DNA Base Composition of *Rickettsia tsutsugamushi* Determined by Reversed-Phase High-Performance Liquid Chromatography

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The DNA base composition of *Rickettsia tsutsugamushi* was determined by reversed-phase high-performance liquid chromatography and compared with that of *Rickettsia rickettsii*. The G+C contents were 28.1 to 30.5 mol% for *R. tsutsugamushi* and 32.1 mol% for *R. rickettsii*.

Classification of rickettsiae has been based primarily on phenotypic characteristics. These characteristics include the ecological, biological, and immunological properties of these organisms and the diseases which they produce. The genus *Rickettsia* has been divided into the typhus, spotted fever, and scrub typhus groups (9).

The G+C contents of rickettsiae have been utilized as an objective index of taxonomical relatedness among rickettsiae (3, 8). These values have been determined by the buoyant density method (8) and the thermal denaturation method (3, 8) and have clearly shown that the organisms are taxonomically related. However, *Rickettsia tsutsugamushi*, a rickettsia of the scrub typhus group, was not included in the previous studies, and the genetic relatedness of this organism to other rickettsiae remains to be determined.

In this study, the DNA base composition of *R. tsutsugamushi* was determined by reversed-phase high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

**Rickettsiae.** *R. tsutsugamushi* strains Gilliam, Karp, Kato, Kawasaki, Kuroki, and Shimokoshi were propagated by using L929 or Vero cells. The standard strains (strains Gilliam, Karp, and Kato) were repeatedly plaque cloned before propagation (4). *Rickettsia rickettsii* strain Bitterroot was propagated by using L929 cells. These rickettsiae were purified by density gradient centrifugation with Percoll (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) (7).

**Preparation of rickettsial and cellular DNAs.** A suspension of purified rickettsial cells was treated with 0.1 mg of DNase I (Sigma Chemical Co., St. Louis, Mo.) per ml for 1 h at 37°C. After the DNase was inactivated, the rickettsial cells were collected and treated for 1 h at 56°C with 0.1 mg of proteinase K (Merck & Co., Inc., Rahway, N.J.) per ml in a solution containing 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.6% sodium dodecyl sulfate. Uninfected L929 and Vero cells were collected and treated with proteinase K as described above. The DNA was extracted with phenol and precipitated with ethanol. The resulting DNA pellet was dissolved in a 10 mM Tris-HCl (pH 7.5)–5 mM EDTA solution and treated with a mixture of RNase A type III (0.1 mg/ml; Sigma) and RNase T1 (2 μg/ml; Sigma) for 1 h at 37°C. The DNA was subjected to another cycle of phenol extraction and ethanol precipitation and then redissolved in distilled water at a concentration of 1 mg/ml.

**Determination of molar ratio by HPLC.** The HPLC analysis was performed by using the method of Tamoka and Komagata (6), with slight modifications. A DNA solution was boiled for 5 min and chilled. The resulting denatured DNA preparation received an equal volume of a nuclelease P1 (Sigma) solution (0.1 mg/ml of nuclease P1 in 40 mM sodium acetate–2 mM ZnSO4 [pH 5.3]) and was incubated for 1 h at 50°C. The DNA digest was dephosphorylated with alkaline phosphatase (100 U of calf intestine alkaline phosphatase [Takara Shuzo, Kyoto, Japan] per ml in 0.17 mM MgCl2–0.33 M Tris-HCl [pH 8.1]) for 1 h at 37°C. The resulting hydrolysate was analyzed by HPLC by using a model 655 system (Hitachi, Ltd., Tokyo, Japan); an aliquot of the hydrolysate was applied to a COSMOSIL 5 C18 column (Nacalai Tesque, Kyoto, Japan) and eluted with a mixture of 0.2 M NH4H2PO4 (pH 4.5) and acetonitrile (20:1, vol/vol) at a flow rate of 1 ml/min. The A270 was monitored. The relative amount of each nucleoside was calculated on the basis of the peak area in the HPLC elution profile. Bacteriophage λ DNA (G+C content, 49.8 mol%; Takara Shuzo) was used as a standard to determine the base ratios of rickettsial DNAs.

RESULTS

**DNA base composition.** Authentic deoxyribonucleosides eluted from a reversed-phase column in the following order: deoxyctytidine, deoxyguanosine, deoxythymidine, and deoxyadenosine (6). An elution profile of a hydrolysate of highly purified λ DNA contained four peaks corresponding to these deoxyribonucleosides (Fig. 1a). When monitored at 270 nm, the areas of the peaks paralleled the molar ratio of deoxyribonucleosides.

The deoxyribonucleosides from cellular and rickettsial DNAs were clearly separated by their elution profiles as was the case with the highly purified λ DNA (Fig. 1b through d). Pretreatment of the DNAs with a combination of RNases A and T1 was indispensable for eliminating the ribonucleosides from RNA that contaminated the original DNA preparations. Thus, the amount of each deoxyribonucleoside was accurately determined on the basis of the peak area. The values obtained are shown in Table 1. The G+C content of *R. rickettsii* Bitterroot was 32.1 mol%. This value is very similar to the values of 32.4 and 32.0 mol% determined by the buoyant density method and the thermal denaturation method, respectively (8). Under the same conditions, the G+C content of *R. tsutsugamushi* ranged from 28.1 to 30.5 mol%. There was a small but significant difference in G+C content between *R. rickettsii* and *R. tsutsugamushi*.

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HPLC technique

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and fungi

alkaline phosphatase and analyzed by reversed-phase HPLC. The
sources of DNA were bacteriophage λ (a), L929 cells (b), Vero cells
c), R. tsutsugamushi Karp (d), R. tsutsugamushi Kawasaki (e), and
R. rickettsii Bitterroot (f).

FIG. 1. Elution profiles of hydrolysates of DNAs. Approximately 2 µg of DNA was treated with nuclease P1 and calf intestine alkaline phosphatase and analyzed by reversed-phase HPLC. The sources of DNA were bacteriophage λ (a), L929 cells (b), Vero cells (c), R. tsutsugamushi Karp (d), R. tsutsugamushi Kawasaki (e), and R. rickettsii Bitterroot (f).

DISCUSSION

The DNA base ratios of microorganisms are specific to
individual species and have been used to identify and clas-
sify organisms. The DNA base ratio approach has also been
applied to the classification of rickettsiae (3, 8). However,
previous studies did not include R. tsutsugamushi strains,
probably because of the technical difficulty in purifying
them.

Determining the DNA base ratio of R. tsutsugamushi with
an accurate method should be very important, and the
HPLC technique (2) can do this. In fact, this technique has
been used in taxonomical studies of bacteria (6) and fungi
(1). In these studies, the base ratios were calibrated on the
basis of the absorption of known concentrations of standard
doxyribonucleosides. In our study, we used λ DNA as a
standard to calculate the base ratios. Our method guarantees
the accuracy of the molar ratio for each deoxyribonucleo-
side, because the standard deoxyribonucleosides were com-
ponents of a DNA whose molar ratio was determined on the
basis of sequence data (5).

The G+C content of R. rickettsii determined by reversed-
phase HPLC was identical to the G+C contents determined
by the classical methods (3, 8). This proves the usefulness of
the HPLC technique for determining the G+C contents of
rickettsiae. Under our conditions, the G+C contents of R.
tsutsugamushi ranged from 28.1 to 30.5 mol%. These values
do not overlap the value obtained for R. rickettsii (a member
of the spotted fever group) but coincide with the values
obtained for rickettsiae belonging to the typhus group (3, 8).
However, rickettsiae with the same G+C contents are not
necessarily identical. Further taxonomical study of the spe-
cies or strains will require DNA homology tests.

TABLE 1. G+C contents of rickettsial DNAs

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Host cells</th>
<th>G+C content (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. tsutsugamushi</td>
<td>Karp</td>
<td>L929</td>
<td>30.5 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Gilliam</td>
<td>L929</td>
<td>30.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Kato</td>
<td>L929</td>
<td>29.7 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Kawasaki</td>
<td>L929</td>
<td>29.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Kawasaki</td>
<td>Vero</td>
<td>29.5 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Kuroki</td>
<td>Vero</td>
<td>29.3 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Shimokoshi</td>
<td>L929</td>
<td>28.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Shimokoshi</td>
<td>Vero</td>
<td>28.5 ± 0.1</td>
</tr>
<tr>
<td>R. rickettsii</td>
<td></td>
<td>Bitterroot</td>
<td>32.1 ± 0.0</td>
</tr>
</tbody>
</table>

*G+C contents of rickettsiae were determined by reversed-phase HPLC. Bacteriophage λ DNA was used as a standard (C+C content, 49.8 mol%) to calculate the base ratios.

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