of the coding sequence. It is this part of the gene that the Tn5-5 insertion disrupts, and transcripts from this mutant that originate at the normal promoter (if they occur) would encode a GlpD protein that lacks the carboxy-terminal coding information. Nucleotide sequence data⁽²³⁾ suggest that the amino-terminal portion of the protein contains a flavin-binding domain, while a putative glycerol-3-phosphate binding site has been found in the carboxy-terminal half. Expression of the portion of *glpD* distal to the Tn5-14 insertion appears to be sufficient for production of a protein that provides dehydrogenase activity. Alternatively, the incomplete protein might associate with a moiety (protein or otherwise) capable of providing the necessary electron transfer function that a non-flavin-containing enzyme would lack. In either case, the protein is not entirely normal, as evidenced by the partial mutant phenotype it conveys upon the cell. These results confirm that insertion mutations are not always null mutations.

This REP-Tn5 PCR-based technique for rapid mapping and characterization of insertion mutations can be applied to a wide range of insertion mutation studies in *E. coli*, *S. typhimurium*, and other organisms for which repeat ele-

ments (such as REP sequences) have been defined. As new repeat sequences are discovered in diverse bacterial species, the utility of this technique will likely increase. REP-like and ERIC-like sequences are conserved in many diverse eubacterial species.⁽⁹⁾ For any transposable element for which the sequence is known, a primer specific to its end may be synthesized and used in an initial REP-Tn PCR screen. Caution must be exercised when designing primers to the ends of the transposable element. The Tn5 primer used in these experiments was later found to contain similarity to an *E. coli* chromosomal DNA sequence by performing a computer-aided search of the GenBank and EMBL databases with the FastA algorithm.⁽²⁷⁾ In addition, it is important to be sure that the strain utilized for mapping does not contain endogenous IS sequences with similarity to the primer binding site. If repetitive sequences are indeed present in the genome of interest, the principal limitation is the need for proximity of the insertion to the repetitive sequence. The fact that most transposons have a twofold axis of symmetry enables detection of a nearby repetitive sequence on either side of the transposon insertion. Presently maximum amplifiable distances using REP primers alone are in the range of 5 kb of DNA, but this limit appears to be a function of the processivity of the DNA polymerase under specific conditions.

Probing a library blot with the PCR products quickly enables one to obtain clones covering a small, defined portion of the genome. The mutation then can be mapped as described here if a known locus is found in the interval covered by the Kohara clones or by using the PCR product as a probe to identify restriction fragments of interest within the λ clones. In this manner, several new insertions in known loci can be identified, and new genes may be isolated and characterized within a period normally required to define a single mutation by traditional mapping methods.

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