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Detection in Soil of a Deletion in an Engineered DNA Sequence by Using DNA Probes†

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Two *Pseudomonas* strains were engineered to contain the *nptII* gene and plasmid vector sequences in their chromosomes. After incubation of these strains in nonsterile soil, total bacterial DNA was isolated and analyzed by Southern blot hybridization with the *nptII* gene and the plasmid vector as probes. In addition to the expected bands of hybridization, a new band corresponding to the loss of vector sequences from the chromosome while retaining the *nptII* gene was observed for one of the strains. The more stressful conditions encountered in soil appeared to increase the frequency of loss of the vector sequences from this strain.

Interest in the environmental safety of genetically engineered organisms has highlighted the question of how stable engineered DNA sequences are in natural communities. Previously, methodology did not exist to measure changes in gene structure in an indigenous soil population. But, coupling the ability to extract and purify DNA from soil (4) with size separation of endonuclease-digested DNA and DNA probe methodology, it should be possible to detect these changes in natural communities. Rearrangement, deletion, and transfer are the more common examples of mechanisms which would affect the fate of engineered DNA sequences. In this report, we describe how an alteration in an engineered DNA sequence was first detected in soil with DNA probes.

To study the fate of engineered DNA sequences in soil, we first constructed model genetically engineered microorganisms by the addition of a gene foreign to members of the soil bacterial community. We chose pseudomonads to study because they are of major interest for environmental biotechnology applications. The two strains we engineered were a nalidixic acid-resistant strain of *Pseudomonas cepacia* PCO1224 (provided by Ron Olsen, University of Michigan) and a rifampin-resistant strain of *Pseudomonas* sp. strain B8 (provided by Lloyd Elliott, Washington State University). *P. cepacia* strains are being used as benchmark organisms by the Environmental Protection Agency in testing risk assessment methods. *Pseudomonas* sp. strain B8 is a nonfluorescent, aggressive root-colonizing bacterium that inhibits root growth of winter wheat (3).

Our approach to construct the model engineered microorganisms was to insert the foreign gene into the chromosome since DNA inserted in this manner is thought to be more stable and less subject to gene transfer when compared with sequences on autonomously replicating plasmids. We selected the *nptII* gene to insert because it has a negligible background in the soil bacterial community (4) and it confers a useful selectable marker, kanamycin resistance. The strategy for insertion relied on homologous recombination between the chromosome of the organism and a plasmid carrying a cloned segment of chromosomal DNA from the

organism into which the *nptII* gene was inserted. The mobilizable plasmid vector pRL425 (1) was chosen for the constructions because it contained the ColE1 origin of replication and thus could not replicate in the *Pseudomonas* strains used. The constructed plasmids, pJJ5 and pJJ6, containing chromosomal segments of *Pseudomonas* sp. strain B8 and *P. cepacia* PCO1224, respectively (Fig. 1), were introduced into the corresponding strains by triparental mating with the helper plasmid pRK2013 (2). Potential recombinants were selected by resistance to kanamycin; clones selected for further study are designated B8-1 and PC-1, respectively.

Since pRL425 encodes ampicillin resistance and both *Pseudomonas* strains were naturally ampicillin resistant, it was necessary to screen for the presence of pRL425 sequences by Southern hybridization (9). This was accomplished by using ³²P-labeled pRL425 DNA to probe total genomic DNA isolated from the recombinant strains. Both engineered strains were found to contain pRL425 sequences. To verify that pRL425 sequences were actually incorporated into the chromosome, the total genomic DNA of each of the transconjugants was digested with a restriction endonuclease with only one recognition site in the entire recombinant plasmid sequence (*Bst*EII for pJJ5 and *Bgl*II for pJJ6, Fig. 1), size fractionated by electrophoresis, and subjected to Southern transfer to cellulose nitrate. In both cases, two bands were observed rather than a single band as would be predicted from the linearization of the circular plasmid vectors. We conclude that the plasmid (pJJ5 or pJJ6) has been integrated into the chromosome by a single-crossover recombination event. We tried to obtain spontaneously derived mutants in which the vector sequences had been lost and the *nptII* gene retained in the chromosome (Fig. 2) by incubation in medium containing no antibiotics and then screening by colony hybridization (7) for loss of pRL425-derived sequences with pRL425 as the probe, but we were unsuccessful after screening 500 individual colonies of each strain.

The engineered strains PC-1 and B8-1 were inoculated singly or together into nonsterile Capac loam soil (Aeric Ochraqualf, pH 6.8, 2.2% organic carbon). The bacterial inoculum was grown in King B liquid medium (5) containing rifampin (100 µg/ml) and kanamycin (200 µg/ml) for strain B8-1 or nalidixic acid (200 µg/ml) and kanamycin (200 µg/ml) for strain PC-1. The cultures were grown to an optical density (640 nm) of 1.0 and harvested by centrifugation (10

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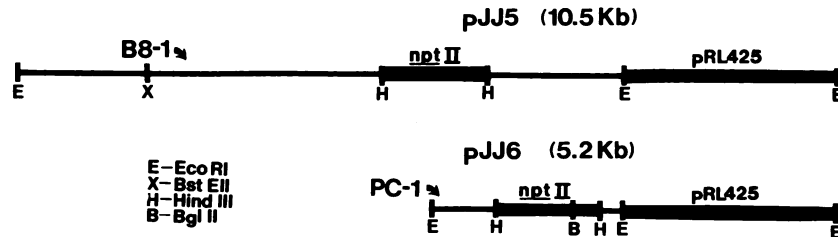


FIG. 1. Restriction map of plasmids pJJ5 and pJJ6. Narrow solid lines indicate cloned *Pseudomonas* DNA; wide solid lines indicate the *nptII* gene and vector sequences as designated. Kb, Kilobases.

min at $8,000 \times g$). The cells were washed in 0.01 M sodium phosphate buffer (pH 7.0), resuspended in one-half the original volume of the same buffer, and incubated at 25°C for 9 h to starve them before inoculation into soil.

The bacterial suspensions were inoculated into soil by spraying them uniformly over the soil surface, using a syringe with an 18-gauge needle. The density of cells added was 10^9 cells per g (dry weight) of soil in the first experiment and 10^7 cells per g (dry weight) of soil in the subsequent experiment to minimize loss to predation. The indigenous bacterial population of this soil, as determined by acridine orange direct counts, was approximately 10^{10} cells per g (dry weight) of soil. The soil was then mixed thoroughly and moistened to field capacity (23% moisture). Inoculated soil and uninoculated controls were incubated in plastic bags in the dark at 25°C.

After 1 day of incubation, total bacterial DNA was isolated from 50-g soil samples as previously described (4). Bacterial DNA was isolated from liquid cultures by the procedure of Marmur (8).

The restriction enzyme *ClaI*, which had no recognition site in either of the engineered sequences, was used to digest DNA isolated from both soil and liquid cultures. This allowed the engineered sequences of each organism to be detected as a single, unique fragment of a predicted size. The digested DNA was size fractionated on a 0.7% agarose gel and then transferred to a cellulose nitrate filter by the method of Southern (9). The filter-bound DNA was hybridized with ^{32}P -labeled single-stranded probe for the *nptII* gene as described previously (4). DNA isolated from soil inoculated with either strain individually generated a band of predicted size when compared with control DNA from pure

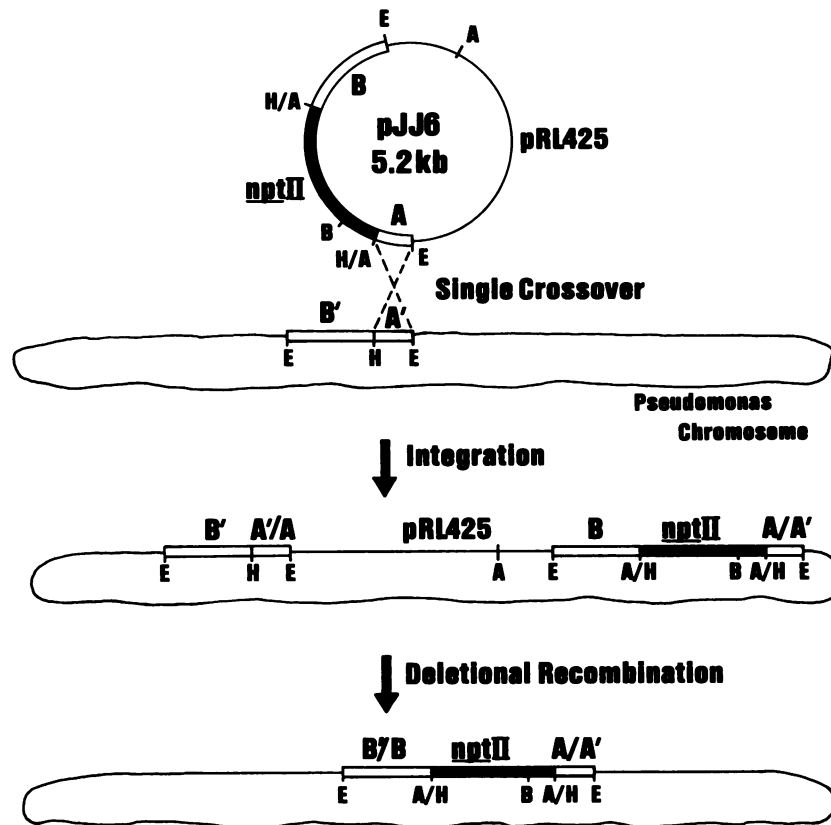


FIG. 2. Graphic representation of integration of pJJ6 into the *P. cepacia* chromosome and subsequent deletion recombination of the integrated pRL425 sequences. Restriction sites designated by small capital letters are as follows: E, *EcoRI*; A, *AluI*; H, *HindIII*; B, *BglII*. Large capital letters, A, B, A', and B' represent *P. cepacia* chromosomal fragments. *nptII*, Neomycin phosphotransferase II gene insertion. kb, Kilobases.

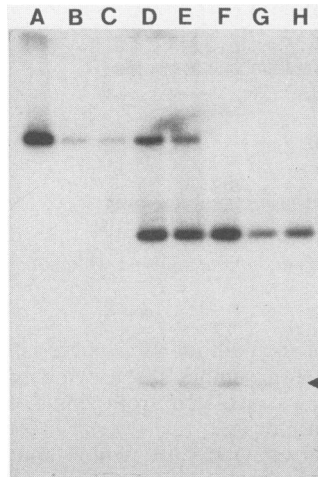


FIG. 3. Autoradiogram from Southern blot of *Clal*-digested total bacterial DNA isolated from soil samples that were inoculated with PC-1 and B8-1. Lanes A and H contain genomic DNA isolated from liquid cultures of B8-1 and PC-1, respectively. Lanes B through G contain DNA isolated from soil inoculated with B8-1 alone (B and C); PC-1 alone (F and G), or B8-1 plus PC-1 (D and E). The lower band in lanes D through F represents a deletion recombination derivative of PC-1 (arrowhead).

culture (Fig. 3, lanes A, B, C and F, G, H). The two pseudomonads could readily be distinguished from each other in a single soil sample based on the difference in size of the *Clal* restriction fragments containing the *npfII* gene sequences (Fig. 3, lanes D and E).

An unanticipated result was the appearance of an anomalous band after Southern blot hybridization of DNA isolated from soil that was inoculated with PC-1 either singly or together with B8-1 (Fig. 3, arrowhead, lanes D, E, F, and G). Comparison with DNA size standards indicated that this band was of the size predicted for a double-crossover recombination event resulting in integration of only the *npfII* gene (Fig. 2); the single-crossover structure in the chromosome of PC-1 had spontaneously resolved by a deletion event into the structure predicted for a double-crossover event.

Genomic DNA isolated from a liquid culture of PC-1 did not contain this anomalous band (Fig. 3, lane H). This led us to evaluate further whether the deletion event was more prevalent in soil. Kanamycin-resistant colonies isolated from the inoculated soil were screened by colony hybridization for deletion of the pRL425 vector segment with pRL425 as a probe (Fig. 4). Of 400 colonies screened, 4 were found to contain no pRL425 sequences. Colonies from liquid culture controls were also screened for the deletion event, but of 200 colonies screened from the inoculum used plus 500 from mid-log-phase cultures, none were found to have lost the vector sequences. We were, however, able to isolate similar deletion derivatives from inoculated minimal medium held at 4 or 40°C. These extreme conditions are far from optimal for growth of PC-1 and thus may have resulted in a stress-induced deletion. The strains used here, when added to soil, likely did not grow to a significant extent since we observed a steady decline in numbers over several weeks of incubation in previous studies (data not shown). Therefore, this study may represent a case in which stress, most likely due to the limited carbon in soil, caused a genomic rearrangement. This resulted in the deletion of vector sequences,

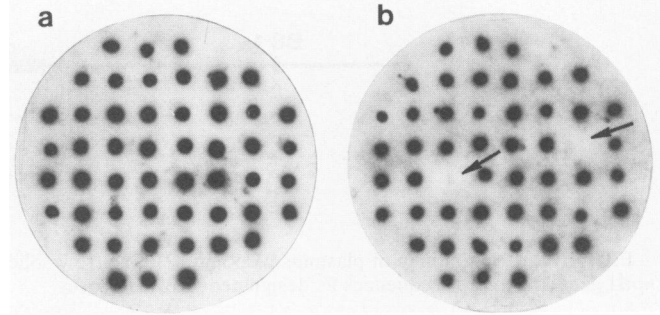


FIG. 4. Autoradiogram from colony hybridization analysis with ^{32}P -labeled pRL425 as a probe. (a) Colonies isolated from original inoculum; (b) colonies isolated from soil on solid King B medium containing kanamycin (500 $\mu\text{g}/\text{ml}$), nalidixic acid (400 $\mu\text{g}/\text{ml}$), and cycloheximide (200 $\mu\text{g}/\text{ml}$). Arrows indicate Kan^r colonies not hybridizing with the pRL425 probe.

possibly owing to the proximity of identical chromosomal sequences (Fig. 2).

Subsequent experiments showed that the deletion event could sometimes be observed in DNA from both the original inoculum and a stationary-phase culture incubated at room temperature if larger amounts of DNA were used. Thus, variants with the deletion may have always been present in the inoculum but at a much lower frequency than found in soil, especially since the target DNA was diluted by DNA from the total soil community.

The single-crossover insertion of vector DNA was less stable in PC-1 than in B8-1 even though PC-1 had much shorter regions of homologous chromosome than did B8-1. The lower stability of our PC-1 construct may be due to the fact that nalidixic acid-resistant (Nal^r) mutants are known to have a higher frequency of deletion and recombination because the Nal^r of the mutation affects DNA gyrase (6). We cannot rule out the possibility that a similar deletion also occurred in B8-1, but if so, it was at a frequency below detection.

While the deletion of vector sequences under stress conditions may not be surprising, what is important is the demonstration of the ability to detect changes in the structure of DNA sequences in natural communities. This is particularly important in risk assessment of engineered microorganisms proposed for environmental use. Southern blots were advantageous in that they were capable of detecting recombinants in soil at frequencies lower than could be practically determined by colony hybridization. The principle of detecting genomic rearrangements by restriction fragment length polymorphism applies whether the rearrangement is within the genome of an individual organism or results from gene transfer between organisms.

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