Deoxyribonucleic Acid Homologies Among Three Immunological Types of *Corynebacterium renale* (Migula) Ernst

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Deoxyribonucleic acid hybridization among three immunological types of *Corynebacterium renale* (Migula) Ernst was carried out. The data indicated that hybridizations between the different types were lower than those obtained in the homologous systems and that the three types of *C. renale* are not too closely related.

*Corynebacterium renale* (Migula) Ernst is a species which causes pyelonephritis and cystitis in cattle. Three types of *C. renale* were identified in our laboratory by the antigenicities of their cell wall extracts by immunodiffusion (25). Differences in some biochemical properties were also shown: xylose was fermented only by the strains of type III, and nitrate was reduced only by the strains of type II (25). Further differences between the three types were found: the strains of type III required more growth factors and amino acids than the strains of other types (13); the strains of type II had numerous pili, whereas those of type I and usually those of type III had a small number of pili (26, 27); lysogeny was found only in strains of type I (28). Virulence for cows was also different between the three types: the strains of type III caused severe hemorrhage, epithelial degeneration, and ulceration of the mucous membrane in the urinary bladder. The strains of type I caused cystitis less severe than those of type III, and the strains of type II were least in virulence (14). Differences in their deoxyribonucleic acid (DNA) base compositions were also confirmed (17). Averages of the guanine plus cytosine (GC) contents expressed in percentage were 56.7 ± 1.1 (type I strains including ATCC 10848 and ATCC 19412), 57.9 ± 1.9 (type II strains including strains 45 and 46), and 53.5 ± 0.9 (type III strains including strains 42 and 43).

The present study was undertaken, using strains employed in previous studies, to determine what relationships exist, if any, among the three types of *C. renale* by the DNA hybridization technique.

**MATERIALS AND METHODS**

**Bacterial strains.** Strains of *C. renale* used in the present study were American Type Culture Collection (ATCC) 10848, ATCC 19412 (type I), strains 45 and 46 (type II), and strains 42 and 43 (type III), all isolated from cows with signs of pyelonephritis. The typing of these organisms was done by immunodiffusion (25). Radiolabeled DNA was extracted from ATCC 10848 (type I), strains 46 (type II), and 42 (type III). Control strains were *C. pseudotuberculosis* (ATCC 809), which, on the basis of a numerical taxonomy study is not too closely related to *C. renale* (10), and *Escherichia coli* O-111, which is known to be taxonomically remote from *C. renale*. Two strains of *E. coli* (Hfr 30 RT" and Hfr 30 RT") were used only in the test of thymidine kinase activity.

**Media and cultural techniques.** Cells were grown in nutrient broth or in a Casamino Acids medium containing 0.5% Casamino Acids (Difco) and 0.5% NaCl. Nutrient agar with 0.5% glucose was also used. The pH of the media was 7.2. The organisms were grown at 37°C for 48 h, except for *E. coli*, which was grown at 37°C for 24 h.

**Enzymes and radioisotopes.** Ribonuclease (RNase) (EC class 2.7.7.16) and deoxyribonuclease (DNase) (EC class 3.1.4.5) were purchased from Sigma Chemical Corp. (St. Louis, Mo.). 3H-thymidine (5.0 Ci/mmol, 20.5 mCi/mg) and 3H-uridine (5.0 Ci/mmol, 20.6 mCi/mg) were purchased from the Radiochemical Centre (Amersham, England).

**Preparation of labeled DNA.** As described below, the DNA of the strains of *C. renale* was not labeled when cultivated in the presence of 3H-thymidine. Therefore, 3H-uridine was used for labeling the bacterial DNA. Incorporation of tritium from 3H-uridine into DNA has been reported (1). Labeled DNA was extracted from the organisms, which were
cultivated in the broth containing 1 to 1.5 μCi of
3H-uridine per ml and harvested immediately before
the stationary phase and purified as described above.
The purified, labeled DNA was further treated two or
three times with RNase.

Immobilization of DNA on nitrocellulose filters. The
DNA was diluted in standard saline citrate (SSC: 0.15 M NaCl plus 0.015 M sodium citrate) and
denatured by boiling for 6 min with quick cooling.
The denatured DNA in SSC was slowly passed through
a membrane filter (Millipore Corp., 100-nm pore size,
VCWP2500). The filter was washed with distilled
water before use. After application of DNA, the filter
was dried overnight at room temperature and then in a
vacuum oven at 80°C for 2 h. Approximately 30 μg of
DNA, calculated from optical density absorption at
260 nm of the DNA solution before and after
filtration, were usually immobilized on each filter.

DNA-DNA hybridization. A filter on which un
labeled DNA was immobilized was incubated in a
scintillation solution containing 1 ml of preincuba
tion mixture (PM) at 65°C for 6 h according to Denhardt
(7). The filter was then placed in another vial con
taining 1 ml of 6 × SSC, to which was added about
3 μg of labeled and sheared DNA, and incubated at 67
to 68°C for 12 h. After the hybridization was
completed, the filter was carefully washed with 30 ml
of 0.1 × SSC on each side. It was then dried at room
temperature, placed in a vial containing a toluene
scintillation solution, and assayed for radioactivity in a
liquid scintillation counter (Horiba, Kyoto). As it was
impossible to immobilize the same amount of the
unlabeled DNA on each filter, the labeled DNA was
hybridized with the unlabeled DNA in unequal
amounts. The labeled DNA hybridized with the
unlabeled DNA, therefore, was determined by the
count per 1 μg of unlabeled DNA. The amount of
DNA on the filter was estimated after each hybridiz
ation by using Burton's modification of the dipheno
ylamidine reaction (5). (The DNA on the filter was first
hydrolyzed by heating the filter at 70°C for 30 min in
0.5 N perchloric acid.)

RESULTS

Lack of thymidine kinase activity in C. renale. C. renale strains ATCC 10848 (type I),
46 (type II), and 43 (type III), and E. coli strains H/r 30 RT⁺ and H/r 30 RT⁻, as
controls, were cultivated at 37°C for 72 h in 1
ml of the Casamino Acids medium containing 1
μCi of 3H-thymidine per ml. The cells were
harvested by centrifugation. The radioactivity
incorporated into the cells per milliliter of
culture was 83 to 127 counts/min for the
strains of C. renale, whereas it was 22,714 to
29,148 counts/min for the strains of E. coli.
Thus 3H-thymidine was incorporated into the
cells of E. coli but not into the cells of C.
renale. This fact suggests that C. renale may not
possess thymidine kinase activity. Thymidine
kinase activity of C. renale was, therefore,
measured by the method of Bresnick and
Thompson (4). The results (Table 1) indicate
that the thymidine kinase activity of C. renale
is extremely low, whereas that of E. coli and of
dog kidney cells infected with infectious canine
hepatitis virus is high. The lack of thymidine
kinase activity in cells of C. renale has thus
been demonstrated.

Incorporation of 3H-uridine into the DNA of
C. renale. A 10-μCi amount of 3H-uridine was
added to 1 ml of the Casamino Acids medium
in which C. renale ATCC 10848 was then
inoculated. The radioactivity incorporated into
the cells of ATCC 10848 was 415,700 counts
per min per cell. Incorporation of 3H-uridine
into the DNA was then determined as follows.
Purified DNA was obtained from the cells of
ATCC 10848 grown in 400 ml of broth
containing 3H-uridine (1 μCi/ml), using the
purification procedure involving RNase treat
ment. The radioactivity of the purified DNA
solution, which was RNase (100 μg/ml) resist
ant, was 1,693 counts per min per 25 μlitters.
The labeled DNA was, however, extremely
sensitive to DNase; the radioactivity decreased
to one-tenth after the treatment with DNase
(20 μg/ml; for 1 h at 37°C). The results indicate
that the DNA was labeled with tritium from
3H-uridine. The specific activity of the labeled
DNA of ATCC 10848 (type I) was 1,963
counts per min per μg, that of strain 46 (type II)
was 1,022 counts per min per μg, and that of
strain 42 (type III) was 430 counts per min
per μg. In the following hybridization experi
ments, therefore, the DNA of C. renale labeled
with 3H-uridine was used.

Determination of the ratio on the filter of
labeled DNA to unlabeled DNA. The ability of

<table>
<thead>
<tr>
<th>Cells</th>
<th>Thymidine kinase activity a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min (counts/min)</td>
</tr>
<tr>
<td>C. renale</td>
<td>ATCC 10848 (type I)</td>
</tr>
<tr>
<td>Strain 46 (type II)</td>
<td>237</td>
</tr>
<tr>
<td>E. coli</td>
<td>145</td>
</tr>
<tr>
<td>Infectious canine hepatitis virus-infected dog kidney cells b</td>
<td>229</td>
</tr>
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</table>

a Expressed as 3H-thymidine incorporated into thymidylate.
b Forty hours after infection.
labeled DNA to hybridize with unlabeled DNA on filters was tested. When unlabeled DNA immobilized on the filter was fixed at 35 μg, the rate of hybridization increased in proportion as labeled DNA was increased to the amount of 4.1 μg. When labeled DNA added to unlabeled DNA was fixed at 4 μg, the rate of hybridization was increased in proportion as unlabeled DNA was increased to the amount of 44.5 μg. From this, it was determined that the rate of hybridization is highest when the ratio of labeled DNA to unlabeled DNA on the filter is 1:10.

**DNA hybridization among the three types of C. renale.** The labeled DNA from each strain of three types of C. renale was hybridized respectively with unlabeled DNA from six strains of C. renale. The DNA of *C. pseudotuberculosis* and *E. coli O-111* were used as controls. The results are given in Table 2.

The hybridization between different types was always lower than that obtained in the homologous system. Type I (ATCC 10848) had a mean of 45% DNA binding with type II (strain 46) and a 55% DNA binding with another strain (strain 45) of type II. Type I and type II were found to be, therefore, not so closely related. Type I had only a mean of 15% and a 20% DNA binding with type III strains 42 and 43, respectively, indicating that type I and type III were unrelated. Type II (strain 46) had a mean of 66% DNA binding with type I (ATCC 10848) and a mean of 71% DNA binding with type III (strain 42). Hence, the indications were that type II might have common DNA, partly with type I and partly with type III. Type III (strain 42) had a mean DNA homology of 47% with type I ATCC 10848 and a mean 57% with type II strain 46; hence, a not too close relationship was indicated.

*C. pseudotuberculosis* was unrelated to C. renale, as was *E. coli*. It has been reported generally that the same species have approximately more than 80% DNA homology (8, 16, 24). From this standpoint, types I, II, and III of C. renale might be of different species.

**DISCUSSION**

The DNA hybridization technique has been used to study genetic relationships among many bacterial groups and among yeasts, such as the *Enterobacteriaceae* (3, 20), *Bacillus* (23), *Pseudomonas* (6), *Pasteurella* (22), *Streptococcus* (24), *Neisseria* (16), *Agrobacterium* (12), *Brucella* (15), *Candida* (2), *Mycoplasma* (21), *Leptospira* (9), *Mycobacterium* (8), and *Clostridium botulinum* (18, 19).

**TABLE 2. DNA homologies** in the three types of C. renale

<table>
<thead>
<tr>
<th>Unlabeled DNA</th>
<th>Labeled DNA (%)</th>
<th>C. renale type I ATCC 10848</th>
<th>C. renale type II strain 46</th>
<th>C. renale type III strain 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. renale</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I ATCC 10848</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66 (61, 63, 72)</td>
<td>47 (35, 60)</td>
<td></td>
</tr>
<tr>
<td>Type I ATCC 19412</td>
<td>80</td>
<td>100 (40, 46, 48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type II strain 46</td>
<td>55</td>
<td>71 (12, 14, 17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type III strain 45</td>
<td>15</td>
<td>71 (67, 70, 77)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type III strain 43</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. pseudotuberculosis ATCC 809</td>
<td>9</td>
<td>33</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>E. coli O-111</td>
<td>1</td>
<td>3</td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed in terms of percentage binding for heterologous reactions relative to those of the homologous reactions.

<sup>b</sup> The radioactivity (counts per minute) of each homologous reaction was 989 (type I), 1,315 (type II), and 306 (type III).

<sup>c</sup> Not done.

<sup>d</sup> Average of the percentage of the repeated experiments shown in parentheses.
In this paper, the DNA hybridization technique was applied to determine the relationships among members of Corynebacterium renale. The results indicated that types I, II, and III were not so closely related. The results of the DNA homology test may suggest that types I, II, and III should be recognized as different species. However, the taxonomic relationship of the three types of Corynebacterium renale will be determined after a numerical taxonomy study of this species, which is now in progress.

There was a lack of concurrence in the data on the cross-homology studies. For example, type I DNA only absorbed to a level of 15% to type III DNA, whereas type III DNA absorbed to type I DNA to a level of 47%. This was the most striking example. Peterson and Pollock (21) reported that the lack of concurrence in the data in their cross-homology studies was due to difference of genome size of each DNA. This might also be the case in our study.

Corynebacterium pseudotuberculosis was not closely related to the strains of Corynebacterium renale from the viewpoint of DNA homology. This is in accord with the results of the numerical taxonomy study of corynebacteria by Harrington (10).

It is interesting to note that thymidine was not incorporated into the DNA of Corynebacterium renale due to the lack of thymidine kinase activity, but the DNA of Corynebacterium renale was labeled with the tritium from H-uridine. The uridine tritiated in the 5' position was originally used as the RNA precursor because the tritium in the 5' position is lost under biological conditions when uridine is methylated to thymidine (11). In the present study, however, the tritium of H-uridine was incorporated into the bacterial DNA. Presumably, the labeled uridine was converted to deoxyuridine derivatives after phosphorylation, and was incorporated into the DNA. Much higher specific activity might be obtained if labeled adenine were used instead of H-uridine.

Tritium from H-uridine was incorporated in the DNA of type III strains in smaller amounts than that incorporated in types I and II. Thus, it may be surmised that type III may possibly be different from types I and II in the metabolic pathway of nucleic acid.

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REPRINT REQUESTS

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LITERATURE CITED


