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Parallel and Divergent Genotypic Evolution in Experimental Populations of *Ralstonia* sp.

CINDY H. NAKATSU,* RYSZARD KORONA,† RICHARD E. LENSKI, FRANS J. DE BRUIJN, TERENCE L. MARSH, AND LARRY J. FORNEY‡

NSF Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824

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Genetic rearrangements within a population of bacteria were analyzed to understand the degree of divergence occurring after experimental evolution. We used 18 replicate populations founded from *Ralstonia* sp. strain TFD41 that had been propagated for 1,000 generations with 2,4-dichlorophenoxyacetic acid (2,4-D) as the carbon source. Genetic divergence was examined by restriction fragment length polymorphism analysis of the incumbent plasmid that carries the 2,4-D catabolic genes and by amplification of random regions of the genome via PCR. In 18 evolved clones examined, we observed duplication within the plasmid, including the *tfdA* gene, which encodes a 2,4-D dioxygenase that catalyzes the first step in the 2,4-D catabolic pathway. In 71 of 72 evolved clones, a common 2.4-kb PCR product was lost when genomic fingerprints produced by PCR amplification using degenerate primers based on repetitive extragenic palindromic (REP) sequences (REP-PCR) were compared. The nucleotide sequence of the 2.4-kb PCR product has homology to the TRAP (tripartite ATP-independent periplasmic) solute transporter gene family. Hybridization of the 2.4-kb REP-PCR product from the ancestor to genomic DNA from the evolved populations showed that the loss of the PCR product resulted from deletions in the genome. Deletions in the plasmid and presence and/or absence of other REP-PCR products were also found in these clones but at much lower frequencies. The common and uncommon genetic changes observed show that both parallel and divergent genotypic evolution occurred in replicate populations of this bacterium.

Experimental evolution of bacteria has been an instrumental approach used to gain a greater understanding of the fundamental processes involved in adaptive evolution (7, 19, 23, 43, 44). These studies have shown that phenotypically similar populations evolve but underlying genetic divergence may exist because different adaptive mutations can result in similar phenotypic changes. The similarity or difference in genetic changes among replicate populations during adaptive evolution is a function of both the number of different possible adaptive mutations and the frequency at which they occur (21, 22, 44, 46). There are many potential sources of genetic variations (e.g., point mutations and genomic rearrangements) (14, 29, 32), and thus the likelihood of truly parallel (identical) genetic changes occurring would appear to be small, given the uncertainties of mutation and fixation. By observing the genetic changes that occur in experimentally evolved populations, we should be able to gain a better understanding of the genetic mechanisms used in adaptive evolution.

The objective of this study was to examine genetic changes that occurred in laboratory-evolved replicate populations of *Ralstonia* sp. strain TFD41 to determine if changes could be observed and, if so, whether these changes can be correlated with the observed phenotypic changes (23, 24). This is a potentially difficult process because improved fitness can result from genetic changes associated with a number of different aspects of bacterial growth, including but not limited to the phases of bacterial growth in batch culture (lag, logarithmic, stationary, and death), a particular cell structure (e.g., cell wall or ribosome), or a particular aspect of nutrient utilization (uptake or catabolism). To facilitate the search for genetic changes, we have pursued a top-down analysis, looking for genetic changes resulting from genome rearrangements. The approaches for genetic analysis were chosen because recent studies suggest that the role of recombination and genetic rearrangements in adaptive mutation and gene evolution may be greater than previously realized (3, 4, 18, 32, 35).

The genes (*tfdA* through *tfdF*) required for the catabolism of the carbon source 2,4-dichlorophenoxyacetic acid (2,4-D) (13, 31, 41) are plasmid encoded; therefore, we first examined rearrangements of the plasmid by restriction fragment length polymorphism and hybridization with the gene probes. Our second analysis was to look for genomic changes without regard to a particular locus by genome fingerprinting using degenerate primers based on repetitive extragenic palindromic (REP) sequences and PCR (REP-PCR) (12, 45). Potentially any number of different genetic changes could be responsible for improved fitness, and focusing on a single specific locus might cause one to overlook major genetic changes. This technique is simple, such that many replicate assays can be performed, and unbiased, such that random regions of the bacterial genome can be sampled. REP elements are just one example of many highly repeated sequences that are distributed throughout the genome of phylogenetically diverse bacteria (28). There are 581 copies of the REP sequence distributed throughout the *Escherichia coli* chromosome (8), allowing random amplification of the genome.

In this study, we demonstrated that genetic changes after experimental evolution of populations of *Ralstonia* sp. strain TFD41 can be observed by the various methods that we used. With the genetic information obtained from comparative analysis of plasmid fingerprints and REP-PCR-generated genomic

* Corresponding author. Present address: Department of Agronomy, Purdue University, West Lafayette, IN 47907-1150. Phone: (765) 496-2997. Fax: (765) 496-2926. E-mail: cnakatsu@purdue.edu.

† Present address: Institute of Environmental Biology, Jagiellonian University, 30-060 Krakow, Poland.

‡ Present address: Department of Microbiology, University of Groningen, Kerklaan 30, 9750 AA Haren, The Netherlands.

fingerprints, we were able to show parallel and divergent evolution in the genotype of replicate populations of a bacterium.

MATERIALS AND METHODS

Experimental evolution conditions. *Ralstonia* (formerly *Alcaligenes*) sp. strain TFD41 was isolated from an agricultural soil and shown to utilize 2,4-D as a sole carbon source (42). This strain was formerly called *Comamonas* sp. strain TFD41, but 16S rRNA sequence determination shows that it is a *Ralstonia* strain (29b). The protocol used to experimentally evolve *Ralstonia* sp. strain TFD41 has previously been described (24). Briefly, 18 independent populations initiated from a single ancestral clone were grown for 1,000 generations (250 days) in minimal defined medium (MMO) (40) with 2 mM 2,4-D as the carbon source. All populations improved in competitive fitness, relative to their common ancestor, by approximately 40% whether they were propagated in mass-action (6 populations in liquid batch) or structured (12 populations on solid agar substrate) environments. The ancestral strain was designated generation 0. Representative samples of each population were harvested every 100 generations through to generation 1000 and preserved as glycerol stocks (15% [final concentration]) at −80°C. Individual clones were randomly chosen for genetic analysis by streaking the glycerol stocks onto 2,4-D–MMO, agar plates. Each clone was grown in 2,4-D–MMO medium and preserved in glycerol at −80°C, allowing us to confirm experiments by performing replicate analysis.

Hybridization analyses. The *tfdA* through *-F* genes carried on plasmid pJP4 of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) JMP134 are involved in the initial steps of 2,4-D catabolism and have been well described (13, 31, 41). *E. coli* strains carrying the cloned structural genes *tfdA* through *-F* were used to generate probes for the hybridization experiments (20). All *E. coli* strains were grown at 37°C in Luria-Bertani broth plus ampicillin (50 mg/liter) (33). Plasmid DNA was isolated by the alkaline lysis method (33) and labeled by using PCR amplification primers and conditions described by Holben et al. (20) except that the standard deoxynucleoside triphosphate mix was replaced with the nonradioactive label, digoxigenin-dUTP–deoxynucleoside triphosphate mixture (Boehringer Mannheim).

Total genomic (5) and plasmid (33) DNAs were isolated from the ancestral clone and from one clone of each population at generation 1000. All cultures of TFD41 used for DNA extraction were grown at 30°C in minimal defined medium A+N (47) with 2 mM 2,4-D as the sole carbon source. Total genomic DNA was digested with the restriction enzyme *Bam*HI; plasmid DNA was digested with *Bam*HI and *Eco*RI (Bethesda Research Laboratories) and then separated by electrophoresis in an 0.8% agarose gel. Ethidium bromide-stained gels were photographed on a UV transilluminator, using type 55 Polaroid film. Plasmid gel photographs were scanned (Umax Astra 1200S) and then analyzed on a Power Macintosh 7500/100 computer, using the public domain NIH Image program (available on the Internet at http://rsb.info.nih.gov/nih-image/) to determine the relative intensities of the plasmid restriction fragments. To estimate larger fragment sizes (>20 kb), plasmid DNA was also resolved by using a CHEF (clamped homogeneous electric field) mapper system (Bio-Rad Laboratories). In this case, a 1.0% agarose gel buffered with 0.5× Tris-borate-EDTA (33) was run at 6 V/cm, with a 0.22- to 8.533-s switch time and 120° angle, at 14°C. The digested fragment sizes were determined based on comparative electrophoretic mobility vis a vis molecular markers of known sizes. Hybridization experiments were performed on DNA samples that were immobilized onto Hybond nylon membranes (Amersham) by Southern transfer (39). Hybridizations were carried out under high-stringency conditions (17). Detection was carried out according to the instructions supplied by the manufacturer (Boehringer Mannheim).

Genome fingerprinting. The genomic organization of ancestral and evolved populations of TFD41 was determined by DNA fingerprinting using REP-PCR. The fingerprints of four clones from each population were determined after 1,000 generations and compared to that of the ancestral strain. To determine if trends in genetic changes could be observed and if the small sample size was representative of the populations, an additional four clones from every 100 generations of populations 5 and 15 were analyzed, except at generations 500 and 1000, where 50 clones were examined. One microliter of frozen glycerol stock of each clone was amplified in a Perkin-Elmer Gene Amp 9600 system, using conditions and reagents described by de Bruijn (12). Fingerprint patterns were confirmed by repeating the amplification at least twice.

Genetic distances. Genetic distances between pairs of the four clones analyzed by REP-PCR from each of the 18 evolved populations after 1,000 generations of experimental evolution ($73 \times 72/2 = 2{,}628$ pairs), as well as the ancestral strain, were determined. The genetic distance between clones based on the REP-PCR fingerprints was calculated by determining the number of different PCR fragments (either present or absent) between every pair of clones and then dividing by the total number of PCR fragments observed. The average genetic distance was determined for three different comparisons: (i) among clones from the same evolved population ($4 \times 3 \times 18/2 = 108$ pairs), (ii) among clones from two different evolved populations ($4 \times 4 \times 18 \times 17/2 = 2{,}448$ pairs), and (iii) between the ancestor and evolved clones ($1 \times 72 = 72$ pairs). For comparisons i and iii, confidence limits (95%) were calculated based on *t* distribution using 17 degrees of freedom ($n - 1$, where $n = 18$ independently evolved populations). To obtain the confidence limits for comparison ii, we used the jackknife technique (37) to determine the contribution of each population to the overall average. All values are reported as the mean ± standard error of the mean.

Cloning and nucleotide sequencing of the 2.4-kb REP-PCR fragment. Genomic DNA from the ancestral strain was amplified by REP-PCR, and the resulting DNA fragments were separated on a 1.5% low-melting-point agarose gel (SeaPlaque; FMC). The 2.4-kb amplified fragment that was lost in the majority of evolved clones was cut out of the gel, purified by using a Gene Clean kit (Biolabs 101), inserted into the pCRII cloning vector (Invitrogen), and introduced into *E. coli* as instructed by the manufacturers. Recombinant plasmid DNA was isolated by the alkaline lysis technique (33), digested with *Eco*RI, and resolved by agarose gel electrophoresis. The 2.4-kb fragment was purified (Gene Clean) and labeled with digoxigenin-dUTP according to the instructions supplied by the manufacturer (Boehringer Mannheim). The 2.4-kb REP-PCR fragment was hybridized to genomic DNA digested with *Bam*HI and prepared as described above. Hybridization to the REP-PCR fingerprints was also determined by the method described above. Nucleotide sequence of the cloned fragment was determined by using Amersham's ThermoSequenase fluorescent primer sequencing kit and an ALFexpress automatic sequencer (Pharmacia). Nucleotide sequences were compared to entries in the available databases at the National Institutes of Health, using the BLAST (basic local alignment search tool) program (2).

Nucleotide sequence accession number. The nucleotide sequence of the 2.4-kb REP-PCR product has been deposited in the GenBank database under accession no. AF045553.

RESULTS

Plasmid and 2,4-D catabolic gene analyses. The ancestral strain of *Ralstonia* sp. strain TFD41 carries a large plasmid (~200 kb), designated pTFD41 that carries the genes required for catabolism of 2,4-D, the carbon source used in these experiments. Restriction enzyme digestion of the plasmid and fragment density plots revealed 14 *Bam*HI fragments, consisting of 12 unique and two duplicated sizes (Fig. 1). The three largest fragments (66.8, 29.6, and 27.2 kb) were resolved by pulsed-field electrophoretic separation (CHEF mapper system). The density plot of the *Bam*HI digest of the ancestral plasmid (Fig. 1B, lane 1) illustrates that the peak height and area of the 6.7- and 3.6-kb fragments were greater than those of the preceding fragments (8.6 and 3.7 kb, respectively) of larger size, suggesting greater numbers of copies of these fragments. All 18 clones from the evolved populations at generation 1000 maintained the catabolic plasmid. In each of the evolved plasmids examined, the 6.7- and 3.6-kb *Bam*HI fragments also had a greater peak height and area relative to the adjacent fragments (Fig. 1, lanes 2 to 6). In addition, the peak height and area of the 10.5-kb fragment were similar to those of the 9.9-kb fragment in the ancestral strain but noticeably higher than those in the evolved plasmids; therefore, this fragment may have been duplicated.

The *tfdA* through *-F* structural genes from *R. eutropha* JMP134 hybridized under high-stringency conditions to the 10.5-kb (*tfdA*), 9.9-kb (*tfdBDEF*), 8.5-kb (*tfdBCDEF*), and 6.7-kb (*tfdB*) *Bam*HI fragments of the plasmids in the ancestral and evolved clones. In total (chromosome and plasmid) genomic DNA preparations, only the fragments corresponding to the plasmid hybridized, demonstrating that copies of the *tfd* genes were not present on the chromosome. The 10.5-kb *Bam*HI fragment that apparently duplicated in the evolved plasmids hybridized to the *tfdA* gene. To differentiate the duplication of the *tfdA*-carrying fragment from the coincidental appearance of a new fragment of the same size, plasmids were digested with *Eco*RI because this site is present in the gene and hybridized with the *tfdA* gene probe. The ancestral plasmid was digested into approximately 33 *Eco*RI fragments ranging in size from 16 kb to less than 0.8 kb (data not shown). In the evolved plasmids, the 5.9-, 4.7-, and 3.0-kb *Eco*RI fragments had a greater relative intensity, and the 4.7- and 3.0 kb-fragments hybridized to *tfdA*, supporting the hypothesis that this region of pTFD41 had become duplicated.

There were also more obvious changes in the *Bam*HI re-

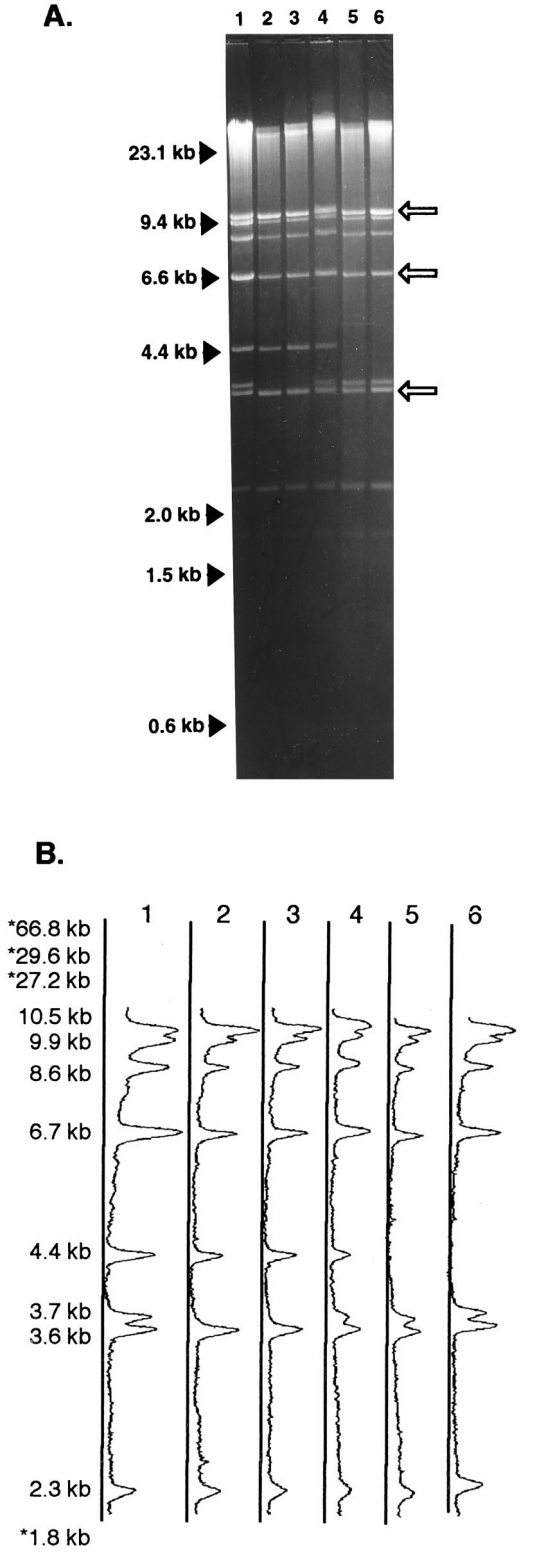

FIG. 1. (A) Restriction enzyme digest fingerprint of pTFD41 from ancestral and evolved clones of *Ralstonia* sp. strain TFD41. Lanes 1 to 6, *Bam*HI digests of pTFD41 from the ancestral strain, 15-1-1000 (population-clone-generation), 1-1-1000, 13-1-1000, 16-1-1000, and 18-1-1000, respectively. The fragments with greater intensity (10.5, 6.7, and 3.6 kb) are marked with open arrows. (B) Density plot of the *Bam*HI digest fingerprints from panel A. Plasmid fragment sizes are listed on the vertical axis. Asterisks indicate fragments not included on the density plot because they were either above or below the resolution level of the program. Lanes correspond to those in panel A.

striction enzyme digestion fragment patterns in four of the evolved plasmids when compared to the ancestral plasmid. Plasmids from two clones (from populations 1 and 13) were missing the 3.7-kb *Bam*HI fragment, and two other clones (from populations 16 and 18) were missing the 4.4-kb *Bam*HI fragment. In these plasmids there were no observable size changes relative to the other *Bam*HI fragments. However, digestion with *Eco*RI showed the loss of an 8.6-kb restriction fragment in populations 16 and 18 and a gain of an 8.0-kb fragment. In populations 1 and 13, the 7.6-kb *Eco*RI fragment was lost and an 8.2-kb fragment was gained. Additionally, in population 1, a 16-kb fragment was also lost and a 13-kb fragment was gained, demonstrating that the changes to these two plasmids were not identical. These deletions and additions did not involve any DNA fragments carrying catabolic genes.

REP-PCR fingerprint analyses. Genome organization of ancestral and evolved clones of *Ralstonia* sp. strain TFD41 were determined by REP-PCR fingerprint analyses (Fig. 2A). Nine unique REP-PCR fingerprint patterns were observed from the 337 evolved clones examined. These unique genotypes were distinguished by the presence or absence of specific REP-PCR amplification products. Of the 337 clones examined, 296 (88%) had REP-PCR fingerprints different from those of their ancestor. The majority of clones with ancestor-like REP-PCR fingerprints were from generation 500 or earlier (28% [37 of the 134 clones] versus 2% [4 of 171 clones] at generation 1000). Ten REP-PCR-amplified fragments were common to all clones examined. Table 1 characterizes the four REP-PCR genotypes that were detected most frequently during this study, including the ancestral genotype. They varied in the presence of the 2.4-, 2.0-, and 1.9-kb amplification products. Seventy-two evolved clones were analyzed after 1,000 generations of experimental evolution, and 48, 7, 15, 1, and 1 clones had genotypes I, II, III, ancestral, and unique, respectively. Note that 71 of the 72 clones lacked the 2.4-kb fragment. Evidently, in all 18 independently evolving populations of *Ralstonia* sp. strain TFD41, one or more genotypes that lacked the 2.4-kb REP-PCR fragment appeared and achieved high frequency.

To characterize the molecular events that led to loss of the 2.4-kb DNA fragment in REP-PCR fingerprints, the 2.4-kb fragment was cloned and used to probe REP-PCR-amplified DNA, plasmid DNA, and total genomic DNA from the ancestral and evolved populations. The cloned 2.4-kb DNA fragment strongly hybridized to the 2.4-kb REP-PCR fragment of the ancestral strain, one evolved strain from generation 1000 (clone 3 of population 15), and representative strains from two earlier generations (300 and 500) (Fig. 2B). In the genotypes where the 2.4-kb DNA fragment was absent, hybridization to any of the DNA preparations was not observed, verifying that the correct fragment was cloned. Two weak hybridization signals corresponding to DNA fragments of 3.0 and 0.6 kb were observed; since no corresponding fragments are visible in the REP-PCR fingerprint, these 3.0- and 0.6-kb fragments may represent PCR artifacts. The loss of the 2.4-kb REP-PCR-amplified DNA fragment apparently arose from chromosomal deletions in the evolved populations. The 2.4-kb DNA fragment hybridized only to the genome of the ancestral strain of TFD41 and to clone 3 of population 15 (Fig. 3). Only these isolates had a 2.4-kb REP-PCR amplification product. The probe hybridized to the same location in the genomes of both clones, to two *Bam*HI fragments 7.8 and 2.2 kb in size. The 2.4-kb clone failed to hybridize to restriction enzyme digests of the ancestral and evolved plasmids, confirming that the deletion occurred in the chromosome (data not shown).

The frequency at which specific genotypes were observed during experimental evolution was determined in two popula-

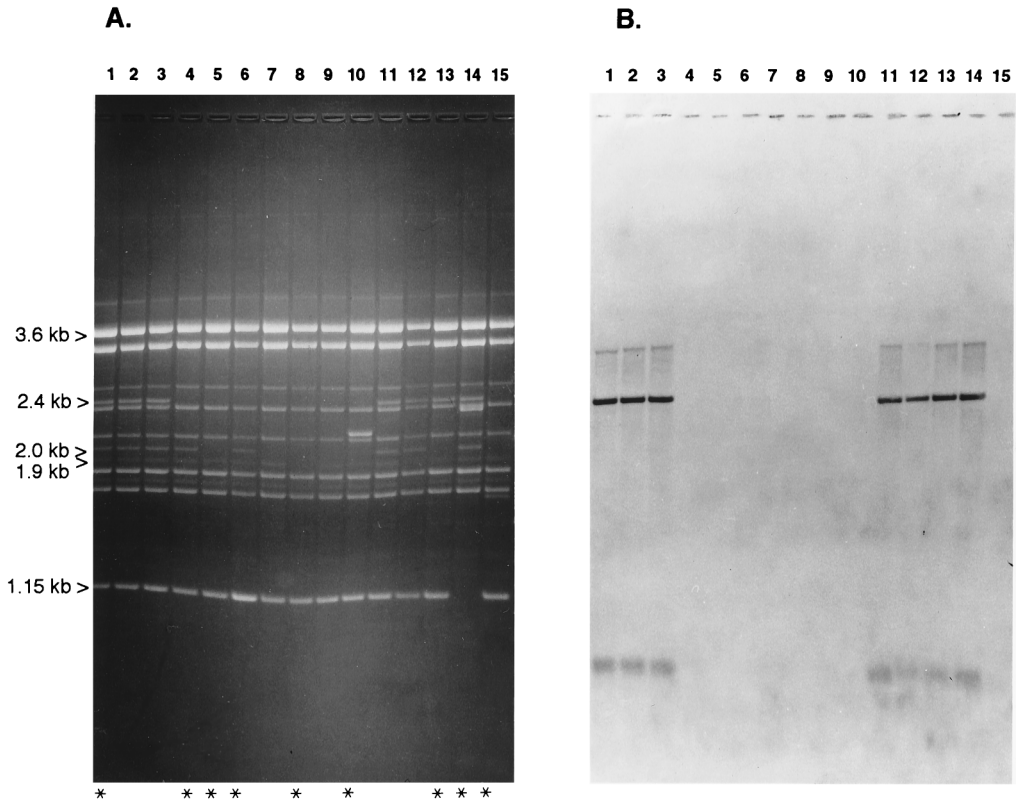

FIG. 2. (A) Comparison of REP-PCR fingerprints of ancestral and evolved strains of *Ralstonia* sp. strain TFD41. Lanes 1 to 3, ancestral strains not antibiotic resistant, streptomycin resistant, and nalidixic acid resistant, respectively; lanes 4 to 15, evolved clones 1-1-1000 (population-clone-generation), 2-3-1000, 5-1-1000, 6-1-1000, 8-1-1000, 11-1-1000, 14-1-1000, 15-3-1000 5-1-300, 5-2-300, 5-23-500, and 15-1-300, respectively. Asterisks indicate the nine different REP-PCR fingerprints that were observed. (B) Hybridization of cloned 2.4-kb fragment to REP-PCR-amplified DNAs from ancestral and evolved clones of *Ralstonia* sp. strain TFD41. Lanes correspond to those in panel A.

tions (5 and 15) by REP-PCR analyses of more than 50 clones from each population after 500 and 1,000 generations (Fig. 4). Population 5 had been propagated in liquid medium, whereas population 15 was propagated on agar surfaces. In both populations, evolved genotype I (which lacks the 2.4-kb REP-PCR fragment) largely displaced the ancestral genotype within the first 500 generations. In population 5, evolved genotype II (which lacks both the 2.0- and 2.4-kb fragments) had also become fairly common by 1,000 generations (in 15 of 53 clones). Samples (four clones for each population) taken at 100-generation intervals are consistent with these trends (data not shown).

TABLE 1. Most common REP-PCR genotypes of *Ralstonia* sp. strain TFD41 detected during long-term experimental evolution[a]

Genotypic designation	Presence of indicated approximate fragment size[b]			No. observed[c]	Frequency (%) observed
	2.42 kb	2.01 kb	1.90 kb		
Ancestral	+	+	+	1	1.4
I	−	+	+	48	66.7
II	−	−	+	7	9.7
III	−	−	−	15	20.8
Other				1	1.4

[a] Five other distinct REP-PCR genotypes were detected, but none of them were represented more than once among the >300 clones analyzed.

[b] All four genotypes listed also possess fragments of sizes 3.56, 3.28, 2.62, 2.37, 2.12, 1.83, 1.74, 1.66, and 1.15 kb. Values to only one decimal place are used in the text.

[c] For the 72 clones examined at generation 1000.

Genetic distances. Genetic distances between pairs of the four clones analyzed by REP-PCR from each of the 18 evolved populations after 1,000 generations of evolution as well as the ancestral strain were determined (summarized in Fig. 5). The average genetic distances determined were (i) 0.333 ± 0.094, among clones from the same evolved population; (ii) 0.848 ± 0.077, among clones from two different evolved populations; and (iii) 1.528 ± 0.161, between the ancestor and evolved clones. The larger values for genetic distances indicate a greater genetic divergence between the populations being compared. As one would expect, there was greater divergence between clones from different evolved populations (0.848 ± 0.077) than between clones from a single population (0.333 ± 0.094). One would expect that as independent populations evolve, the genetic distance between these populations (0.848 ± 0.077) would be greater than the distance between the evolved clones and their ancestor (1.528 ± 0.161). However, the values were opposite, suggesting that the independently evolved populations underwent parallel evolution. Most of this evolutionary convergence can be attributed to the loss of the 2.4-kb fragment; therefore, when it is excluded from these analyses, the average genetic distance between the evolved and ancestral genotypes is reduced by almost 1 (to ~0.5), whereas the average genetic distance between clones from different populations is hardly changed (~0.8). Then the average genetic distance between clones from two independently evolved populations was substantially greater than the

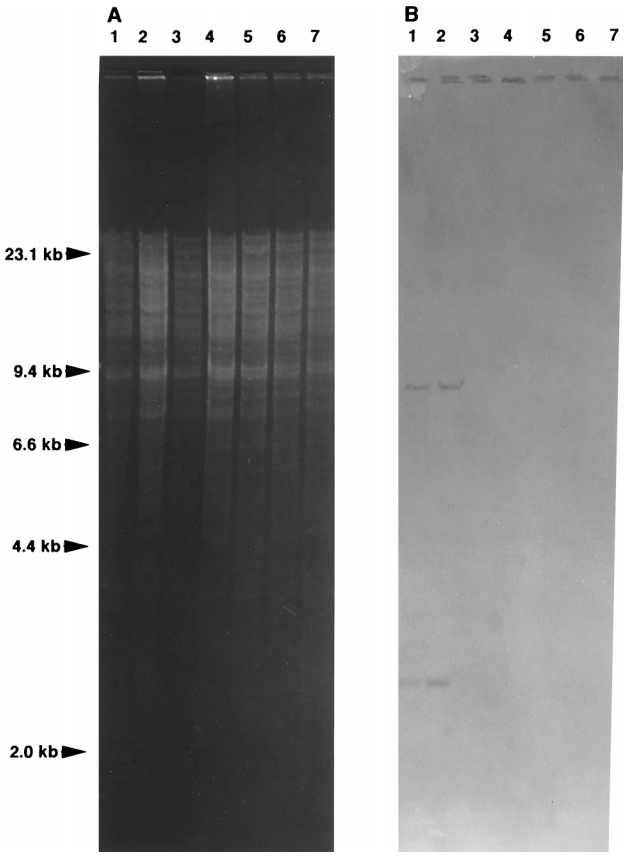

FIG. 3. (A) Ethidium bromide-stained agarose gel of total genomic DNAs from ancestral and evolved clones of *Ralstonia* sp. strain TFD41. Lanes 1 to 7, *Bam*HI-digested genomic DNAs from ancestral strain, 15-3-1000 (population-clone-generation), 15-1-1000, 1-1-1000, 2-1-1000, 13-1-1000, and 14-1-1000, respectively. (B) Hybridization of the 2.4-kb REP-PCR-amplified DNA fragment to ancestral and evolved clones of *Ralstonia* sp. strain TFD41. Lanes correspond to those in panel A.

average distance between evolved clones and their common ancestor, consistent with expectations for phylogenetically informative molecular characters.

Nucleotide sequence analysis of the 2.4-kb REP-PCR fragment. Comparison of the DNA sequence of the 2.4-kb (actual length, 2,420 bp) REP-PCR fragment nucleotide sequence to the GenBank database revealed homology to tripartite ATP-independent periplasmic transporter genes identified in *Rhodobacter capsulatus* and deduced to exist in *E. coli*, *Salmonella typhimurium*, *Haemophilus influenzae*, and *Synechocystis* spp. (16). The greatest amino acid sequence similarity is to the deduced YiaN (58% identical and 78% similar) protein in *E. coli* (8) and YiaM (33% identical and 62% similar) and YiaO (39% identical and 59% similar) in *H. influenzae* (15, 36). The termini of the 2.4-kb PCR product were located within the genes with sequence similarity to *yiaN* and *yiaO*. This finding is unusual because the REP sequences are typically extragenic. The REP nucleotide sequences delineating the PCR products were not found in the homologous sequences from the database search.

DISCUSSION

After propagation under identical environmental conditions for 1,000 generations (250 days), replicate populations of a 2,4-D-degrading environmental isolate, *Ralsonia* sp. strain

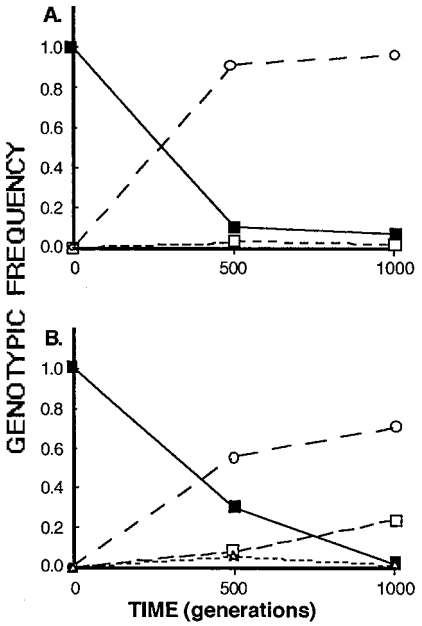

FIG. 4. Changes in relative frequency of REP-PCR genotypes of *Ralstonia* sp. strain TFD41 observed during experimental evolution. Ancestral genotype (■) and evolved genotypes I (○), II (□), and III plus other (△) are described in Table 1. (A) Population 5 propagated in the liquid (mass-action) habitat. (B) Population 15 propagated on the surface (physically structured) environment. Each of the 500- and 1,000-generation sample frequencies is based on >50 clones.

TFD41, were found to undergo both parallel and divergent evolution. Phenotypic analysis suggested that parallel evolution had occurred because of the systematic increases in competitive mean fitness relative to that of the common ancestor of each of the evolved populations (24). At the same time, divergence was observed by the variance of mean fitness values and changes in colony and cell morphology (23, 24). In this study, we were able to show that the organization of the genome had been altered and could be detected in these populations by

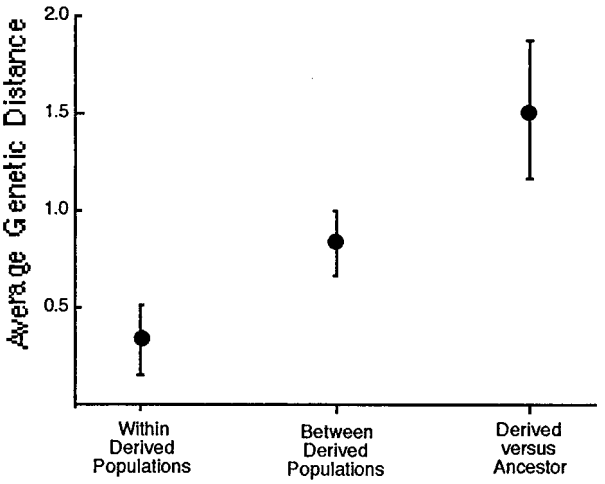

FIG. 5. Average genetic distances of ancestral and evolved clones of *Ralstonia* sp. strain TFD41 based on differences in REP-PCR fingerprint patterns. The first entry is the average distance among clones taken from the same evolved population. The second entry is the average distance among clones taken from two different evolved populations. The third entry is the average distance between the ancestral and evolved clones. Error bars indicate 95% confidence limits.

using relatively simple genetic analysis techniques. The genotypic analysis showed that common genetic changes occurred in these populations but the resulting genotypes were not identical.

Parallel genotypic evolution was suggested by the duplication of a common segment of the plasmid and by the deletion of a common fragment in the fingerprint pattern of the REP-PCR amplification products. Using DNA hybridization experiments and restriction digest analyses, we determined that there is an apparent duplication in the plasmid that occurred in all of the clones examined, suggesting that it is a determinant of competitive fitness in these populations. It may first appear unusual that the duplication in the plasmid is not accompanied by the addition of a new fragment. However, preliminary evidence indicates that the region is over 10 kb in size and is flanked by insertion sequence (IS) elements (10). We believe that recombination between the IS elements created the duplication and an additional copy of the IS element; therefore, the restriction fragments between and within the IS elements have been duplicated but no new fragments are created because the region external to the elements remains unchanged. By digestion with *Xba*I, an enzyme without a restriction site in the IS element, a unique fragment is observed in the evolved plasmids (29a).

It was not surprising to find that the apparent duplication in the plasmid included the *tfdA* gene, encoding the first enzyme in the 2,4-D catabolic pathway. DNA duplication not only is common in bacteria but has been observed frequently in many other organisms (9, 25, 27, 30). Duplications are believed to arise as a primitive regulatory mechanism to increase metabolism under extreme conditions (32). If the duplicated *tfdA* genes in the evolved *Ralstonia* populations are functional, then their fitness could be improved by increased enzyme activity. However, the duplicated region is large; therefore, in addition to the *tfdA* gene, other genes unrelated and related to 2,4-D utilization (e.g., gene regulation and substrate transport) are likely to be present in this region. This observation is similar to those of Sonti and Roth (38), who found that under limited-carbon conditions, large regions of the *S. typhimurium* chromosome were duplicated. The region duplicated included the permease genes for the carbon sources tested, but the duplication was also large and included other unknown genes.

Less expected, but most important and revealing, was the loss of a common 2.4-kb PCR amplification product in almost all (71 of 72) of the evolved clones examined (Fig. 2 and 3). The results were not expected because it seemed very unlikely that the same region of the chromosome would be deleted in virtually every genotype in each population. These findings are important because this technique allowed the screening of a great number of clones from independently evolved populations; therefore, it seems certain that the loss of the 2.4-kb REP-PCR fragment is causally related to the improvements in competitive fitness demonstrated in our previous study (24). The loss of the 2.4-kb product from REP-PCR fingerprints resulted from a deletion in the chromosome and not a point mutation or rearrangement into an alternate locus as shown by the lack of hybridization of the 2.4-kb PCR product to total genomic DNA from these evolved strains. All 18 experimental populations independently led to genotypes that had lost this fragment, and in all cases these genotypes reached high frequencies. We stress that these changes in the REP-PCR fingerprint must have evolved independently in each case because each population was founded from a single colony (and hence a single cell) of the ancestral strain. Moreover, we can exclude the possibility that the parallel changes in the 2.4-kb fragment resulted from cross-contamination among the experimental populations because the replicate populations possessed different antibiotic resistance markers that were strictly alternated during the serial propagation of cultures and remained distinct from one another (24). It is possible that the parallel changes at this locus indicate that it is hypermutable (6, 11, 29), but the fact that the deletion mutation achieved high frequency in every population implies that the deletion was under strong positive selection during the evolution experiment.

Nucleotide sequencing revealed that the 2.4-kb fragment carries genes related to the tripartite ATP-independent periplasmic transporter genes, encoding a high-affinity transport system for the C_4 dicarboxylates malate, succinate, and fumarate (16). We do not know if these genes are functional in *Ralstonia*; the REP primer sequences were intragenic and may have disrupted the genes. Also, the extent of the genomic deletion associated with the loss of the 2.4-kb PCR fragment is unknown; therefore, other genes that contribute to the fitness changes may have been lost. The deletion of this region can improve fitness if genes carry detrimental information or provide no selective advantage but are a costly genetic load (1). Further research is required to determine why and how this genetic change affects fitness.

It is important to recognize the fact that although common genetic events were observed, the resultant populations were not genetically identical. Genetic diversification was indicated by the different REP-PCR fingerprints observed in clones at generation 1000 and as early as generation 100. These differences showed that genetic divergence occurred both within and between the independently evolved populations. Genetic diversification was also indicated by the deletions that were observed in 4 of the 18 plasmids from evolved populations. These changes may be less frequent and/or provide less of a selective advantage than loss of the 2.4-kb DNA segment. Future research can elucidate if these genetic differences contribute to the variation in mean fitness observed within populations, produce an alternate phenotype (e.g., physiology or morphology), or are neutral mutations.

The results of this study suggest that the role of genetic rearrangements in adaptive evolution may be greater than is presently realized. The parallel genotypic changes observed in this study arose from some form of genetic recombination (26, 32, 34) that resulted in both duplications and deletions of relatively large spans of DNA sequences. Finding two genotypic changes associated with improved fitness reinforces the fact that a number of physiological factors affects the fitness of a population and that studies are not complete when single gene changes are found. More importantly, the study shows that genetic analysis of experimentally evolved populations with a known ancestor provides a means to begin interpreting genetic events that led to organisms as we see them now and to understand the underlying genetic mechanism.

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