Transformation Frequency of a mariner-Based Transposon in Rickettsia rickettsii

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Received 11 May 2011/Accepted 30 June 2011

Transformation frequencies of a mariner-based transposon system in Rickettsia rickettsii were determined using a plaque assay system for enumeration and isolation of mutants. Sequence analysis of insertion sites in both R. rickettsii and R. prowazekii indicated that insertions were random. Transposon mutagenesis provides a useful tool for rickettsial research.

Rickettsiae are Gram-negative bacterial obligate intracellular parasites that are the etiologic agents of several significant vector-borne diseases. The pathogenic rickettsiae are comprised of the spotted fever and typhus groups. Within the spotted fever group are Rickettsia rickettsii, the etiologic agent of Rocky Mountain spotted fever, R. conorii, causing Mediterranean spotted fever, and several other species which exhibit various degrees of virulence (7, 11, 14, 20). Spotted fever group rickettsiae are typically associated with tick vectors, although some species, such as R. akari, are associated with mites. The typhus group rickettsiae include R. prowazekii and R. typhi, the louse-borne and flea-borne causative agents of epidemic typhus and murine typhus, respectively (2).

The spotted fever group of rickettsiae may be distinguished from the typhus group based upon the antigenicity of their lipopolysaccharides (LPS) (1, 19). Biologically, a major distinction between the groups is the ability of spotted fever group rickettsiae to co-opt an actin-based motility to promote inter- and intracelluar spread (8, 22). In contrast, the typhus group rickettsiae stimulate little or no actin polymerization (8, 18) and thus do not disseminate as rapidly to adjacent cells (23). Related to the ability to directly spread from cell to cell is the ability of spotted fever group rickettsiae to readily form visible plaques in cell culture, while the typhus group does not (4, 12, 15).

Until recently, the use of genetic tools to understand the molecular basis of virulence in rickettsiae was severely limited by the inability to stably introduce DNA. The development of molecular genetics has led to a number of recent advances (3, 5, 9). The development of transformation systems for rickettsiae was accomplished in R. prowazekii but necessitated extensive cell culture passage and selection for clonal isolation by limiting dilution (10, 16, 17). As a consequence, transformation frequency could not be calculated. The plaque-forming ability of the spotted fever group rickettsiae permits determination of transformation frequency and is reported here.

The R. rickettsii R strain was grown in Vero cells as previously described (6) and partially purified by differential centrifugation followed by centrifugation through 30% Renografin (21). The rickettsiae were then washed three times with 250 mM sucrose. Approximately 3 × 10^9 PFU of rickettsiae were suspended in 100 μl of 250 mM sucrose and transformed by electroporation with 5, 10, or 20 μg of pMW1650 (10) in a Bio-Rad GenePulser XL electroporation system (field strength = 2.5 kV/cm, pulse time = 5 ms, resistance = 200 Ω).

Transformation Frequency of a mariner-Based Transposon System

**FIG. 1.** Effect of DNA concentration on frequency of transformation of R. rickettsii. The results plotted are the means ± SEMs of results from three independent experiments. Based upon these results, calculated with 5 μg of DNA electroporated, the transformation efficiencies were 57 transformants per μg of DNA.
TABLE 1. Selection of R. rickettsii transformants by rifampin

<table>
<thead>
<tr>
<th>Preparation stage or type of plasmid DNA</th>
<th>Presence of rifampin (200 ng/ml) (h after plating)</th>
<th>Mean no. of PFU ± SEM (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preelectroporation*</td>
<td>−</td>
<td>3.1 × 10^6 ± 7.4 × 10^6 (3)</td>
</tr>
<tr>
<td>pMW1650</td>
<td>−</td>
<td>2.5 × 10^6 ± 4.6 × 10^6 (9)</td>
</tr>
<tr>
<td>pMW1650</td>
<td>+ (24)</td>
<td>283 ± 88 (9)</td>
</tr>
<tr>
<td>pMW1650</td>
<td>+ (6)</td>
<td>339 ± 84 (6)</td>
</tr>
<tr>
<td>pMW1630b</td>
<td>+ (24)</td>
<td>11 ± 11 (3)</td>
</tr>
</tbody>
</table>

* Direct particle counts for the preelectroporation rickettsial preparation equaled 5.3 × 10^9 ± 3.6 × 10^9 particles/ml.

b Negative control.

c n, number of independent transformations.

capacitance = 25 μF). Immediately after electroporation, rickettsiae were diluted in brain heart infusion (BHI) broth, plated on Vero cell monolayers, incubated at 34°C in a 5% CO₂ atmosphere for 30 min to allow attachment, and overlaid with medium 199, containing 5% fetal bovine serum and 0.5% agarose. Rifampin was added to 200 ng/ml at 24 h postinfection, and incubation continued at 34°C for 7 days before counting and/or isolation of plaques.

Over the range of 5 to 20 μg of plasmid DNA, there was little difference in the numbers of rifampin-resistant transformants recovered (Fig. 1). In nine independent transformations, the numbers of rifampin-resistant transformants recovered ranged from >100 to 733 PFU over all DNA concentrations tested, with an average of approximately 283 ± 88 PFU (mean ± standard error of the mean [SEM]) per transformation or a frequency of approximately 1.1 × 10⁻⁷ (Table 1).

Transformation with a negative-control plasmid, pMW1630 (10), which does not encode the transposon or transposase, yielded only a single visible plaque from all three experiments. Spontaneous resistance to rifampin did not, therefore, appear to be a significant obstacle. In three independent experiments using both 10 and 20 μg/ml of DNA, addition of rifampin at 6 h after plating did not significantly change the frequency of recovery, with 339 ± 84 PFU per transformation.

Although the measured transformation frequency is relatively low, the ability to stably introduce DNA in a reproducible fashion offers the potential for the use of modern technologies to understand the basis of virulence in rickettsiae. The experiments described above were designed expressly to determine frequency of transformation using the Himar1 system. In practice, the culture from a routine transformation experiment is plated to recover the maximum number of recombinants. Typically, 2 × 10⁹ PFU are electroporated in 100 μl of 250 mM sucrose and brought to 3.6 ml with BHI. Aliquots of 100 μl are then plated on each of 36 wells of 6-well tissue culture plates and overlaid and incubated as described above for 7 to 10 days. In two examples in which total numbers of transformants were enumerated, recovery ranged from 18 to 140 PFU/plate, or a total of 205 to 718 transformants per experiment. Plating at lesser dilutions appears to reduce yield, presumably from the toxicity of the high numbers of rickettsiae in the inoculum.

A limiting factor in the study of rickettsial pathogenesis is the obligate intracellular nature of the bacterium. Recovery of rickettsial transformants from plaques remains a tedious and time-consuming process. A spotted fever group rickettsial plaque typically requires approximately 7 days of incubation before it is of a size that can be readily visualized and recovered. Small-plaque mutants may take longer. Numbers of viable rickettsiae recovered from plaques are low and require replaquing to ensure clonality, as well as multiple cycles of expansion before sufficient DNA can be recovered for analysis. Because rifampin is bacteriostatic to rickettsia rather than bactericidal, each plaque is likely contaminated with untransformed organisms. Typically, three rounds of plaque purification are required to ensure clonality. Expansion for archiving clones and DNA isolation require several additional passages in cell culture. The constraints upon rickettsial genetics thus are largely due to the time-consuming tissue culture practices.

![FIG. 2. Diagrams of R. rickettsii and R. prowazekii chromosomes depicting the insertion sites for each of the mutants generated by transposon mutagenesis in several independent experiments. The R. rickettsii map was derived from three independent experiments using either the R or Iowa strain (3, 9). The map was constructed using R. rickettsii Iowa coordinates, and insertion sites determined in experiments 1 (9) (arrowheads labeled “1”), 2 (3) (arrowheads labeled “2”), and 3 (arrowheads labeled “3”) are indicated. For R. prowazekii, insertion sites determined in experiments 1 and 2 are shown and mapped against R. prowazekii Madrid E coordinates. Maps were generated using Geneious v2.5 (A. J. Drummond, J. Heled, T. Thierry, B. Ashton, A. C. Wilson, and S. Stones-Havas, 2006).](http://jb.asm.org/Downloaded from http://jb.asm.org/).
required for their manipulation. The cumulative results of analyzing insertion sites of pMW1650 indicate that transposition is random in the genomes of R. rickettsii (3, 9) and R. prowazekii (Fig. 2; see also Tables S1 and S2 in the supplemental material). No identical insertion sites were observed in any experiment. We analyzed bias in an 18-bp sequence flanking the TA integration sites in both R. rickettsii and R. prowazekii by comparing the observed frequency to those from 10,000 random computer-simulated insertions from the respective genomes. Statistical significance was assessed using a χ² test, thus confirming that the transposon insertion sites were random.

The reduced genome of rickettsiae may contribute to the observed low frequency of transformation. Because of their extreme host dependency, rickettsiae are believed to be evolving toward a minimal genome; thus, a high percentage of genes are likely essential (13). Saturation of the rickettsial genome to define essential genes may be feasible but technically demanding. With our demonstrated ability to select transposon mutants (9), to complement defective genes (3), and to select directed mutants (5), the potential for the advancement of our knowledge of rickettsial pathogenesis is encouraging.

This work was supported by the Intramural Research Program of the NIAID at the NIH and by Public Health Service grant AI20384 to D.O.W.

We thank A. Omsland, R. Heinzen, and A. Tucker for critical review of the manuscript.

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