NOTES

Transposon Mutagenesis in *Acinetobacter calcoaceticus* RAG-1

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Molecular genetic studies of *Acinetobacter* spp. have been greatly limited by the lack of a method for transposon mutagenesis. In this study, a genetically engineered derivative of Tn10, mini-Tn10PttKm, was conjugally transferred in plate matings from *Escherichia coli* SM10[(λ pir)(pLOFPttKm)] to *Acinetobacter calcoaceticus* RAG-1. Transfer frequencies were dependent on mating ratios and varied from 7.9×10^{-8} to 3.4×10^{-7} per recipient cell. The 27 lipase-deficient transconjugants which were isolated exhibited several different phenotypes, including gelatinase mutants, esterase mutants, and putative auxotrophs. Southern blot analysis confirmed the insertion of the mini-Tn10PttKm transposon in single, unique sites in five transconjugants. Four of five lipase mutants contained single insertions of mini-Tn10PttKm in the same chromosomal restriction fragments. To our knowledge, this is the first report of the use of a transposon for direct, generalized mutagenesis in *Acinetobacter* spp.

Molecular genetic studies of the hydrocarbon-degrading bacterium *Acinetobacter calcoaceticus* RAG-1 (6, 8, 11), as well as of other *Acinetobacter* spp., have been hampered by the lack of a suitable method for transposon mutagenesis. The transposon Tn3171 was found to have several sites for insertion in the chromosome of *A. calcoaceticus* EBF65/65 (17), but the distribution of insertions was considered to be nonrandom because of the existence of an insertional "hot spot" in a gene encoding arginine biosynthesis. Similarly, Tn5 was reported to insert in a single site in *Acinetobacter* strain HO1-N and in two sites in *A. calcoaceticus* BD413 (13). Tn5 has been used to generate insertion mutations in cloned fragments of *Acinetobacter* chromosomal DNA (3, 5, 10), which indicates that generalized mutagenesis will occur in the absence of hot spots. However, the insertional specificity of Tn5 and Tn3171 for sites in the *Acinetobacter* chromosome precludes their use in systems for direct mutagenesis in *Acinetobacter* spp.

The objective of this study was to investigate the use of a genetically engineered derivative of Tn10, which will be referred to as mini-Tn10PttKm (constructed by Herrero et al. [7]), in transposon mutagenesis of *A. calcoaceticus* RAG-1. Previously, Tn10 was introduced into *A. calcoaceticus* BD413 (4), but no attempt was made to determine whether chromosomal insertion sites were specific or not. We report here that mini-Tn10PttKm has several insertion sites in the *A. calcoaceticus* RAG-1 chromosome, on the basis of (i) Southern hybridization analysis, (ii) the isolation of transposon mutants of several different phenotypes, and (iii) the ability to isolate mutants which contain a transposon in a specific gene, in this case, the lipase gene.

Bacterial strains and plasmids. *Escherichia coli* SM10 (λ pir) (*thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km^r*

λ pir) containing the plasmid pLOFPttKm was kindly provided by V. de Lorenzo. pLOFPttKm (7) is a transposon delivery plasmid which contains the mini-Tn10PttKm transposon.

A. calcoaceticus RAG-1 (ATCC 31012) was obtained from the American Type Culture Collection (Rockville, Md.). Strains M1, M2, M3, M4, M5, L10, L20, L27, L47, and L55, generated in this study, are RAG-1 transconjugants which contain chromosomal mini-Tn10PttKm insertions. RAG-1 and its derivatives exhibit moderately high-level resistance to ampicillin and chloramphenicol (MICs > 100 μ g/ml).

Media and growth conditions. *A. calcoaceticus* strains and *E. coli* SM10[(λ pir)(pLOFPttKm)] were grown in LB medium (LB) (12) at 30 and 37°C, respectively. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used at the following concentrations: ampicillin (AP), 150 μ g/ml; chloramphenicol (CM), 25 μ g/ml; and kanamycin (KM), 75 μ g/ml. Isopropyl- β -D-thiogalactopyranoside (IPTG) was obtained from Sigma.

Conjugations. Donor *E. coli* SM10[(λ pir)(pLOFPttKm)] cells were streaked on LB+AP+KM plates and incubated overnight at 37°C. Recipient *A. calcoaceticus* RAG-1 cells were streaked on LB+CM plates and incubated overnight at 30°C. From these plates, seven LB+CM plates of *A. calcoaceticus* RAG-1 and two LB+AP+KM plates of *E. coli* SM10[(λ pir)(pLOFPttKm)] were inoculated with cotton-tipped applicators to form bacterial lawns. Lawn plates were allowed to grow for 16 h at the appropriate temperatures. Cells from all plates were collected with a spatula, mixed on the surface of an LB plate containing 500 μ M IPTG, and incubated at 30°C for 8 h in a humid environment. A total of five mating plates were prepared. Cells from the mating plates were collected and resuspended in 1 ml of MgSO₄ (10 mM). The cell suspension was diluted with phosphate-buffered saline and plated onto LB, LB+AP+KM (to enumerate donor cells), and LB+CM (to enumerate recipient

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cells). Transconjugants were selected for by plating 0.1 ml of the cell suspension onto LB+CM+KM plates containing 500 μ M IPTG and incubating them at 30°C for 6 days.

From each mating plate, the number of donor cells ranged from 3.25×10^{10} to 4.25×10^{10} CFU and number of the recipient cells ranged from 1.57×10^9 to 5.43×10^9 CFU. Km^r transconjugants were detected at frequencies ranging from 7.9×10^{-8} per recipient cell (when the donor:recipient ratio was 2.4:1) to 3.4×10^{-7} (when the ratio was 6.9:1). Spontaneous Km^r mutants of RAG-1 were not observed (frequency, $<1.7 \times 10^{10}$).

Characterization of lipase-negative transconjugants. A total of 24,645 Km^r transconjugants were obtained from five mating mixtures. 10,000 transconjugants were picked and transferred to LB+CM+KM plates. After 4 days of incubation at 30°C, colonies were replica plated onto Spirit Blue Agar (16). Twenty-seven phenotypically lipase-negative mutants were isolated on the basis of their inability to produce the dark blue halo which is indicative of lipase activity. All 27 mutants displayed normal growth in nutrient broth compared to wild-type RAG-1. Eight putative auxotrophic mutants failed to grow in a standard mineral base (15) with any of the seven carbon sources tested. Fourteen mutants produced no gelatinase or produced less gelatinase than wild-type RAG-1 on gelatinase plates (1), indicating probable defects in the genes encoding protein export. Five of these 14 mutants also displayed reductions in esterase activity on Tween 40 plates (1). The loss of lipolytic activity by the 22 putative auxotrophs and gelatinase- and esterase-deficient mutants was ascribed to pleiotropic mutations occurring outside the lipase gene.

The five remaining lipase mutants, L10, L20, L27, L47, and L55, were gelatinase and esterase positive and exhibited normal growth with eicosane, hexadecane, cetyl alcohol, ethanol, palmitic acid, and potassium acetate as sole carbon sources in the standard mineral base. All five were incapable of utilizing the lipid tripalmitin as a carbon source. These strains were believed to contain mutations within a lipase gene or genes, rather than pleiotropic mutations at other loci, and were therefore selected for additional study.

Analysis of mini-Tn10PttKm insertions. Further experiments were done with the five lipase mutants L10, L20, L27, L47, and L55 and five randomly selected transconjugants, designated M1, M2, M3, M4, and M5. A rapid method of plasmid DNA preparation was used to screen transconjugants for loss of plasmid DNA (12). Insertion of the mini-Tn10PttKm transposon into the chromosome of the transconjugants was analyzed by Southern hybridizations (14) in which the probe pLOFPttKm was labeled with [14-biotin]dATP (BioNick Labeling System; Gibco-BRL, Gaithersburg, Md.) and hybridized to *Hind*III-digested genomic DNA. Subsequent staining of nitrocellulose blots (Bio-Rad Laboratories, Richmond, Calif.) with the Blue-Gene Nonradioactive Nucleic Acid Detection System (Gibco-BRL) was carried out according to manufacturer's recommendations with some modification (9). Extraction and purification of plasmid and chromosomal DNA, restriction enzyme digestions, and agarose gel electrophoresis were done according to methods described previously (2, 18).

Southern hybridization analysis (Fig. 1) revealed that RAG-1 transconjugants M1, M2, M3, M4, and M5 contain the transposon mini-Tn10PttKm inserted into unique, single sites within the chromosome. The multiple bands which are visible in the blot of strain M4 DNA in Fig. 1, lane 7, result from incomplete restriction digestion by *Hind*III; only the two lowest-molecular-weight junction fragments were visible on previous blots.

FIG. 1. Southern hybridization analysis of total DNA from *A. calcoaceticus* RAG-1 transconjugants, probed with biotinylated pLOFPttKm plasmid DNA. Hybridizing DNA was detected with alkaline phosphatase-streptavidin conjugate after transfer to nitrocellulose. Lanes: 1, biotinylated λ -*Hind*III molecular size markers; 2 to 8, *Hind*III-digested DNA (2, pLOFPttKm plasmid DNA; 3, *A. calcoaceticus* RAG-1; 4, M1; 5, M2; 6, M3; 7, M4; 8, M5). Note: digestion of the pLOFPttKm probe at the *Hind*III site in the PttKm transposable element generates two junction fragments.

The Southern blot of chromosomal DNA isolated from lipase mutants (Fig. 2) also shows insertion into single sites by the transposon. L10, L20, and L27 contain insertions which map to the same two *Hind*III fragments. L55 shares one of the *Hind*III fragments in common with L10, L20, and L27, indicating that the site of insertion of the transposon in L55 is close in proximity to the insertion sites for those strains. L47 shows no band in common with the other four lipase mutants, so the transposon may or may not be located near the insertion sites for the other four lipase mutants.

Results from hybridization analyses also provide evidence

FIG. 2. Southern hybridization analysis of total DNA from *A. calcoaceticus* RAG-1 transconjugants, probed with biotinylated pLOFPttKm plasmid DNA. Hybridizing DNA was detected with alkaline phosphatase-streptavidin conjugate after transfer to nitrocellulose. Lanes: 1 to 6, *Hind*III-digested DNA (1, L10; 2, L20; 3, L27; 4, L47; 5, L55; 6, *A. calcoaceticus* RAG-1); 7, biotinylated λ -*Hind*III molecular size markers; 8, *Hind*III-digested pLOFPttKm plasmid DNA. Note: digestion of the pLOFPttKm probe at the *Hind*III site in the PttKm transposable element generates two junction fragments.

that transposon insertion occurred by true transposition and not by insertion of the plasmid. Digestion of pLOFPttKm with *Hind*III yields four restriction fragments (7) with molecular sizes of 0.7, 1.0, 2.0, and 7.1 kb. The 7.1- and 0.7-kb fragments, neither of which contain any portion of a transposon DNA sequence, are absent from *Hind*III digests from all of the 10 transconjugants (Fig. 1 and 2), which strongly suggests that plasmid insertion did not occur.

Significance. In summary, the results presented here provide the first published evidence of the use of direct transposon mutagenesis in *Acinetobacter* spp. to produce mutants of several different phenotypes as well as those containing mutations in a specific gene. Mini-Tn10PttKm and possibly other Tn10 derivatives appear to be useful tools for the genetic study of *Acinetobacter* spp. A viable method of transposon mutagenesis should contribute to a greater understanding of the molecular basis of *Acinetobacter* pathogenesis in humans and of the genetic and physiological aspects of the degradation of hydrocarbons by these bacteria.

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