Genetics of *Treponema*: Relationship Between *Treponema pallidum* and Five Cultivable Treponemes

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Three genetically distinct groups of treponemes have been identified by saturation reassociation assays using $^{125}$I-labeled treponemal DNAs. The three groups are (i) virulent *Treponema pallidum* (Nichols strain), (ii) *T. phagedenis* and its biotypes Reiter and Kazan 5, and (iii) *T. refringens* biotypes Nichols and Noguchi. There is no detectable DNA sequence homology (<5%) among the three groups. The groups have distinct guanine + cytosine contents: 52.4 to 53.7% for *T. pallidum*, 41.5% for *T. refringens*, and 38 to 39% for *T. phagedenis*.

*Treponema pallidum* was identified as the causative agent of syphilis in 1905 (21). Since then, many pathogenic isolates have been found, among which are the Nichols pathogenic, Truffi, Gand, Gent, and A mi strains (25). The one that has been the most extensively studied is the Nichols pathogenic strain. It was originally isolated in 1912 from the cerebrospinal fluid of an individual with secondary syphilis (18) and has since been passaged in rabbit testes. Even after 65 years it has remained pathogenic for man (4, 8). To date, neither this strain nor any other pathogenic strain of *T. pallidum* has been cultivated in vitro.

In contrast, the many strains of treponemes presumably isolated from cases of human syphilis (25) and successfully cultivated in vitro have proven to be nonpathogenic in animals. Among these are the Nichols nonpathogenic strain, the Reiter and Noguchi strains, and numerous Kazan strains (25). These particular strains are now classified as biotypes of the genital treponemes *T. phagedenis* (Reiter, Kazan) or *T. refringens* (Nichols, Noguchi) (22). The Nichols nonpathogenic strain was reputedly obtained by in vitro cultivation of the Nichols pathogenic strain (15). Some of the cultivable strains, notably *T. phagedenis* biotype Reiter and *T. refringens* biotypes Nichols and Noguchi, were reported originally to have been pathogenic but to have lost their virulence upon long-term in vitro cultivation (15, 19, 20).

Although the cultivable strains are nonpathogenic, they have continued to be of interest, since it has been repeatedly shown that they cross-react immunologically with *T. pallidum*. The one shared protein antigen is common to many treponemes, including *T. pallidum*, *T. phagedenis* biotype Reiter, and *T. denticola*, as well as to *Spirochaeta zuelzeriae* (5). It therefore appears to be nonspecific. Antibodies to this antigen are present in sera from both normal and syphilitic individuals (14), and absorption of sera with either a sonic extract or the autoclaved culture fluid from *T. phagedenis* biotype Reiter removes antibodies against the antigen (5, 23).

Although *T. pallidum* appears to be antigenically related to some of the cultivable treponemes, the exact genetic relationship between it and the nonpathogenic treponemes is still unclear. It is possible that certain of the cultivable treponemes are genetically related to, and do represent, nonpathogenic forms of *T. pallidum*. If so, then it might be possible to develop a vaccine against syphilis using an easily produced, stable nonpathogenic organism. On the other hand, the cultivable treponemes may be genetically distinct organisms that were contaminants in the original clinical specimens. Since there are many cultivable strains of treponemes, a systematic and efficient experimental approach is needed to determine genetic relationships.

In the past, the determination of genetic relationships among the treponemes was hindered by the inability to introduce radioactive label into the treponemal DNA. We have labeled purified treponemal DNAs with $^{125}$I to a specific activity in excess of $3 \times 10^6$ cpm/µg of DNA and have determined the genetic relationship between pathogenic *T. pallidum* (Nichols strain) and five cultivable nonpathogenic treponemes. The data show that there are at least three distinct genetic groups of treponemes: *T. pallidum*, *T. phagedenis* and its biotypes Reiter and Kazan 5, and *T. refringens* biotypes Nichols and Noguchi. The applicability of these techniques for the elucidation of genetic relationships between any two organisms is discussed.
MATERIALS AND METHODS

Chemicals. Cortisone acetate was purchased from Upjohn Co.; sodium diatrizoate (Hypaque) was from Winthrop Laboratories; NaCl was from New England Nuclear; and hydroxyapatite was from Bio-Rad Laboratories. Nucleoside (EC 3.1.4) from Miles Laboratories was dissolved in 0.01 M potassium phosphate (pH 6.5)–50% (vol/vol) glycerol and stored at −20°C. Salmon sperm DNA was obtained from Calbiochem, and Escherichia coli DNA was from Worthington. The following solutions were used: SSC, 0.15 M NaCl-0.015 M sodium citrate; PBS, phosphate-buffered saline (6); PBS-G, PBS with 0.6 mg of reduced glutathione per ml (1); S1 buffer, 0.03 M NaCl-0.03 M sodium acetate (pH 4.3)–1.5 mM ZnCl2; PB, equimolar NaH2PO4 and Na2HPO4 at the molarities specified. The scintillation fluid consisted of Lixi-fluor (New England Nuclear) in toluene. All glassware was treated for at least 1 h with dimethyldichlorosilane (Sigma Chemical Co.).

Animals. New Zealand white male rabbits were purchased from LIT, Apts., Calif.; 3- to 4-kg animals that were VDRL negative were used throughout. They were housed individually at 16 to 18°C.

Treponemes. The bacterial strains were obtained in the following: virulent T. pallidum (Nichols) from James N. Miller, University of California, Los Angeles; T. phagedenis biotype Reiter from Paul H. Hardy, Jr., Johns Hopkins University, Baltimore, Md.; T. refringens biotypes Nichols and Noguchi and T. phagedenis and its biotype Kazan 5 from A. W. Hanson of the Center for Disease Control, Atlanta, Ga.

Propagation and purification of T. pallidum. T. pallidum was propagated in VDRL-negative adult male rabbits, as previously described (7). Infected rabbits were given daily injections of cortisone acetate, according to the procedure of Hardy and Nell (12). Treponemes were harvested aseptically 13 to 14 days postinfection at the peak of infection, as already described (7). The total yield of treponemes per rabbit was 10^10 to 3 × 10^10. Cellular debris and sperm were removed by centrifugation of the treponemal suspension at 500 × g for 5 min. Treponemes were concentrated by centrifugation at 12,000 × g for 10 min in a Sorvall SS34 rotor and resuspended in PBS-G. They were purified by band sedimentation through a discontinuous Hypaque gradient (1). After being collected from the gradient, they were diluted with PBS, pelleted as above, and resuspended in PBS. Before DNA extraction, 1/10 volume of 100 mM MgCl2 was added, and the treponemal suspensions were treated with 100 µg of deoxyribonuclease I per ml for 60 min at 37°C, after which the treponemes were pelleted and suspended in SSC at 4°C.

Growth of cultivable treponemes. Lyophilized cultures were rehydrated as described by Hanson and Cannefax (10). No motile organisms were observed when the cultures were rehydrated. Several weeks of incubation at 33°C were required before visible growth was observed. All cultures were maintained at 33°C by weekly passage in fresh Hanson's medium (10) with 0.001% resazurin. A 3- to 4-day logarithmic-phase culture was used as the inoculum for batch cultures prepared in 500-ml screw-cap Erlenmeyer flasks. Cultures were gently agitated at 24-h intervals. After 4 days of incubation for the T. phagedenis biotypes and 5 to 7 days of incubation for the more slowly growing T. refringens biotypes, the treponemes were harvested by sedimentation at 12,000 × g, washed with PBS, pelleted, and stored at −20°C. They were resuspended in SSC for DNA extraction.

DNA purification. DNA was purified by the procedure of Marmur (16) or by the phenol-chloroform procedure. There was no difference in the ultraviolet absorption profile or the reassociation kinetics of DNA purified by the two procedures. The DNA preparations were dissolved in 0.085% NaCl and stored at 4°C. For radiolabeling and reassociation assays, DNAs were sheared to 150,000 single-strand molecular weight by boiling in 0.3 N NaOH for 20 min (17). Sheared DNAs were neutralized with 3 N HCl and stored at 4°C.

Determination of G+C content. The DNA base composition of all the treponemes except T. refringens biotype Nichols was determined from thermal denaturation profiles of double-stranded DNA. DNA samples were heated in 0.01 M sodium phosphate (pH 7.0)–1 mM EDTA, using a GCA/McPherson model EU-707K spectrophotometer-recorder with a P120 electronic temperature programmer. The temperature was raised 18°C/min. Each assay included an internal standard of E. coli DNA. The guanine + cytosine (G+C) content was calculated from the Tm values by taking E. coli DNA as a standard of 50% G+C and using the relationship 0.41°C/1% G+C.

Preparation of [125I]DNA. [125I]DNA was prepared as previously described (17) except that it was separated from unreacted radioisotope by binding to and elution from hydroxyapatite columns. [125I]DNA solutions were heat denatured in 0.05 M PB and quickly cooled to 4°C. Single-stranded [125I]DNA was bound to 1-ml columns of hydroxyapatite in 0.05 M PB at 60°C. Unreacted [125I]I was removed by washing the columns with 30 ml of 0.14 M PB, and the single-stranded [125I]DNA was eluted with 8 ml of 0.14 M PB. All preparations of single-stranded [125I]DNA were rendered acid soluble (>95%) by treatment with 1,600 U of S1 per ml for 2 h at 37°C and were insensitive to ribonuclease A. The specific activities of the [125I]DNAs ranged from 3.6 × 10^8 to 2.9 × 10^8 cpm/µg, assuming complete recovery of the DNA.

DNA-DNA reassociation kinetics. Reassociation kinetic reactions were performed as previously described (17). A trace amount of [125I]DNA was added to a reaction mixture containing 6.4 absorbancy units at 260 nm (A260)/ml of unlabeled homologous DNA either in 0.4 M PB–10 mM NaCl–0.03 M EDTA (pH 6.80), if the products were to be analyzed on hydroxyapatite columns, or in a buffer consisting of 0.6 M NaCl–0.01 M tris(hydroxymethyl)aminomethane - hydrochloride–1 mM EDTA (pH 6.80), if the products were to be analyzed by S1 digestion. The DNA solutions were heat denatured and quickly cooled to 4°C. Reactions were begun by rapidly raising the solution temperature to 60°C. Solutions were overlaid with mineral oil to prevent evaporation. At intervals, 50-µl samples were removed, chilled, and diluted into cold 0.14 M PB for hydroxyapatite chromatography or into 2 ml of cold S1 buffer for analysis with S1. The total radioactivity
for each 50-μl sample ranged from 1,000 to 8,000 cpm. The data have been corrected to the rate of reassociation in 0.18 M Na⁺ (3).

**Saturation reassociation.** Saturation reassociation assays were performed as previously described (17). In the standard assay, 10 μl of [125I]DNA was used in a total reaction volume of 50 μl. Reactions were run for 72 h at 60°C in one of the buffer solutions described above, depending on the method of product analysis. Simultaneous reactions were performed in the presence of increasing amounts of test DNA. Reactions were terminated as described above, and the products were analyzed with hydroxypatite or S1. In all cases, the test DNA concentration was sufficient to reach plateau levels of reassociation with homologous [125I]DNA sequences. The total radioactivity per sample was 1,500 to 15,000 cpm. Sealed, silanized microcapillary tubes were used as reaction vessels to prevent evaporation. The data have not been corrected for viscosity differences.

**Analysis of reaction products.** The extent of reassociation of [125I]DNA was determined by hydroxypatite chromatography or by digestion with nuclease S1. The method of analysis on hydroxypatite columns has been described (17). The percent reassociation was calculated as the ratio of labeled double-stranded DNA to the total labeled DNA in each sample. For analysis with S1, 50-μl reaction samples were diluted into 2 ml of cold S1 buffer containing 1,000 U of S1 per ml. The mixture was incubated in a 37°C water bath for 2 h. Acid-precipitable [125I]DNA was collected on filters, and the percent reassociation was calculated as the ratio of S1-resistant radioactivity (counts per minute) to the total counts per minute in each sample. Total counts per minute was the acid-precipitable radioactivity in a duplicate sample without S1 in the digestion mixture. All samples were counted in a Beckman liquid scintillation counter.

**RESULTS**

**DNA base composition.** The base compositions of all the treponemes except *T. refringens* biotype Nichols are shown in Table 1. The treponemes tested fall into three groups according to their G+C content. *T. phagedenis* and its biotypes contain 38 to 39% G+C, and *T. refringens* biotype Noguchi contains 41.5% G+C. In contrast, *T. pallidum* contains 52.4 to 53.7% G+C, which is much higher than that of the cultivable nonpathogenic treponemes. *T. pallidum* DNA was also compared with normal rabbit testes DNA. Normal testes DNA was extracted from a VDRL-negative male rabbit by the phenol-cresol technique. In simultaneous assays, the base compositions of *T. pallidum* and rabbit testes DNA were 52.5 and 42.2% G+C, respectively. All of the DNA species gave smooth thermal denaturation curves, with no indications for the presence of satellite DNAs.

**Kinetics of reassociation.** The reassociation kinetics of DNA from pathogenic *T. pallidum* and three cultivable nonpathogenic treponemes were determined by measuring the reassociation of trace amounts of [125I]-labeled DNA with known concentrations of uncleaved, totally homologous DNA. [Abbreviations used are: Tp, *T. pallidum* (Nichols); Pd, *T. phagedenis*; Re, *T. phagedenis* biotype Reiter; K5, *T. phagedenis* biotype Kazan 5; Ng, *T. refringens* biotype Noguchi; Ni, *T. refringens* biotype Nichols; and C8, product of DNA concentration and length of the reaction.] The reassociations of Tp, Re, and Ng DNAs are shown in Fig. 1. The data have been expressed in the usual form, C8.t (2). In each case, the C8.t values are those for the uncleaved homologous DNA. The calculated concentration of [125I]DNA was ±8 × 10⁻⁴ A260/ml; the concentration of unlabeled DNA was 6.4 A260/ml. The maximum C8.t value for the [125I]DNA alone was 0.02 mol-s/l when the C8.t value for unlabeled DNA was 1,000 mol-s/l. The reassociation of [125I]DNA is, therefore, totally dependent on the reassociation of the un-

**TABLE 1. G+C content of DNAs**

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>% G+C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. pallidum</em> (Nichols)</td>
<td>52.4-53.7</td>
</tr>
<tr>
<td><em>T. phagedenis</em></td>
<td>39.0</td>
</tr>
<tr>
<td><em>T. phagedenis</em> biotype Reiter</td>
<td>39.0</td>
</tr>
<tr>
<td><em>T. phagedenis</em> biotype Kazan 5</td>
<td>38.0</td>
</tr>
<tr>
<td><em>T. refringens</em> biotype Noguchi</td>
<td>41.5</td>
</tr>
<tr>
<td>Rabbit testes</td>
<td>42.2</td>
</tr>
<tr>
<td><em>E. coli</em> (standard)</td>
<td>50.0</td>
</tr>
</tbody>
</table>

* The *T*ₘ of each DNA species was measured against that of *E. coli*. The G+C content was then calculated from the relationship 0.41°C/1% G+C, with *E. coli* DNA defined as 50.0% G+C.

**Fig. 1. Reassociation kinetics of treponemal DNAs.** Trace amounts of [125I]-labeled DNAs were reassociated in the presence of excess unlabeled, totally homologous DNAs in 0.4 M PB at 60°C. At intervals, samples were diluted to 0.14 M PB with water and analyzed on hydroxypatite columns. C8.t values are for the unlabeled DNA and have been corrected to standard conditions of 0.18 M Na⁺. C8,1/2 values were: 29 (Tp, △), 50 (Re, ○), and 7.5 (Ng, □) mol-s/l. Each point represents a total of 1,500 (Tp), 2,500 (Re), and 2,000 (Ng) cpm.
labeled, totally homologous DNA species. The maximum level of reassociation was 85 to 90%, and the base line level was 2 to 5% for Tp and Re DNAs. The data show that the Cx1/2 values are different for each of the DNA species tested. The smallest value, 7.5 mol-s/l, was for Ng DNA. The Cx1/2 values for Tp and Re DNAs were 29 and 50 mol-s/l, respectively. All these reactions follow second-order kinetics. The reactions were analyzed using hydroxyapatite.

Figure 2 shows the results of reassociation reactions analyzed with S1. Again, trace amounts of [125I]DNAs were added to a vast excess of unlabeled, totally homologous DNA. It can be seen that the kinetics of reassociation for Re DNA are identical whether the products are analyzed on hydroxyapatite (Cx1/2, 50 mol-s/l; Fig. 1) or by resistance to S1 digestion (Cx1/2, 52 mol-s/l; Fig. 2). The Cx1/2 value for Pd DNA was 28 mol-s/l, which was identical to that for Tp DNA (Fig. 1).

All the reassociation reactions followed second-order kinetics when analyzed by either hydroxyapatite or S1. The maximum levels of reassociation ranged from 85 to 90% of the total DNA and occurred at Cx values of 300 (Ng), 600 (Tp, Pd), and 1,000 (Re) mol-s/l under our conditions.

Saturation reassociation. The maximum extent of DNA sequence homology between the five cultivable nonpathogenic treponemes and pathogenic T. pallidum (Nichols) was determined by saturation reassociation assays. In such an assay, a constant amount of 125I-labeled probe DNA is permitted to reassociate for a fixed length of time such that no self-reassociation occurs. Simultaneous reactions, run in the presence of increasing amounts of test DNA, are used as a direct measure of the extent of sequence homology between the two DNA species. The results showed that after 72 h of incubation in the standard reaction mixture, there was <3% self-reassociation in the absence of added unlabeled DNA. The levels of self-reassociation were 2% (Tp, Re, K5) and 1% (Pd, Ng). These results match the base line levels previously determined in the reassociation kinetic studies.

Saturation reassociation assays were performed with Tp [125I]DNA and unlabeled DNA from the five cultivable treponemes as well as salmon sperm (Fig. 3). In the presence of salmon sperm DNA, the level of reassociation was 2%. The maximum level of reassociation was 85%, which occurred with about 0.1 A260 unit of added Tp DNA. The level of reassociation with the five cultivable treponemes was 5%. The total radioactivity per sample was 1,500 cpm. The level of resolution in this case was about 5%. Reaction products were assayed on hydroxyapatite columns.

The genetic relationships among the cultivable treponemes were then determined and analyzed by S1 digestion. Only DNAs from the five cultivable treponemes were used as test DNAs.

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** Reassociation kinetics of Re and Pd DNAs. Trace amounts of [125I]-labeled DNAs were added to an excess of unlabeled, totally homologous DNAs, as described in the legend for Fig. 1. Samples were analyzed by S1 digestion. The Cx1/2 values were 52 (Re, ◯) and 28 (Pd, △) mol-s/l. The total radioactivity in each sample was 15,000 (Re) and 8,600 (Pd) cpm. Data have been corrected to standard conditions of 0.18 M Na+.

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** Saturation reassociation of Tp DNA. [125I]-labeled Tp DNA was reassociated in 0.4 M PB at 60°C to a Cx value of <0.02 mol-s/l. Simultaneous reactions were run in the presence of increasing concentrations of added unlabeled DNAs. All reactions were analyzed as described for Fig. 1. The base level of reassociation was 2%. Unlabeled DNAs were Tp (○), salmon sperm (○), Re (●), Pd (△), K5 (□), Ng (■), and Ni (○). Each point represents a total of 1,500 cpm.
The level of reassociation of Re $[^{125}\text{I}]$DNA in the presence of salmon sperm DNA was 2% (Fig. 4). The plateau level of reassociation in the presence of totally homologous Re DNA was 91%. This same level was reached with unlabeled K5 DNA, indicating that K5 possesses all the DNA sequences present in Re. The plateau level of reassociation of Re $[^{125}\text{I}]$DNA in the presence of Pd DNA was 54%, whereas the level in the presence of Ng or Ni DNA was only 5%. The total radioactivity per sample was 15,000 cpm. These results were confirmed by measuring the saturation reassociation of K5 $[^{125}\text{I}]$DNA. The data (Table 2) indicate 93% homology between K5 and Re but no homology between K5 and either Ng or Ni. In addition, Pd DNA possesses only 57% of the sequences in K5 DNA. The total radioactivity for each sample was 8,500 cpm. The degree of DNA sequence homology among the T. phagedenis biotypes was completed by using Pd $[^{125}\text{I}]$DNA as probe. The data indicate that Re contains all the sequences of Pd and that K5 contains at least 93% of the sequences of Pd: the total radioactivity for each sample was 12,000 cpm. There was no homology between Ng $[^{125}\text{I}]$DNA and Re, K5, or Pd DNA. Ni DNA has at least 90% of the sequences present in Ng DNA; the total radioactivity for each sample was 1,500 cpm.

![Graph](image)

**Fig. 4.** Saturation reassociation of $^{125}\text{I}$-labeled Re DNA. $^{125}\text{I}$-labeled Re DNA was reassociated in 0.6 M NaCl-0.01 M tris(hydroxymethyl)aminomethane-hydrochloride-1 mM EDTA (pH 6.80) at 60°C to a C$_s$ value of $<0.02$ mol-s/l. Simultaneous reactions were run in the presence of added unlabeled DNAs. Reactions were analyzed by S$^1$ digestion. The base level of reassociation was 2%. Unlabeled DNAs and symbols were the same as described for Fig. 3. Each point represents a total radioactivity of 15,000 cpm.

### DISCUSSION

The data clearly show that of the treponemes studied, there were at least three genetically distinct groups that shared no sequence homology ($<5\%$) with each other. The three groups are T. pallidum, T. phagedenis and its biotypes, and the T. refringens biotypes. The results are not an artifact due to differences in genome molecular weights or in the kinetics of reassociation, since the C$_s$ value for each of the unlabeled treponemal DNA species tested was sufficient to give complete reassociation of all homologous sequences. Under our conditions, with an unlabeled DNA concentration of 0.3 A$_{260}$/50-μl reaction volume, the relative C$_s$ value under standard conditions (0.18 M Na$^+$) would be $>1,000$ mol-s/l, which is sufficient to yield complete reassociation of all of the treponemal DNAs tested. The complete reassociation of each $[^{125}\text{I}]$DNA with unlabeled homologous DNA in saturation reassociation and reassociation kinetics assays shows that the $[^{125}\text{I}]$DNAs are accurate probes. In addition, the shape of the reassociation kinetics curve of the T. pallidum DNA indicates that there was little or no contamination with other DNAs, such as rabbit testes DNA. This was confirmed by the fact that no spectrophotometically detectable amounts of any contaminating DNA were observed in the thermal denaturation assays.

The genome molecular weight of each treponemal group can be calculated from the C$_s$ curves (2), since none of the treponemal genomes contain repeated sequences. The estimated double-strand molecular weight of the T. pallidum genome is $9.05 \times 10^9$; that of E. coli is approximately $2.5 \times 10^9$. T. phagedenis also has a genome size of $9.05 \times 10^9$ daltons, which is 56%

| Table 2. Maximum levels of reassociation of $[^{125}\text{I}]$-labeled DNAs and unlabeled test DNAs $^*$ |
|---------------------------------|-----|-----|-----|-----|-----|-----|-----|
|                                | SS$^+$ | Tp  | Re  | K5  | Pd  | Ng | Ni |
| $[^{125}\text{I}]$DNA           |       |     |     |     |     |    |    |
| Tp                              | 2    | 100 | 5   | 5   | 5   | 5  | 5  |
| Re                              | 2    | ND$^*$| 100 | 100 | 54  | 5  | 5  |
| K5                              | 2    | ND  | 93  | 100 | 57  | 2  | 2  |
| Pd                              | 1    | ND  | 100 | 93  | 100 | 1  | 1  |
| Ng                              | 1    | ND  | 1   | 1   | 1   | 100| 90 |

$^*$ Saturation reassociation assays were performed with each $[^{125}\text{I}]$-labeled DNA and the indicated test DNAs. The maximum level of reassociation in each assay is shown. The level of reassociation with the totally homologous test DNA, which ranged from 85 to 97%, was taken as 100%. In all cases, a plateau level of reassociation was reached.

$^*$ SS, Salmon sperm. All other abbreviations are noted in the text.

$^*$ ND, Not done.
that of *T. phagedenis* biotype Reiter (1.62 × 10^{10} daltons). On the other hand, the estimated genome size of *T. refringens* biotype Noguchi is identical to that of *E. coli*. The difference in the sizes of the genome of *T. phagedenis* and its biotype Reiter is extraordinary. The genome sizes calculated from the C_{t}t curves are in agreement with the relative sizes calculated from the saturation reassociation data. *T. phagedenis* contains only 54 to 56% of the sequences present in biotypes Reiter and Kazan 5, whereas both biotypes contain all the sequences present in *T. phagedenis*. Since there are no repeated sequences in the genomes of these treponemes, the results can mean only that the molecular weight of the *T. phagedenis* genome is 56% that of the biotype Reiter genome.

The total lack of homology between *T. pallidum* and the five nonpathogenic treponemes supports the hypothesis that the nonpathogenic treponemes were contaminants in the original clinical specimens and have no function in the pathogenicity of *T. pallidum*. The lack of homology would appear to be in conflict with the antigenic relationships among the treponemes. This is not the case when we consider that the limit of resolution with the *T. pallidum* [^{125}I]-DNA preparations (1,500 cpm total) is 5% of the entire genome or 4.5 × 10^{8} daltons, which is enough DNA to code for approximately 450 proteins. Therefore, the sequences coding for the shared protein antigen may not be detected by this procedure. Even with biotype Reiter DNA (15,000 cpm total), the limit of detection is about 0.5%, or 8 × 10^{7} daltons of DNA, which could code for 80 proteins. It is evident that with treponemes, DNA-DNA hybridization is not as sensitive as immunological assays in detecting a very small degree of relatedness. However, it is quantitative, easily interpreted, and gives the total extent of homology; also, the reagents (DNA) are much easier to isolate in pure form than are protein antigens. There has been only one case in which [^{125}I] labeling has interfered with DNA-DNA reassociation. This involved the biotype Nichols DNA. Although the unlabeled biotype Nichols DNA could reassociate with [^{125}I]-labeled DNA from biotype Noguchi, it was found that when biotype Nichols DNA was labeled with [^{125}I], there was incomplete reassociation (51% maximum) with totally homologous unlabeled DNA. With this exception, the techniques described here have been used successfully with viral (17) and procaryotic DNAs. Since the technique presented here involves the introduction of [^{125}I] into purified DNA, the procedure can be applied to the labeling and analysis of any DNA species and presents a powerful tool for the genetic classification of organisms.

Our data should not be interpreted to mean that no genetic relationship exists between *T. pallidum* and any of the other numerous cultivable isolates of treponemes. Since the genetic relationships among other cultivable treponemes are still unknown and since there are vast differences in DNA sequences between the two groups of cultivable treponemes that we have studied, there may still exist cultivable treponemes that have a significant degree of sequence homology with *T. pallidum*. It is evident that studies of other pathogenic treponemes must be performed. There are four distinct human diseases caused by treponemes which are so similar that they cannot be morphologically or immunologically distinguished. The organisms and the diseases they cause are *T. pallidum* (venereal syphilis, nonvenereal endemic syphilis), *T. pertenue* (yaws), and *T. carateum* (pinta).

There are basically two theories on the origin of these pathogens. The first theory is that one organism causes all four diseases and that the diseases are clinically distinct due to environmental influences (13). The second theory is that the human pathogens probably arose from an animal infection and that mutants corresponding to the three present-day organisms were selected for by climatic changes. The earliest strain is thought to be *T. carateum*, followed by *T. pertenue*, and finally *T. pallidum* (9). In a slight modification of this theory, Willcox (24) suggested that the selective pressure was the mode of transmission of each pathogen and that the ancestral nonhuman treponeme may have been the free-living *S. zuelzeriae*. This is an interesting concept since we have now found that the G+C content of *T. pallidum* (52.4 to 53.7%) is very close to that of *S. zuelzeriae* (56.1%) and that of *S. litoralis* (50.6%), both of which are anaerobic, free-living marine spirochetes. In addition to the pathogenic human treponemes, there are *T. cuniculi*, which is the causative agent of venereal spirochetalis in rabbits, and various spirochetes that are present but apparently nonpathogenic in certain avian species (11). Any of these organisms, normally not present in man, may represent an ancestral form giving rise to the human pathogen. The evolutionary history of *T. pallidum* can be determined by an analysis of the DNA sequences of these treponemes and spirochetes. A nonpathogen ancestor or variant of *T. pallidum* may be found to occur naturally in nonhuman hosts. Such an organism could be used in the development of a vaccine against syphilis.
ACKNOWLEDGMENTS
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LITERATURE CITED