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Genetic Variation in Clones of *Pseudomonas pseudoalcaligenes* After Ten Months of Selection in Different Thermal Environments in the Laboratory

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Abstract. The random amplification of polymorphic DNA (RAPD) method was used to examine genetic variation in experimental clones of *Pseudomonas pseudoalcaligenes* in two experimental groups, as well as their common ancestor. Six clones derived from a single colony of *P. pseudoalcaligenes* were cultured in two different thermal regimes for 10 months. Three clones in the Control group were cultured at constant temperature of 35°C and another three clones in the High Temperature (HT) group were propagated at incremental temperature ranging from 41 to 47°C for 10 months. A total of 45 RAPD primers generated 146 polymorphic markers. Analysis of molecular variance (AMOVA) revealed mild (11%) but significant ($P < 0.001$) genetic difference between the Control and the HT clones. Phylogenetic analysis based on pairwise genetic distances showed that the HT clones were more divergent from the ancestor and from each other than the Control clones, implying that the HT clones of *P. pseudoalcaligenes* may have evolved faster than the Control clones.

Selection for high temperature tolerance in bacteria provides one of the most popular examples of how directional selection operates on laboratory bacterial populations [2–4, 15, 16]. One exciting development in this field is the probing of the molecular basis of the acquisition of high temperature resistance [5, 22, 29]. A recent study on thermal adaptation of viral populations suggests that convergent and probably adaptive evolution occurred at the level of single nucleotide mutations [5]. Meanwhile, studies on *Escherichia coli* have shown that gene duplication may play an important role in thermal adaptation in bacterial species [22].

In previous studies, we addressed the phenotypic responses in clones of *Pseudomonas pseudoalcaligenes* that had been propagated for 10 months under both normal temperature and incremental high temperature [25, 26]. The fitness components (growth parameters) in the three HT clones improved greatly relative to the three Control clones and the ancestor at the originally stressful high temperature of 45°C [26]. The HT clones also exhibited increased cell sizes relative to the ancestral and the Control clones [25]. These stable phenotypic differences between the Control and the HT clones suggest genetic differentiation between groups. Understanding the genetic variation within and between the HT and the Control clones of *P. pseudoalcaligenes* will facilitate evaluation of the source of the genetic variation and provide insight into the processes that may have accompanied and promoted the selection response.

RAPD (random amplification of polymorphic DNA) is a rapid method for genome characterization and detection of DNA sequence variation [30]. RAPD allows random amplification of genomic regions without prior knowledge of the target DNA sequence. The method rapidly became popular because of its simplicity and applicability to any genome [20]. It has been used to study genetic structure and evolution of bacterial pop-

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ulations [8, 19, 21, 32] and to discriminate between different strains [7]. Therefore, RAPD was adopted to probe the potential genomic difference occurring in the *P. pseudoalcaligenes* genomes after 10 months of experimental evolution.

Two hypotheses can be put forward regarding the patterns of changes in genetic diversity in clones of *P. pseudoalcaligenes* that had been selected at different thermal environments for 10 months. First, genetic difference between the treatments is expected because of different selection pressures. Second, the genomes of the HT clones may show more genetic variation than the Control clones, because the increased temperature under which the HT clones had evolved would enhance the mutation rate and increase the genetic variation [12, 27]. The goal of this study was to test these two predictions.

Materials and Methods

Strains. Six clones of *P. pseudoalcaligenes* derived from a single clone (common ancestor) were propagated at different thermal environments for 10 months [26]. Three of them were cultured under normal temperature (35°C) as Control clones; the other three were propagated at gradually increased high temperature (41–47°C) as the HT clones and ultimately tolerated the originally stressful high temperature (45°C). Each Control clone was transferred to fresh Luria-Bertani (LB) agar medium independently every 12 h, and experienced a total of 600 transfers during the 10 months. The HT clones experienced a total of 420 transfers because of the variation in the growth rate during the experiment [26]. This study examined genetic variation at a genome-wide scale among clones both within groups (C1, C2, and C3: Control; HT1, HT2, and HT3: HT), and between groups (Control and HT).

DNA extraction. As the main purpose of this study was to examine the genetic variation at the genomic level among the test clones, it was important to discriminate between the different sources of variation, i.e., the nucleus DNA variation or plasmid DNA variation. Since many pseudomonad species contain plasmid DNA, it was thus necessary to check whether the seven tested clones of *P. pseudoalcaligenes* contained plasmid DNA. For this reason, both the plasmid DNA and the genomic DNA of the species were extracted. The plasmid DNA was extracted by an alkaline lysis miniprep procedure [1]. As a result, no plasmid DNA was extracted evidenced by electrophoresis.

To reduce the possible errors resulting from the different DNA extractions, a middle-scale genomic DNA extraction protocol was adopted, so that for each clone the DNA from one extraction was sufficient for all RAPD reactions throughout the study. Genomic DNA was isolated from each *P. pseudoalcaligenes* clone as following: 50 mL LB broth was inoculated with a single bacterial colony and grown to an OD_{600} of 0.5–1.0. Cells were collected by centrifugation at 5000 rpm, 4°C, for 10 min. The pellets were resuspended in 4 mL TEN buffer (0.1 M pH 8.0 Tris-HCl, 0.1 M EDTA, 0.15 M NaCl). Forty microliters of 100 mg/mL lysozyme solution was added to the bacterial suspension and incubated at 37°C for 10 min. This was followed by the addition of 100 μl of 2 mg/mL RNase and incubation at 50°C for 15 min, addition 0.5 mL 10% SDS and 22.5 μL 20 mg/mL proteinase K and incubation at 60°C for 30 min, and addition 1 mL 6 M NaCl, vortexing and centrifugation at 7000 rpm for 30 minutes, then transfer of the supernatant to a new tube and precipitation of the DNA by addition of 8 mL of 100% ethanol. The DNA filament was dried for a few minutes at room temperature and resuspended in 1 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). DNA samples were examined for purity and integrity by agarose gel electrophoresis before storing at –20°C. DNA samples were quantified using a Lambda Bio40 UV/VIS Spectrometer (Perkin Elmer Instruments). If plasmid DNA were present, this method would precipitate both plasmid and genomic DNA.

RAPD. As no plasmid DNA was extracted using the alkaline lysis miniprep protocol, all the RAPDs were carried out using genomic DNA as the template DNA. The parameters of the RAPD assay were optimized first, and then random DNA fragments were amplified in final volumes of 25 μL containing 10 ng of *P. pseudoalcaligenes* DNA, 2.5 μL GeneAmp PCR Gold buffer (Applied Biosystem), 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystem), 3 mM $MgCl_2$ (Applied Biosystem), 200 μM dNTPs, and 0.3 μM of a single primer. In the preliminary experiment, 58 10-mer primers from the Nucleic Acid-Protein Service Unit, The University of British Columbia, were screened. Forty-five primers that produced reproducible amplifications were used in the experiment (Table 1). PCR reactions were carried out in a GeneAmp PCR System 9700 (PE Applied Biosystem). Randomly amplified DNA products were generated by an initial 5 min denaturation at 95°C preceding the cycles, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1 min, and extension at 72°C for 2 min. The reaction was ended by a final extension at 72°C for 7 min. A negative control was included in each set of reactions using primer in the absence of DNA template.

A fraction of the amplification products (7 μL) was subjected to a 1.2% (w/v) agarose (G_{IB} BRL, Spain) gel in 1 × TAE buffer containing 0.3 μL/mL (w/v) of ethidium bromide (EtBr) and separated by electrophoresis at 4 V/cm for 5–6 h. The gels with amplifications were visualized and photographed by UV-transillumination in a MultiImage Light Cabinet (Alpha Innotech). The lengths of fragments were analyzed using the Fluorchem program (Alpha Innotech) by reference to a DNA molecular weight marker, i.e., λ DNA, cleaved with *Eco*RI and *Hind*III (Roche Molecular Biochemicals, Germany).

Data analysis. The RAPD bands were scored as 1 or 0 for presence or absence of a band on a gel. Relative intensity of bands (faint to strong) was not considered as a difference. A matrix of similarities between each pair of individuals was calculated according to Nei's similarity coefficient [18], $S = 2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of fragments shared by the two individuals, and n_x and n_y are the total number of fragments presented in individuals x and y, respectively. The pairwise distance metric used was $D = 1 - S$ for all clone pairs. The distance matrix was used for tree reconstruction with the Fitch-Margoliash (FM) method [10] implemented in the DAMBE program [31]. The FM method was chosen over the simpler UPGMA method because the evolutionary rate, as revealed by RAPD banding patterns, should not be constant. We did not use the neighbor-joining method [24] because it is not a criterion-based method and its advantage of being a fast algorithm is negligible in our case with only a few OTUs (operational taxonomic units).

To assess variance components for RAPD phenotypes and partition, the genetic variance among clones (C1, C2, C3, HT1, HT2, and HT3) within a group (population level) and among groups (Control and HT), two levels of analysis of molecular variance (AMOVA) were carried out using WINAMOVA v.1.55 [9]. AMOVA [9] has been demonstrated to be a powerful method for the analysis of population genetic structure at the molecular level [13, 14, 17]. The distance metric used in AMOVA is $D = 1 - S$ for each pair of individuals, where S is Nei's index of similarity [18]. Levels of significance of

Table 1. Characteristics of the RAPD markers generated by seven clones of *Pseudomonas pseudoalcaligenes* from different experimental groups using 45 random primers. Clones denoted as: A, ancestor; C1, C2, C3, the three 10 month Control clones; HT1, HT2, HT3, the three 10 month HT clones

				No. of fragments								
Primers	Primer sequence (5′–3′)	GC (%)	Patterns	A	C1	C2	C3	HT1	HT2	HT3	Total fragments	Polymorphics
UBC1	CCTGGGCTTC	70	3	10	11	11	12	11	12	11	12	2
UBC2	CCTGGGCTTG	70	5	5	3	4	4	1	5	2	5	4
UBC4	CCTGGGCTGG	80	2	3	4	4	4	4	4	4	4	1
UBC5	CCTGGGTTCC	70	3	3	3	3	3	3	5	1	5	4
UBC6	CCTGGGCCTA	70	1	2	2	2	2	2	2	2	2	0
UBC12	CCTGGGTCCA	70	4	4	6	5	1	1	5	1	6	5
UBC16	GGTGGCGGGA	80	3	11	11	11	11	10	11	8	11	4
UBC17	CCTGGGCCTC	80	2	5	5	4	5	5	5	5	5	1
UBC21	ACCGGGTTTC	60	2	3	6	3	6	6	6	3	6	3
UBC23	CCCGCCTTCC	80	2	5	5	5	5	5	6	6	6	1
UBC25	ACAGGGCTCA	60	5	7	7	6	7	5	8	8	9	4
UBC29	CCGGCCTTAC	70	3	6	6	5	6	5	4	7	7	3
UBC30	CCGGCCTTAG	70	5	7	9	9	9	8	7	2	10	8
UBC31	CCGGCCTTCC	80	2	6	2	2	6	6	6	6	6	4
UBC32	GGGGCCTTAA	60	3	1	2	2	2	3	1	2	3	2
UBC33	CCGGCTGGAA	70	3	8	8	8	8	5	5	9	9	4
UBC34	CCGGCCCCAA	80	2	9	9	9	9	9	9	10	10	1
UBC37	CCGGGGTTTT	60	2	4	4	4	4	4	1	4	4	3
UBC38	CCGGGGAAAA	60	6	13	12	9	7	7	14	14	15	8
UBC39	TTAACCGGGC	60	3	2	3	1	3	3	1	3	3	2
UBC40	TTACCTGGGC	60	4	2	7	2	7	4	7	8	8	6
UBC43	AAAACCGGGC	60	3	10	9	8	8	10	10	10	10	2
UBC51	CTACCCGTGC	70	1	1	1	1	1	1	1	1	1	0
UBC54	GTCCCAGAGC	70	3	4	4	4	4	3	2	3	4	2
UBC56	TGCCCCGAGC	80	1	2	2	2	2	2	2	2	2	0
UBC59	TTCCGGGTGC	70	2	3	3	3	3	2	2	3	3	1
UBC60	TTGGCCGAGC	70	2	4	4	4	4	4	4	1	4	3
UBC63	TTCCCCGCCC	80	5	5	9	9	9	2	4	7	9	7
UBC64	GAGGGCGGGA	80	6	8	8	5	10	8	9	9	10	5
UBC66	GAGGGCGTGA	70	3	8	7	8	7	4	7	8	8	4
UBC71	GAGGGCGAGG	80	3	11	12	8	12	12	12	12	12	4
UBC72	GAGCACGGGA	70	1	7	7	7	7	7	7	7	7	0
UBC73	GGGCACGCGA	80	4	10	10	10	10	9	7	9	10	3
UBC75	GAGGTCCAGA	60	5	8	10	7	6	6	7	13	13	7
UBC77	GAGCACCAGG	70	5	8	9	7	8	11	9	7	11	4
UBC79	GAGCTCGTGT	60	4	5	4	4	4	5	4	6	7	3
UBC82	GGGCCCGAGG	90	1	10	10	10	10	10	10	10	10	0
UBC85	GTGCTCGTGC	70	2	8	3	8	8	8	8	8	8	5
UBC86	GGGGGGAAGG	80	5	7	7	8	8	6	6	7	8	4
UBC89	GGGGGCTTGG	80	2	10	10	10	10	10	10	12	12	2
UBC90	GGGGGTTAGG	70	4	6	5	5	7	5	5	5	8	4
UBC92	CCTGGGCTTT	60	3	4	4	4	4	4	1	2	4	3
UBC96	GGCGGCATGG	80	2	7	8	8	7	8	8	7	8	1
UBC97	ATCTGCGAGC	60	3	3	1	1	1	9	1	3	9	8
UBC98	ATCCTGCCAG	60	3	3	5	5	3	5	1	5	5	4

variance component estimates were computed by nonparametric permutation procedures (i.e.,1000 random permutations).

Results and Discussion

RAPD profile. Of the 58 RAPD primers screened, 45 had reproducible banding patterns. The amplifications generated from the seven clones of *P. pseudoalcaligenes* using 45 random primers are presented in the Appendix. Of the 329 reliable bands generated by the 45 primers, 146 (44%) were polymorphic among the seven clones (Table 1). Considering each clone separately, 85 polymorphic markers (32%) were found in the ancestor, 94 (34%) in C1, 71 (28%) in C2, 89 (32%)

Table 2. The pairwise genetic distances (D) among the six experimental clones and the ancestral clone. Abbreviations of clones as in Table 1

A	C1	C2	C3	HT1	HT2	HT3
0						
0.0862	0					
0.0822	0.0752	0				
0.0812	0.0672	0.0775	0			
0.1179	0.1252	0.1306	0.0977	0		
0.1078	0.1227	0.1318	0.1065	0.1252	0	
0.1054	0.1236	0.1477	0.1188	0.1601	0.1498	0

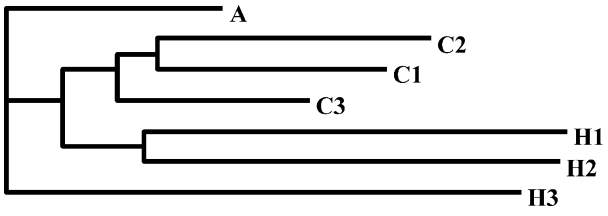

Fig. 1. Dendrogram of clones of *P. pseudoalcaligenes* from different experimental groups generated by the Fitch–Margoliash method, using 146 RAPD markers. Clones denoted as: A, ancestor; C1, C2, C3, the three 10 month Control clones; HT1, HT2, HT3, the three 10 month HT clones.

in C3, 75 (29%) in HT1, 78 (30%) in HT2, and 90 (33%) in HT3.

Of the 45 primers chosen, five produced amplifications with monomorphic bands among all tested clones, and six generated bands with only one polymorphic band among the clones. The remaining 34 primers generated at least two polymorphic bands in their amplified products among clones.

Phylogenetic analysis. The pairwise genetic distance matrix (Table 2) was used for tree reconstruction and AMOVA analysis. The tree reconstructed with the FM method [10] had relatively short branches for the three Control clones and relatively long branches for the three HT clones. This is consistent with one of our two predictions that the HT clones should exhibit more genetic variation as revealed by RAPD banding patterns (Fig. 1). One may note that the three HT clones did not form a monophyletic group, as they should if convergent evolution in the HT clones is largely responsible for the genetic difference between the HT clones and the Control clones (Fig. 1).

AMOVA partition. The two levels (groups and clones) of AMOVA analysis showed that RAPD variation at group level accounted for 11% of the total genetic variation (Table 3), and is highly significant ($P < 0.001$, Table 3). This indicates that selective change might have occurred between the two experimental groups and is consistent with one of our two predictions that the HT clones should have genetic difference from the Control clones. If genetic variance between the two groups was purely due to random genetic drift within each independent experimental clone, a significant genetic difference between the Control and HT groups should not be observed. Thus, the small but significant difference in genetic variance between the two groups was most likely due to positive selection.

AMOVA revealed that among clones within-group variability accounted for 89% of the total genetic variation. Combining the results of pairwise genetic distances and branch lengths of the reconstructed tree, we see that the large amount of within-group variation arose mainly from the great divergence among the three HT clones. Some of the pairwise distances between the HT clones were even larger than that between the HT clone and the Control clone (e.g., D = 0.1601, HT1 vs. HT3; D = 0.1498, HT2 vs. HT3; Table 2). Moreover, all the pairwise distances within the HT clones were larger than the distance between any of them and the ancestor. However, the pairwise distances within the three Control clones were always lower than that between the Control clones and the HT clones or the Control clones and the ancestor. These results suggest that the HT clones result from both convergent and divergent evolution. The divergence among the HT clones may be attributed to the increased mutation rate associated with increased culture temperature [12, 27].

The utility of the RAPD technique in characterizing genomic changes has been described, but it should be kept in mind also that the RAPD technique has its own limitations. For example, RAPD would generally not allow identification of a single mutation or a very small deletion [20].

The molecular events responsible for the genomic changes in the clones of *P. pseudoalcaligenes* have not been determined. The genetic changes underlying the process of adaptation can be point mutation, insertion/deletion events, gene duplications or chromosomal inversions, etc. Previous studies have shown that gene duplication may be an important molecular mechanism among *E. coli* clones genetically adapted to increased

Table 3. Results of analysis of molecular variance (AMOVA) with RAPD bands generated by 45 primers in clones of *P. pseudoalcaligenes*. The two groups are the Control and the high temperature (HT) group, each of which has three clones. Significance levels are based on 1000 permutations (more extreme random value).

	d.f.	MS	Variance	% total variance	*P* value
Control/HT (two levels)					
Among groups	1	0.075	0.0069	11.25	<0.001
Among clones within groups	4	0.055	0.0546	88.75	

Appendix

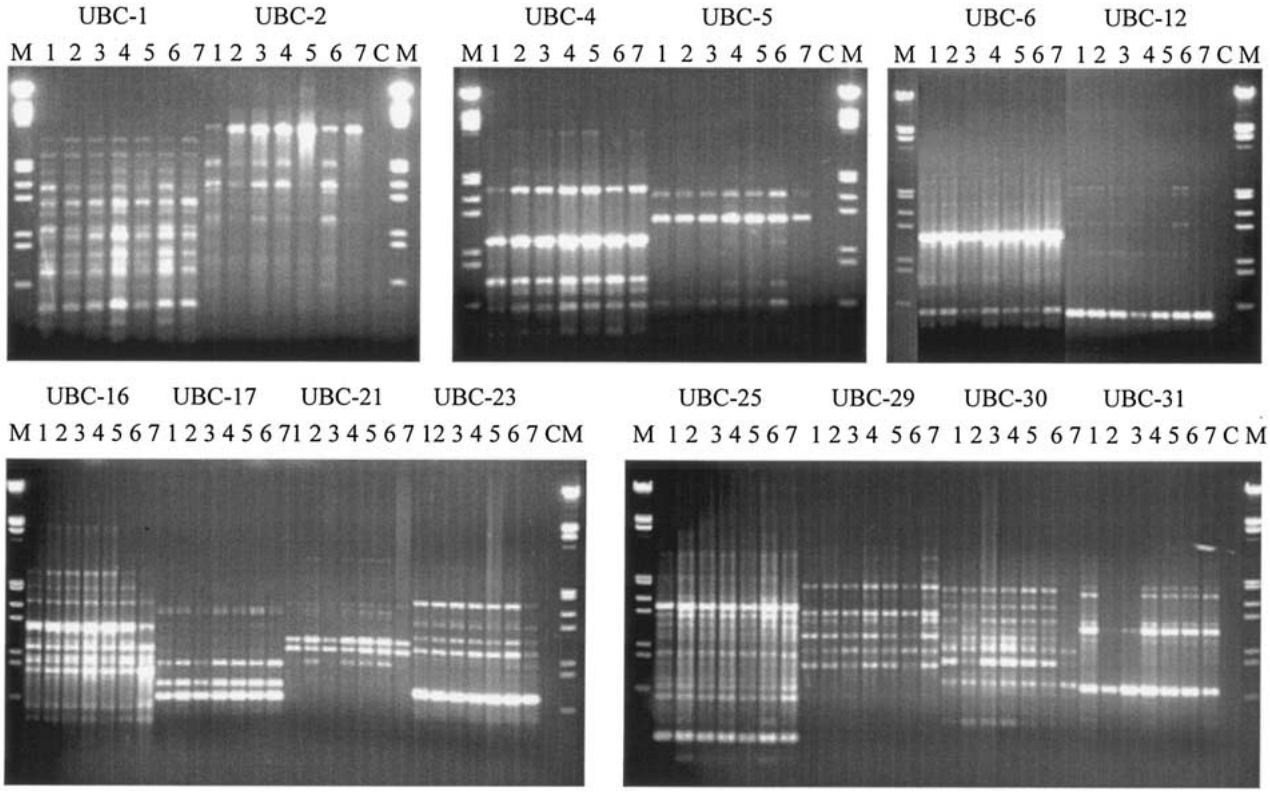

Fig. A1. Banding patterns of RAPD amplifications of *P. pseudoalcaligenes* clones. M, λ DNA *Eco*RI/*Hind*III marker; C, the negative control; lanes 1 to 7, the clones A, C1, C2, C3, HT1, HT2, and HT3, respectively. The upper rows are primers used corresponding to the bands amplified.

culture temperature [22, 23], single mutations have played an important role in virus populations evolving in increasing high temperature [5], and insertion sequence elements have been shown to be important for genomic variation in some bacteria and to impose key roles in genetic adaptation under stress environments [6, 11, 28]. Whether there are events mentioned above that account for the genetic adaptation in the present system are unknown. Other molecular techniques are necessary to answer this question empirically.

In conclusion, the genetic differences between the Control and HT clones *P. pseudoalcaligenes* are presented based on the genomic RAPD variation. The HT clones underwent more rapid molecular evolution than the Control clones, leading to more divergence in their genetic structure during the 10 months of selection in two different thermal environments. The high genetic diversity among the HT clones is likely to be due to the joint effects of selection and genetic drift driving multiple molecular evolution.

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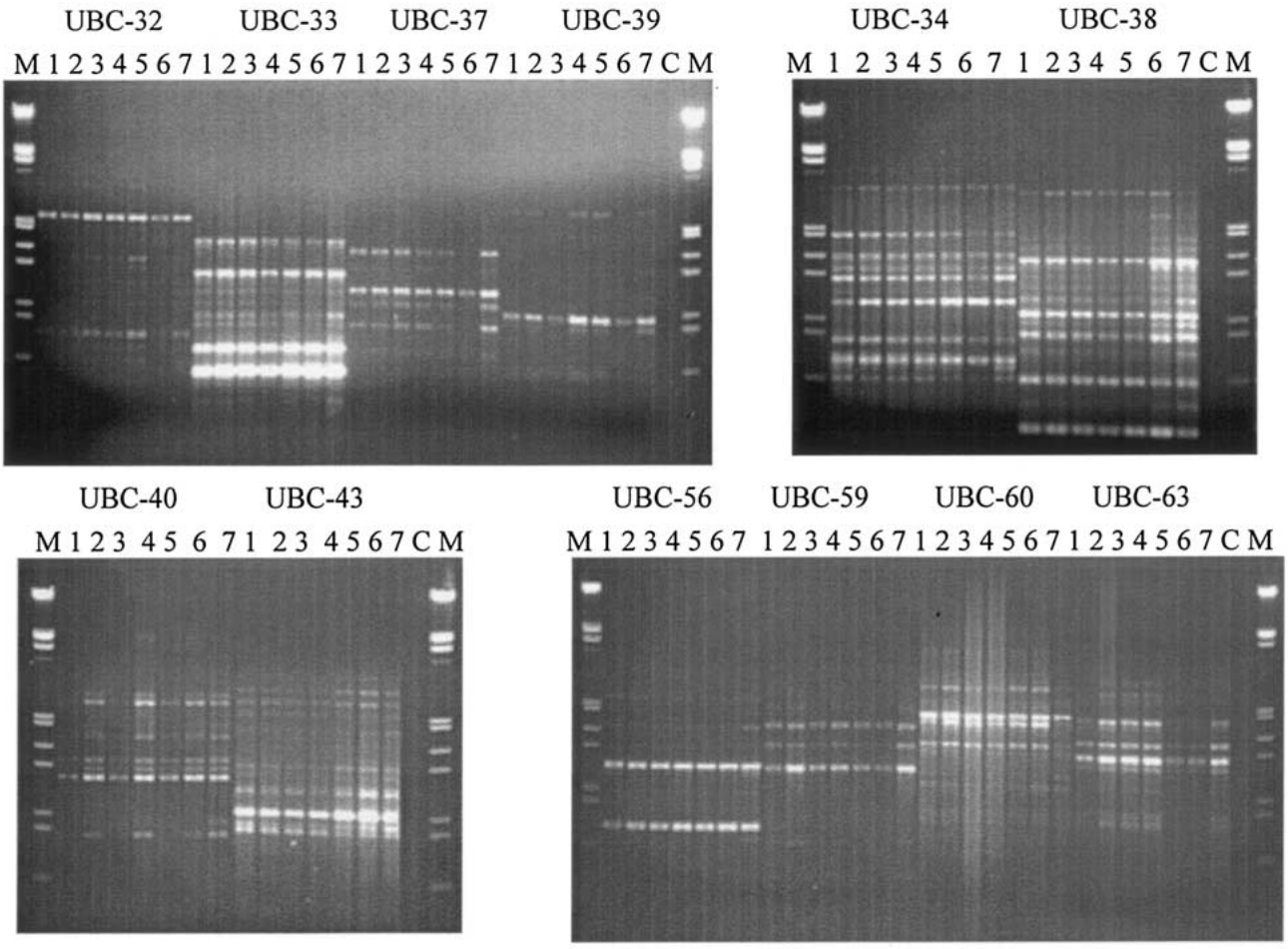

Fig. A1. Continued.

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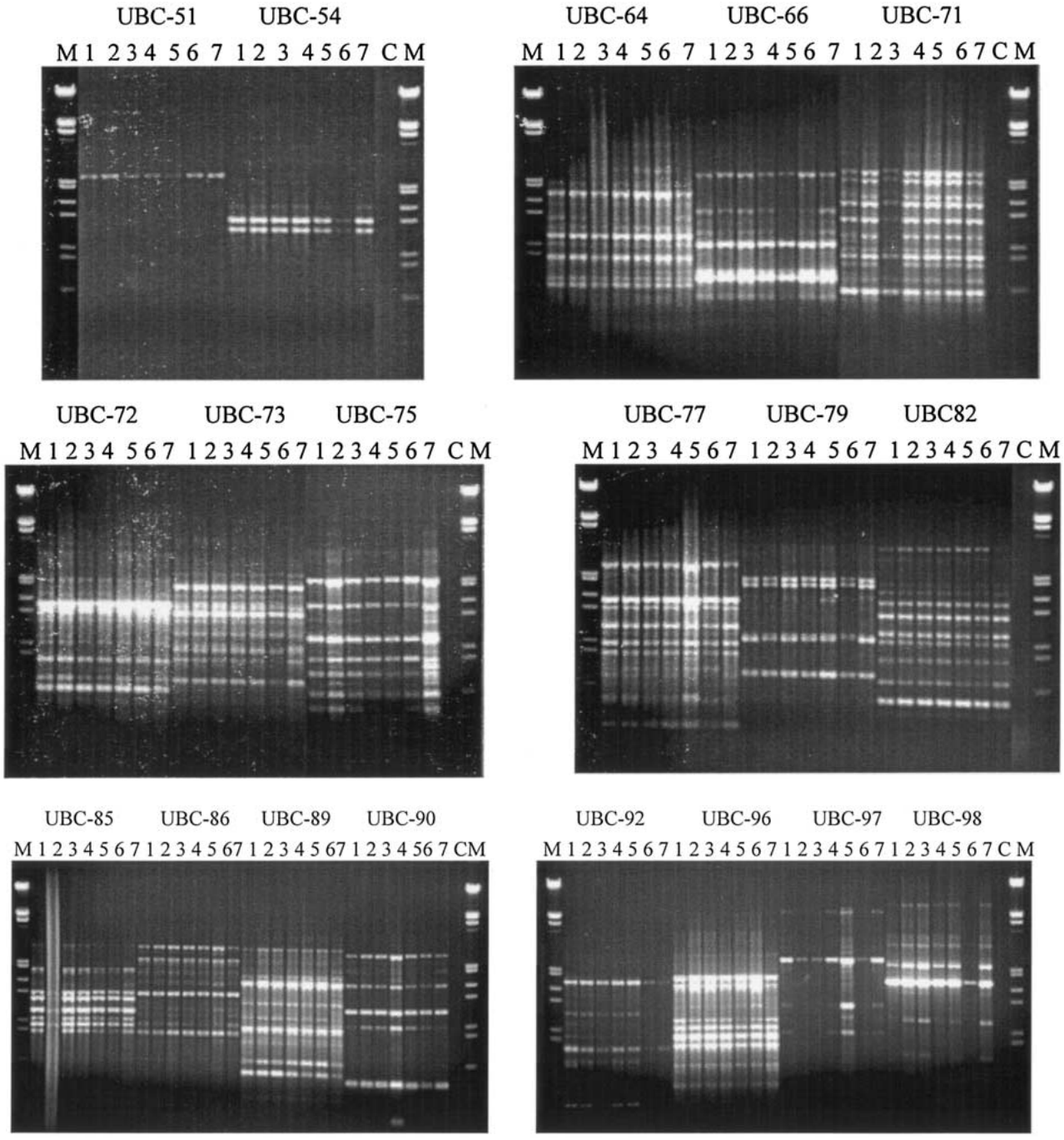

Fig. A1. Continued.

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