Effects of Environmental Stress on Stability of Tandem Repeats in *Escherichia coli* O157:H7

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Multilocus variable-number tandem-repeat analysis (MLVA) is used for source tracking *Escherichia coli* O157:H7 in agricultural environments. Tandem repeats were stable after limited replication but changed after exposure to irradiation, elevated temperatures, and starvation conditions. The pO157 plasmid was frequently lost under these stress conditions. Environmental stresses may increase phylogenetic diversity as measured by MLVA.

*Escherichia coli* O157:H7 is a pathogen causing serious disease that survives in a variety of hosts and environments, many of which are part of our food production system (24). Multilocus variable-number tandem-repeat analysis (MLVA) is an effective, high-throughput typing method that measures hyper-variable tandem repeats (TRs) at various locations in the genome (8, 13, 16, 22). However, any change in the rate of hypervariability in these TRs is problematic when trying to predict the phylogenetic relationships among isolates. Alterations in mutation rates due to stress in several studied systems have been previously reported (2, 6, 9, 10, 15, 20, 25, 28) and recently reviewed (18). Mutation rate changes likely occur through the induction of a general-stress-response sigma factor (rpoS), leading to genome-wide instability through the activity of a strand-slipage mechanism (SSM) during replication (30) and/or to reduced fidelity of repair after DNA damage (SOS response and mismatch repair) (7, 21). Mutation rates have been shown to differ between MLVA loci (13, 16, 22) and between strains (31). Therefore, we hypothesized that environmental stress changes the mutation rate at MLVA TRs.

**Recovery of bacteria stressed in natural environments.** Twenty-eight isolates of *E. coli* O157:H7 strain RM1484 (apple juice outbreak strain) were recovered from *Arabidopsis thaliana* seed after 60 days after 28 independently contaminated plants were planted as previously described (4). Hence, the bacteria survived in the phyllosphere during growth and maturation of the plant and seed. Ninety-six RM1484 isolates were also recovered from creek water (CW) after incubation for 7 days at 15°C. Each strain was analyzed by MLVA for 11 loci as previously published (Table 1) (3). No differences were found at any of the 11 loci in the recovered isolates compared to RM1484 (Table 2). These results are consistent with previous results that showed *Erwinia amylovora* repeat structures to be stable after long-term storage in sterile water at 25°C and passage in plants (26). Additionally, strain RM1484 was recovered from soil after growth of contaminated lettuce for 4 weeks at either 15°C or 23°C; the soil was then allowed to remain fallow and dry for an additional 4 weeks. One isolate from the 23°C soil and two isolates from the 15°C soil contained TR insertions (Table 2). Low nutrient levels in the phyllosphere or CW correlated with decreased DNA replication compared to the rhizosphere results (1). Detection of TR changes exclusively in rhizosphere isolates and not in phyllosphere and CW isolates substantiates the conclusion that replication is important for TR changes.

**The mutation rate can be estimated by monitoring clonal lineages.** The effect of temperature on TR mutation rate was monitored after serial passage of 96 cell lines of *E. coli* O157:H7 (RM1484) at 10, 15, 25, 37, and 43°C on Luria broth agar (LBA). We calculated that each passage of each lineage represented approximately 22.2 generations of growth. Based on evidence indicating that a 2-mm-diameter colony contains about 10⁷ CFU (reference 13 and data not shown), therefore, 96 independent cell lines, each passaged 10 times, represent approximately 20,000 generations. Each line was tested by MLVA before and after all the passages. The mutation rate (total mutations at 11 loci per 20,000 generations) increased as temperature increased (Fig. 1; also see Table S1 in the supplemental material). At 10°C, only 4 mutations were found, and all of them were single-repeat changes in O157-10, the most mutable locus (Table 1). In contrast, at 43°C, 13 mutations were detected for four loci; these mutations included double-step and triple-step mutations, which were previously reported to be less frequent than single-step changes (31). To compare the mutation rates found where larger TR changes occur, it is necessary to adjust the number of mutations by a factor dictated by the reduced likelihood. A model for determining direct-repeat recombination frequency as calculated by Oliveira et al. is approximated by the formula $F_R = (200 + L_3)^{-8.82} (L_R/1 + 2.16L_R + 1.4438L_3)$, where $F_R$ represents the frequency of recombination, $L_R$ represents the length of the repeat unit, and $L_3$ represents the length of the spacer DNA (23).

By this model, double-, triple-, and quadruple-step changes are 8, 15, and 23.5 times less likely to occur than single-step changes, respectively. Using these factors, the numbers of double-, triple-,
and quadruple-step changes were modified to give an adjusted mutation rate at each temperature (Fig. 1). Although the association of temperature with mutation rate is well established (25), the effect of temperature on TRs is less understood. TRs in *Erwinia amylovora* were shown to change only after extended passage at low temperatures or during selection for antibiotic resistance (14). In contrast, we measured a significant increase in the mutation rate as temperature increased.

Similarly, we briefly exposed RM1484 isolates to UV-C (germicidal lamp; 53 µW/cm²/passage) or sunlight (50 µW/cm²/passage) (PMA2100 light meter with PMA2122 or PMA2107 detector; Solar Light Co.) and then tested single colonies from LBA that were grown at 37°C. Single colonies were picked, and the entire process was repeated 10 times for 96 isolates. Mutation rates from UV-C and sunlight exposure were not significantly different than those seen after growth at 37°C in the dark (Fig. 1; also see Table S1 in the supplemental material). Alternately, after being streaked onto membranes (Protran BA85; Schleicher-Schuell), strain RM1484 was starved at 37°C (Fig. 1; also see Table S1 in the supplemental material). More significantly different than a quarter of the 96 clonal lines in the triple-stress experiment. Statistical analysis used a generalized linear model fitted to adjusted totals with the GENMOD procedure according to the instructions provided by the SAS Institute, Inc. (11, 17). A Poisson distribution, data appropriate for counting, and a probability level of 0.05 were employed in testing differences for significance determinations (indicated by letters above the bars). Bars with the same letter represent results that were not significantly different.

### TABLE 1. Names and locations of MLVA loci

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Alternative name(s)</th>
<th>Location</th>
<th>Gene encoding</th>
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</thead>
<tbody>
<tr>
<td>O157-3 Vhec3, TR5</td>
<td>271423 in EDL933</td>
<td>Hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>O157-5 Vhec5</td>
<td>2103941 in EDL933</td>
<td>BigA-like protein</td>
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<tr>
<td>O157-9 Vhec4, TR1</td>
<td>3557714 in EDL933</td>
<td>Not in ORF</td>
<td></td>
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<tr>
<td>O157-10 Vhec1, TR2</td>
<td>3559120 in EDL933</td>
<td>Hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>O157-17 TR3</td>
<td>5456065 in EDL933</td>
<td>Hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>O157-19 TR7</td>
<td>2932247 in EDL933</td>
<td>Hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>O157-25 TR4</td>
<td>1605820 in EDL933</td>
<td>Not in ORF</td>
<td></td>
</tr>
<tr>
<td>O157-34 Vhec2, TR6</td>
<td>5361545 in EDL933</td>
<td>YigL</td>
<td></td>
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<tr>
<td>O157-36 Vhec7</td>
<td>54348 in pO157t</td>
<td>Not in ORF</td>
<td></td>
</tr>
<tr>
<td>O157-37 Vhec6</td>
<td>46468 in pO157t</td>
<td>Hypothetical protein</td>
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<tr>
<td>Vhec6</td>
<td>5454376 in EDL933</td>
<td>Not in ORF</td>
<td></td>
</tr>
</tbody>
</table>

* All loci are designated according to Keys et al. (13), with the exception of Vhec6.
* Vhec loci are from Lindstedt et al. (16); TR loci are from Noller et al. (22).
* Locations are designated with respect to the 5’ end of the repeat sequence.
* ORF, open reading frame.

### TABLE 2. TR changes in RM1484 isolates recovered from stressful environments

<table>
<thead>
<tr>
<th>Conditions</th>
<th>No. of strains</th>
<th>No. of insertions</th>
<th>No. of deletions</th>
</tr>
</thead>
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<tr>
<td>Phyllosphere, 23°C</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Creek water, 15°C</td>
<td>96</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rhizosphere, 15°C</td>
<td>96</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Rhizosphere, 23°C</td>
<td>96</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* Data represent numbers of insertions or deletions of a single TR detected in 96 strains at O157-10. No changes were found at any of the other 10 loci.
* Bacteria were isolated from *Arabidopsis thaliana* seeds.
* Bacteria were isolated from independent soil samples, suspended in phosphate-buffered saline (PBS), and plated on Rainbow agar (Biolog). Isolates were obtained from dry soil at 4 weeks after growth of contaminated lettuce.
high temperature and starvation yielded no colonies, even with high inoculum concentrations (data not shown).

Additionally, 15 of the isolates in the triple-stress experiment had null mutations in loci O157-36 and O157-37 (see Table S1 in the supplemental material). Both O157-36 and O157-37 are located on the pO157 large 92-kb plasmid (Table 1). Purification of plasmids from 15 null strains of the triple-stress experiment indicated that these mutations resulted from plasmid loss (data not shown). Additionally, null mutations were detected in the 10, 15, and 37°C serial-passage experiments, though less often than under triple-stress conditions (27, 29).

The extent to which any set of stress conditions affects isolates in agreement with other reports of plasmid loss under stressful conditions (27, 29). Large shifts in TRs (e.g., >4 repeat units) were not detected even under triple-stress conditions. Admittedly, stresses sustained for long periods (months or years), along with replication, would probably produce changes in TRs much greater than those detected in our study. Nevertheless, for isolates collected in limited spatial and temporal ranges, such as during outbreak investigations, stress-related changes would probably be less extensive than those detected in these experiments. Although studies that correlate relatedness among strains with differences between the numbers of TRs at multiple loci remain valuable (31), environmental stress-related changes should be considered in phylogenetic analysis of MLVA data.

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REFERENCES