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Integration and Excision of a 2,4-Dichlorophenoxyacetic Acid-Degradative Plasmid in *Alcaligenes paradoxus* and Evidence of Its Natural Intergeneric Transfer

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A self-transmissible 2,4-dichlorophenoxyacetic acid (2,4-D)-degradative plasmid, pKA2, has been identified in a new 2,4-D-degrading strain, *Alcaligenes paradoxus* 2811P, isolated from agricultural soil. pKA2 occurred as a 42.9-kb plasmid in strain 2811P. A derivative strain, 2811C, was isolated from a stock culture in which the entire pKA2 plasmid was apparently integrated into the host chromosome without loss of the 2,4-D$^+$ phenotype. This interpretation is based on the disappearance of a free plasmid DNA band, a shift in the *tfdA*-hybridizing band to the chromosome, loss of transmissibility of the 2,4-D$^+$ trait, and appropriate shifts in Southern hybridization bands of plasmid DNA compared with whole-cell DNA. The integrated plasmid of strain 2811C was excised either precisely or imprecisely after continued transfer on 2,4-D-containing medium. This suggests that a chromosome-free plasmid cycle may occur to optimize fitness under conditions of specific resource fluctuation. Another new 2,4-D-degrading strain, *Pseudomonas pickettii* 712, which was isolated from the same field plot but at a different time, was found to carry a plasmid that is nearly identical to pKA2. The plasmid of this strain, pKA4, is 40.9 kb long and has features in common with pKA2, such as high self-transmissibility, hybridization only to the *tfdA* gene among the 2,4-D-metabolic genes of 2,4-D-degradative plasmid pJP4, and similar restriction endonuclease-generated fragments. Furthermore, the genetic homology between the two plasmids was high since all fragments of pKA2 hybridized to pKA4. These results suggest that these two plasmids are closely related and thus their occurrence in two genera in nature is the result of natural horizontal gene transfer.

A number of catabolic plasmids which enable host microorganisms to utilize xenobiotic compounds as carbon and energy sources have been isolated and described. Most catabolic plasmids exist as extrachromosomal genetic elements that replicate autonomously. However, there are reports that catabolic gene recombination occurs between the chromosome and plasmids under selective conditions. This is well documented for aromatic hydrocarbon-degradative genes on the *Pseudomonas* TOL plasmid; these are excised occasionally by reciprocal recombination between the direct repeats (15) and integrated into the host chromosome (10, 20). The integrated genes can be rescued by plasmids R2 and pMG18 to form novel recombinants (10).

Most of the genes coding for herbicide 2,4-dichlorophenoxyacetic acid (2,4-D-degradative enzymes have been reported to be contained on plasmids (3, 4, 16, 17). Unlike the toluene-degradative genes in pseudomonads (1, 10, 20) and the 3-chlorobenzoate-degradative genes in *Alcaligenes* sp. strain BR60 (23), 2,4-D-degradative genes have not been shown to move either between chromosomal DNA and plasmid DNA or between plasmids. Furthermore, there have been no reports that the entire catabolic plasmid can be integrated into the host chromosome and then excised to form a free plasmid. This phenomenon could play an important role in gene persistence and enhanced expression of operons in environments where resources fluctuate.

The increasing use of xenobiotic compounds may stimulate a more widespread dissemination of the corresponding degradative plasmids among microbial populations in nature. The transfer of catabolic plasmids, such as 2,4-D-degradative plasmid pJP4 (5), on agar media is well known. However, naturally occurring horizontal gene transfer between bacterial populations is difficult to detect because of the absence of the appropriate means for investigating such low-frequency events in open environments. The horizontal transfer of genes responsible for 3-chlorobenzoate degradation in a natural aquatic community was suggested in a microcosm study that stimulated conditions in a shallow bay (6). Physically and genetically indistinguishable 2,4-D-degradative plasmids were detected in different *Alcaligenes* species isolated independently from soil (3), which provides indirect evidence of natural gene transfer. Nonetheless, there have been few reports that support degradative gene exchange between different genera in open environments.

In this report, we present evidence of chromosomal integration of an entire 2,4-D-degradative plasmid and demonstrate that it can be excised either precisely or imprecisely during growth on 2,4-D. In addition, we present evidence of natural intergeneric transfer of this plasmid between *Alcaligenes paradoxus* and *Pseudomonas pickettii* strains that were isolated from the same soil at two different times.

MATERIALS AND METHODS

Bacterial strains. Strains 2811P and 712 were isolated from the gene flow plot at the long-term ecological research site at the W. K. Kellogg Biological Station (Hickory Corners, Mich.) by enrichment for growth on 2,4-D as the sole source of carbon. These strains were identified as belonging to *A. paradoxus* and *P. pickettii*, respectively (11). Strain 2811C was

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isolated from a glycerol stock culture of strain 2811P kept at −20°C for 2 years (except when occasionally thawed) and was reconfirmed as *A. paradoxus*. *P. cepacia* DBO1, which was obtained from R. Olsen (University of Michigan), contains transposon Tn*5* and is resistant to kanamycin (75 μg/ml), bacitracin (50 μg/ml), and carbenicillin (50 μg/ml).

Media and growth conditions. Peptone-tryptone-yeast extract-glucose broth consisted of 0.25 g of peptone (Difco Laboratories, Detroit, Mich.), 0.25 g of tryptone (Difco), 0.5 g of yeast extract (Difco), 0.5 g of glucose, 0.03 g of magnesium sulfate, and 0.003 g of calcium chloride. Solid medium containing 1.5% (wt/vol) agar was used for strain purification. Bacterial strains were cultured in Luria broth (14) (to produce cells not induced to metabolize 2,4-D), harvested, and prepared in sodium phosphate buffer as described previously (12). MMO mineral medium (21) plus 2,4-D at 500 ppm was used for the axenic culture studies and cultivation of 2,4-D-degrading strains to detect and isolate plasmid DNA. All cultures were incubated at 30°C. Liquid cultures were aerated by shaking at 200 rpm in a G24 environmental incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J.).

DNA isolation. Plasmid DNA was isolated by using the procedure of Hirsch et al. (8). Chromosomal DNA was isolated by the procedure of Watson et al. (19) and subsequently purified by cesium chloride-ethidium bromide ultracentrifugation (14). For detection of plasmid DNA, cells were lysed by using a modified form (2) of the procedure of Kado and Liu (13). 2,4-D-degrading bacteria were cultivated at 30°C in 5 ml of MMO mineral medium containing 2,4-D at 500 ppm. When cells reached maximum density, they were harvested by microcentrifugation (16,000 × *g* for 1 min), washed with 1 ml of sterilized distilled water, and repelleted in a 1.5-ml Eppendorf microcentrifuge tube. The pellet was resuspended in 30 μl of distilled water and lysed by adding 120 μl of lysing solution (50 mM Tris base, 3% sodium dodecyl sulfate, pH 12.6). The solution was incubated at room temperature for 15 min, heated at 80°C for 1 min in a water bath, and extracted with 1 volume of a phenol-chloroform solution (1:1, vol/vol) saturated with TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0). The lysate was incubated overnight at room temperature. After microcentrifugation (14,000 rpm for 15 min), the aqueous phase was transferred into a new Eppendorf tube and mixed with 1/5 of a volume of 5× loading dye (14). Samples of 110 μl were subjected to electrophoresis in 0.7% (wt/vol) agarose in Tris-acetate buffer (40 mM Tris-acetate, 1 mM Na_2-EDTA). Gels were photographed on a DNA Transilluminator (Fotodyne Inc.) with type 55 positive-negative film (Polaroid Corp.).

Southern hybridization. To study hybridization patterns with 2,4-D-metabolic genes (*tfdA, tfdB, tfdC*, and *tfdD*), plasmid and chromosomal DNAs were prepared in crude lysate as described above, separated in a horizontal 0.7% agarose gel, transferred to nitrocellulose hybridization membranes (14), and hybridized with *tfd* gene probes. To analyze the restriction fragment profile, purified plasmid DNA and chromosomal DNA were digested with appropriate restriction endonucleases, size fractionated, and hybridized as described above. The probes were internal segments of the *tfd* metabolic genes (9) and 23S rRNA gene (18) that had been labeled with ^{32}P with a Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, Ind.). The plasmid DNA probe was labeled with ^{32}P with a Nick Translation Kit (Boehringer Mannheim). The prehybridization, hybridization, and posthybridization washes used have been previously described (11). Hybridization signals were detected by autoradiography with X-Omat AR film (Kodak, Rochester, N.Y.) exposed at −70°C with a Quanta III (Sigma, St. Louis, Mo.) intensifying screen. If necessary, the bound probe was stripped from the membranes prior to rehybridization by washing three times for 15 min each time with boiled distilled water containing 0.1% (wt/vol) sodium dodecyl sulfate.

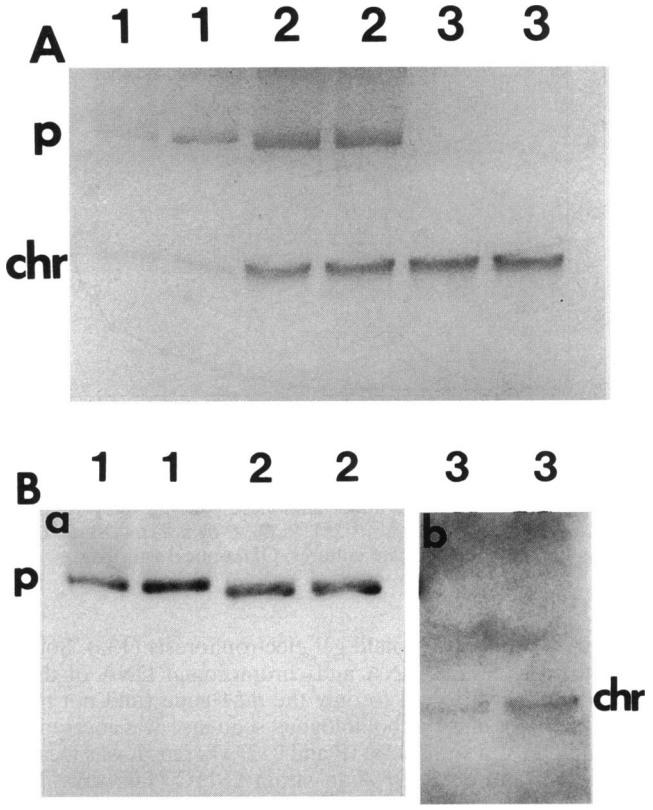

FIG. 1. Resolution of plasmids of 2,4-D-degrading strains. Agarose gel (A) and autoradiogram (B) of the gel after hybridization with the *tfdA* gene as the probe. Lanes: 1, *P. pickettii* 712 containing plasmid pKA2; *A. paradoxus* 2811P containing plasmid pKA2; 2, *A. paradoxus* 2811P containing plasmid pKA4; 3, *A paradoxus* 2811C not containing plasmid DNA. The positions of plasmid and chromosomal and linear (chr) DNAs are shown. The films in panel B were exposed for 20 min (a) and 2 days (b).

Conjugation. Matings were performed on membrane filters as described by Willetts (22). Transconjugants were selected on 2,4-D minimum medium containing MMO mineral medium, 2,4-D at 500 ppm, kanamycin (75 μg/ml), bacitracin (50 μg/ml), carbenicillin (50 μg/ml), and 1.5% Noble agar, and then their plasmid content was analyzed by agarose gel electrophoresis of crude lysates. The donor viable count was measured by plating dilutions on peptone-tryptone-yeast extract-glucose plates and counting the distinctive colonies of the donor. The frequency of transfer was calculated as the number of exconjugants per donor cell.

RESULTS

Properties of strains 2811P, 2811C, and 712. Strains 2811P and 712 were isolated from the same field subplot at different times; they contained 2,4-D-degradative plasmids pKA2 and PKA4 (Fig. 1A), respectively, and grew with 2,4-D as the sole carbon source. Strain 2811C, a derivative of strain 2811P, appeared not to have any free plasmid (Fig. 1A, lane 3). The presence of a free plasmid in 2811C could not be demon-

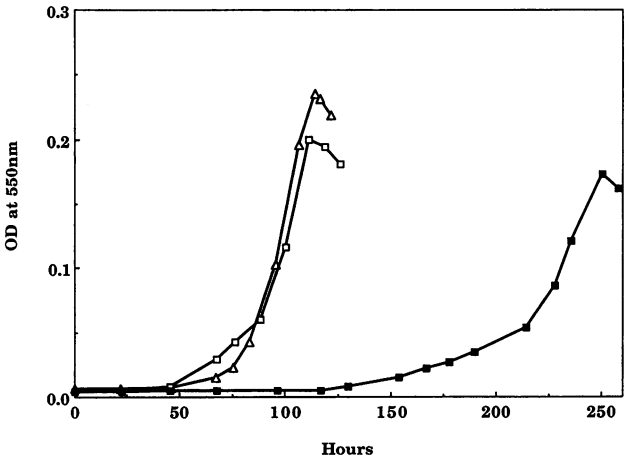

FIG. 2. Growth characteristics of 2,4-D-degrading strains in 2,4-D-containing mineral medium under uninduced conditions. Symbols: △, *P. pickettii* 712; □, *A. paradoxus* 2811P; ■, *A. paradoxus* 2811C. Each point is the mean of replicate cultures. OD, optical density.

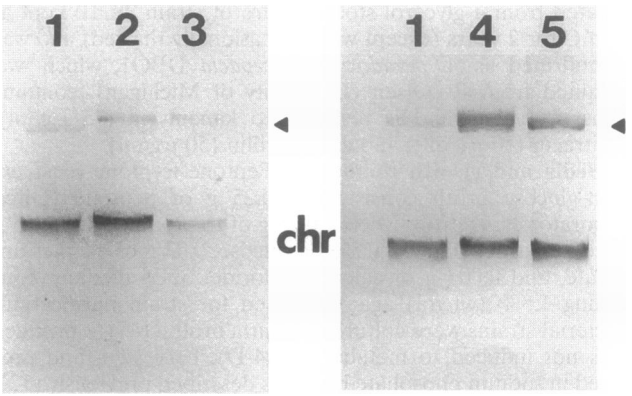

FIG. 3. Agarose gel showing plasmids in the donors, transconjugants, and recipient. Lanes: 1, recipient *P. cepacia* DBO1; 2, transconjugant from 712 × DBO1; 3, donor *P. pickettii* 712; 4, transconjugant from 2811P × DBO1; 5, donor *A. paradoxus* 2811P. The arrowheads show the plasmid conferring the 2,4-D$^+$ phenotype. A cryptic plasmid was also present in the recipient strain.

strated, even by pulsed-field gel electrophoresis (13a). Southern blots of plasmid DNA and chromosomal DNA of these three strains hybridized to only the *tfdA* gene (and not *tfdB*, *tfdC*, or *tfdD*), and the homologous sequence was located on plasmid DNA in strains 2811P and 712, whereas it was located on the chromosomal DNA in strain 2811C (Fig. 1B). This suggested that the plasmid DNA not detected in the agarose gel was integrated into the host chromosome in strain 2811C. A universal probe of the 23S rRNA gene (18) was used as a probe to check whether strains 2811P and 2811C are the same strain. When the total DNAs of strains 2811P and 2811C were digested with two different restriction enzymes (*Eco*RI and *Hin*dIII) and compared on a Southern blot, matching band patterns were observed between these two DNA samples, confirming that these were identical strains.

These three strains showed a relatively long lag time (>50 h) and slow growth in 2,4-D minimal medium when not previously induced to grow on 2,4-D (Fig. 2). Strain 2811C had a much longer lag time than strain 2811P in 2,4-D-containing medium, perhaps because of different gene expression resulting from the chromosomal location of the 2,4-D operon. In contrast, strains 2811P and 712, which were identified as different genera but had nearly identical 2-4-D-degradative plasmids, showed similar and more rapid growth patterns in 2,4-D-containing medium.

Plasmid transfer. The possibility of transfer of the 2,4-D-degradative genes was tested by filter mating. In three independent experiments, transfer of pKA2 from strain 2811P to *P. cepacia* DBO1 was obtained at an average frequency of 7.7×10^{-3} per donor colony formed. Under similar conditions, the 2,4-D$^+$ phenotype was not transferred at a detectable frequency ($<10^{-9}$) from strain 2811C, supporting the interpretation that the 2,4-D genes were not contained on a free conjugative plasmid in this strain. Plasmid pKA4 of strain 712 was transferred at an average frequency of 1.0×10^{-2} per donor cell. Plasmid bands exhibiting identical electrophoretic mobilities were observed in agarose gels containing the transconjugants and their respective donors (Fig. 3).

Physical evidence of integration of the plasmid into the chromosome. To investigate the fate of pKA2 in strain 2811C, plasmid pKA2 and total DNAs of strains, 2811P and 2811C were digested with two different restriction enzymes and analyzed on Southern blots hybridized with ^{32}P-labeled pKA2 plasmid DNA (Fig. 4). As expected, all of the seven hybridized fragments of pKA2 (Fig. 4) were also observed in the *Eco*RI-digested total DNA of strain 2811P, which contained the same plasmid, in addition to an unexpected novel fragment (CA; Fig. 4A). The novel fragment was not observed in the digested plasmid DNA but was observed in the digested total DNAs of both strains 2811P and 2811C. For this reason, we believed it to be derived from the digested host chromosomal DNA. This was further supported by the fact that its radioactive signal was much weaker than the signal obtained with the plasmid DNA fragment of a similar size because of its lower copy number. In *Eco*RI-digested total DNA of strain 2811C, one fragment (ED) disappeared with concomitant appearance of three extra hybridized fragments (arrowheads in Fig. 4A). This observation indicates that the whole pKA2 plasmid was integrated into the host chromosomal DNA without loss of the 2,4-D$^+$ phenotype, although normally the appearance of only two additional bands is expected with the loss of one band with chromosome integrations. The loss of one hybridized fragment (HB) with concomitant appearance of three novel fragments (arrowheads) was also observed in *Hin*dIII digests (Fig. 4).

Excision of plasmid pKA2. The existence of plasmid pKA2 as both an independent plasmid in strain 2811P and a chromosomally integrated plasmid in strain 2811C, together with the observed difference in their growth patterns on 2,4-D-containing medium (Fig. 2), suggested that the population containing a free plasmid might be derived from strain 2811C and that it could be preferably amplified under selection imposed by growth on 2,4-D. To investigate this hypothesis, strain 2811C was cultivated in 2,4-D-containing broth with repeated transfers into fresh medium. After the seventh transfer, a weak plasmid band was detected on the agarose gel. The intensity of this band increased with the increasing number of transfers. Southern hybridization of the gel with *tfdA* exhibited a clear hybridized plasmid band from the seventh transfer, and the radioactive intensity was much increased after the 9th and the 11th transfers (Fig. 5). After longer film exposure, weak hybridized bands were observed in the chromosomal DNA band area on Southern blots of the original culture and in cultures after the 7th, 9th, and 11th transfers (data not shown). After further transfers on 2,4-D-containing medium, a second

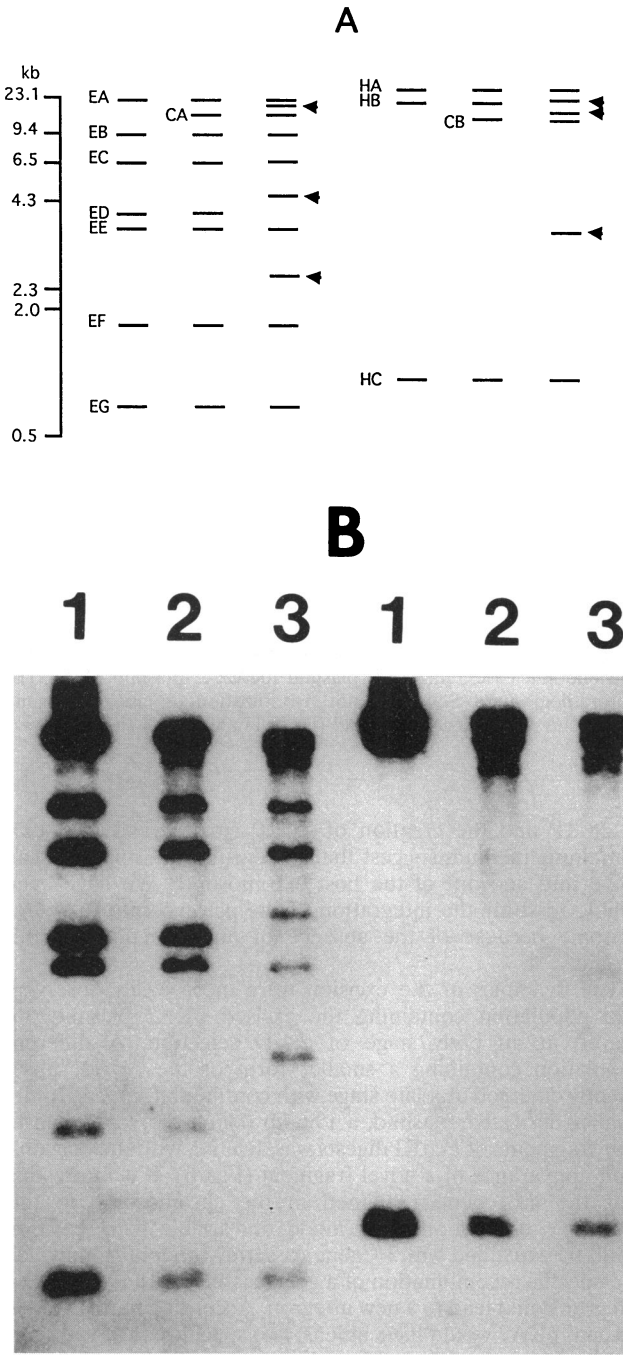

FIG. 4. Line drawing (A) and autoradiogram (B) of a Southern blot hybridization of labeled plasmid pKA2 with *Eco*RI (left) and *Hin*dIII (right) restriction enzyme digests of pKA2 plasmid DNA (lanes 1), total DNA of strain 2811P (lanes 2), and total DNA of strain 2811C (lanes 3). Novel extra bands presumably resulting from the integration process are indicated by the arrowheads in panel A.

plasmid band was detected from the culture of the 20th transfer, suggesting that the integrated plasmid could also be excised in a different way. These results indicated that the initial culture of strain 2811C became a mixed culture of 2811C and at least two other plasmid-containing variants that are probably more fit for growth on 2,4-D.

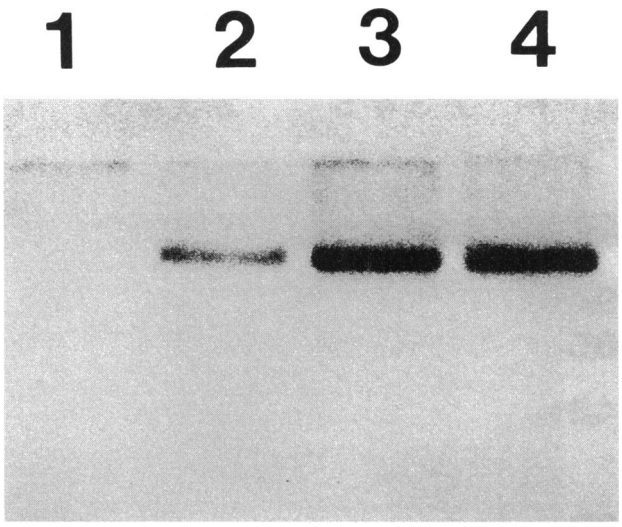

FIG. 5. Excision of plasmid DNA in strain 2811C during continuous growth on 2,4-D-containing medium. Samples (5 ml) of the culture grown initially (lane 1) and after the 7th (lane 2), 9th (lane 3), and 11th (lane 4) transfers were subjected to the Kado procedure and then plasmid and chromosomal DNAs in the lysate were gel separated and analyzed on Southern blots hybridized with the labeled *tfdA* probe. The film was exposed for 4 h.

To estimate the ratio of the 2811C population to the population of the excised-plasmid-containing strains, colonies from cultures after the 8th and 14th transfers were randomly selected, purified on peptone-tryptone-yeast extract-glucose plates, and subjected to the modified Kado procedure. Whereas no plasmid DNA band was detected in the 30 colonies examined from the 8th transfer, clear plasmid DNA bands were observed in 5 of 28 colonies examined from the 14th transfer. This result suggested that the mixed population was composed mainly of strain 2811C, even at the 8th transfer, while plasmid-containing strains gradually became more predominant and made up approximately 18% of the population after the 14th transfer. Plasmid DNAs were isolated from three colonies selected randomly from the five colonies to analyze the fragment profiles of restriction endonuclease digests. Two of the three plasmids showed restriction profiles identical to that of plasmid pKA2 in *Eco*RI and *Hin*dIII digests (Fig. 6), while the third plasmid revealed deletion of four fragments as well as the appearance of an extra, novel fragment (arrowhead) in *Eco*RI digests (Fig. 6). The derivative plasmid may have come from an alternative excision event of the integrated pKA2 plasmid in strain 2811C because the 3.5-kb fragment (the fifth band in the *Eco*RI digest in Fig. 6), which contained the sequences homologous to the *tfdA* gene, remained in the chromosome (data not shown).

Comparison of pKA2 of *A paradoxus* with pKA4 of *P. pickettii*. As stated above, plasmid pKA2 of *A. paradoxus* has properties similar to those of plasmid pKA4 of *P. pickettii* in regard to size, high transmissibility, and hybridization pattern with *tfd* probes. In addition to that, restriction enzyme analysis with *Eco*RI and *Hin*dIII revealed similar restriction patterns for these two plasmids (Fig. 7A). In *Eco*RI digests, only two of the seven fragments (EB and EC) showed altered mobilities in agarose gel electrophoresis while one of the three fragments (HB) revealed different mobility in *Hin*dIII digests (Fig. 7A). When fragments EA, ED, and EE of both plasmids (pKA2 and pKA4) were isolated from the agarose gel and digested with

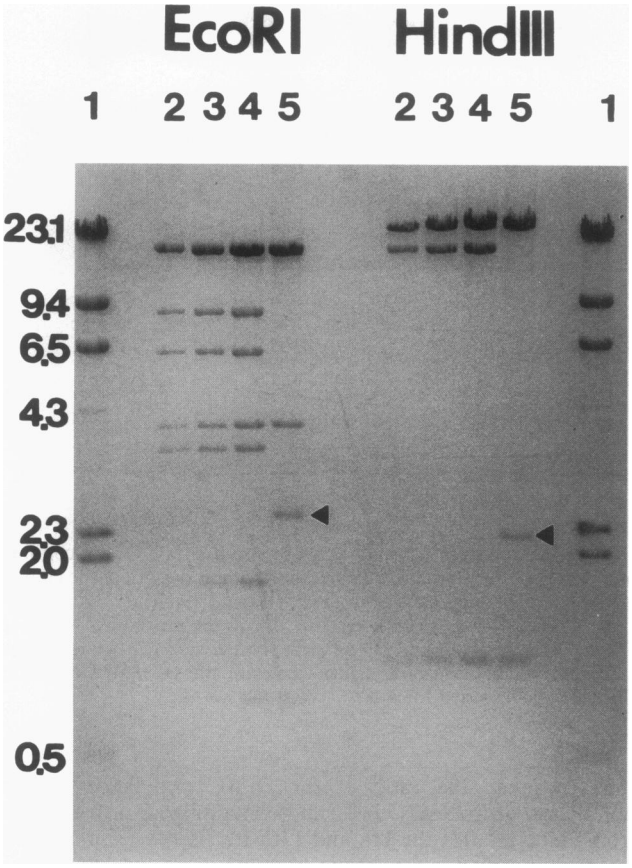

FIG. 6. Agarose gel containing restriction enzyme-digested plasmid DNA. Lanes: 1, lambda DNA digested with *Hin*dIII as size markers; 2, plasmid pKA2; 3, 4, and 5, plasmids isolated from three independent colonies derived from strain 2811C after 14 serial transfers on 2,4-D. The numbers on the left are molecular sizes in kilobases. The arrowheads indicate novel extra bands.

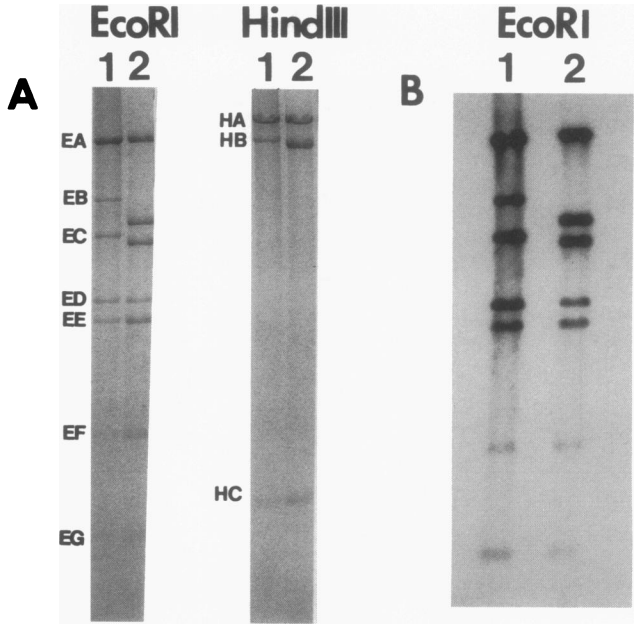

FIG. 7. (A) Agarose gel containing plasmids digested with restriction endonucleases. Lanes: 1, plasmid pKA2; 2, plasmid pKA4. (B) Autoradiogram of Southern blot hybridization of labeled plasmid pKA4 with *Eco*RI-digested pKA2 (lanes 1) and pKA4 (lanes 2).

*Bgl*II, the fragments were identical for each plasmid (data not shown). To determine whether all of the DNA fragments of plasmids pKA2 and pKA4 generated by restriction endonuclease *Eco*RI are homologous to one another, the fragments were analyzed on Southern blots hybridized with pKA4 DNA as the probe (Fig. 7B). All of the fragments of pKA2 were shown to be hybridizable with pKA4. The identical restriction profiles and hybridization patterns demonstrate that pKA2 of *A. paradoxus* and pKA4 of *P. pickettii* are genetically homologous, suggesting that intergeneric gene transfer between these two strains has occurred in nature.

DISCUSSION

The data demonstrate that 2,4-D-degradative plasmid pKA2 can exist as both a plasmid integrated into the host chromosome and a free plasmid. Since most of the 2,4-D-degradative genes have been reported to be carried on plasmids (3, 4, 16, 17), the observation of its chromosomal location was surprising. The integrated plasmid could be precisely excised, reforming the original pKA2 plasmid. The growth advantage of strain 2811P over strain 2811C in 2,4-D minimal medium (Fig. 2) may result from excision of plasmid DNA from the chromosome, with subsequent selection enhancing the plasmid-containing population. The detection of strain 2811C in the stock culture of 2811P and the creation of 2811P from 2811C in 2,4-D-containing medium suggest that the entire pKA2 plasmid can move into and out of the host chromosome. We have been unable to study the integration of the plasmid into the chromosome because of the absence of any selection for this variant.

The dynamics of the excision were more easily observed. The population containing the excised pKA2 plasmid appeared at an early stage of 2,4-D selection. A different population containing a smaller form of the pKA2 subsequently emerged at a late stage with continual transfers. In the smaller derivative plasmid, a 19.6-kb region corresponding to four fragments of *Eco*RI digests was deleted, with the concomitant appearance of a novel fragment (Fig. 6). It is interesting that the *tfdA* gene remained in the chromosome in this derivative population. This mode of variable gene excision could be explained with a "gene cassette" model (7), in which site-specific recombination of a gene cassette with an ancestral integron could lead to a new integron. According to this model, plasmid pKA2 would have at least two specific conserved sites, which recombine either with each other or with the specific site on the chromosome during the insertion and excision events. The observation that a fragment of the digested chromosomal DNA was hybridizable with pKA2 DNA indicates that these two DNA elements shared some conserved sequences.

The location of pKA2 in the chromosome not only resulted in loss of the freedom of its conjugal transfer but also led to slower growth of the host. While chromosomally located plasmid pKA2 seemed to have been excised at an early stage in the serial transfers, the plasmid-containing strain did not rapidly become the dominant type. This result suggests that 2811P could not competitively overcome 2811C within several transfers, although the former grew much more rapidly than the latter in the axenic culture. This observation could be explained by our previous finding that the exchange of cell

products in the mixed-culture environment reduced the lag period of the slowly growing and noncompetitive strain, making it more competitive (12).

We isolated two different 2,4-D-degrading bacteria belonging to the genera *Alcaligenes* and *Pseudomonas* from the same soil at two different times. The observation that plasmid pKA2 of *A. paradoxus*, isolated in 1989, showed a restriction profile nearly identical to that of pKA4 of *P. pickettii*, isolated in 1990, together with their similar physical properties and genetic homology, strongly suggests that intergeneric gene transfer followed by plasmid modification, or vice versa, has occurred between these two species in nature.

While it is not clear how pKA2 is integrated into and excised from the chromosome, our results show that a conjugative plasmid can be integrated in its entirety into the host chromosome and that the chromosomally located plasmid can be excised either precisely or imprecisely. Additionally, we found evidence that intergeneric gene transfer between *Alcaligenes* and *Pseudomonas* species occurred in nature. Future research on the physiological differences between strains 2811P and 2811C on 2,4-D-containing medium, the specific integration sites on pKA2 and the chromosome, and the mechanism of integration and excision should reveal the mechanism and benefit of this genetic flexibility.

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