Studies on the Spectrophotometric Determination of DNA Hybridization from Renaturation Rates

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Summary

The optical method of De Ley et al. (1970) for determining DNA/DNA homologies was reexamined. Several parameters such as incubation temperature, incubation time, DNA concentration and DNA fragment length were checked and the optimal conditions established. Hybridization data of several anaerobic Gram-positive cocci were compared with values obtained by the membrane filter technique. The agreement is excellent above a degree of binding of 25-30%.

Key words: DNA/DNA hybridization – Optical determination of DNA renaturation rates

Introduction

In the last 20 years, DNA/DNA hybridization has proved to be an important tool for taxonomists in clarifying the relationships among bacteria at the species level and partly also at the genus level. Several techniques have been employed: the agar gel (McCarthy and Bolton, 1963) and the membrane filter (Denhardt, 1966) methods with single stranded, high molecular weight DNA bound to agar gel and nitrocellulose filters respectively; the hydroxyapatite method (Brenner et al., 1969) with renatured double stranded DNA bound to hydroxyapatite, and the S1 endonuclease technique (Crosa et al., 1973). All the aforementioned procedures require radioactively-labelled DNA for the detection of the degree of heteroduplex formation.

In 1970 de Ley et al. proposed an optical method for DNA/DNA hybridization, based on the renaturation kinetics of denatured DNA (Marmur and Doty, 1961; Wetmur and Davidson, 1968). Although this method offers some advantages, especially the fact that no labelled DNA is required, it is not as widely used as the membrane filter or hydroxyapatite methods. With the advantage of better instrumentation than originally available, the optical method was reexamined and compared with the membrane filter technique.
Material and Methods

Organisms and Growth Conditions

The organisms used were anaerobic cocci of the genera *Peptococcus* and *Peptostreptococcus* as well as unnamed anaerobic cocci of the Hare Groups I and IX (Hare et al., 1952; Thomas and Hare, 1954). *Escherichia coli* K 12 was included as a reference. All strains, except for *E. coli* K 12, which was grown aerobically in yeast-extract-dextrose-peptone-bouillon, were cultivated anaerobically in a medium described by Kilpper-Biltz and Schleifer (1981) with additional 5 mg/l hemin and 1 mg/l vitamin K₁ (Holdeman et al., 1977) as well as 2.5 g/l glycine and 2.5 g/l glutamic acid. The incubation temperature was 37 °C. Cells were harvested at the end of the exponential growth phase.

DNA Preparation

Extraction of labelled and unlabelled DNA was carried out as previously described by Meyer and Schleifer (1975, 1978). Purified DNA was dialyzed against standard saline citrate buffer (SSC; 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) for the determination of the guanine-plus-cytosine (G + C) content. Fixation of unlabelled DNA was performed according to Gillespie and Spiegelman (1965).

For renaturation experiments the DNA was passed three times through a French Pressure Cell at about 1.5 × 10⁶ Pa. and dialyzed against double strength (2X) SSC. DNA concentration was adjusted spectrophotometrically at 260 nm. Sheared DNA was stored at −20 °Cm while high molecular weight DNA was kept over a few drops of chloroform at 4 °C.

Guanine-plus-Cytosine (G + C) Content of DNA

The determination of the G + C content of the various DNA preparations was carried out in a microprocessor-controlled Gilford 2600 spectrophotometer equipped with a thermoelement including four cuvettes with integrated temperature control, a thermoprogrammer and an automatic cuvette changer. DNA base composition was calculated according to De Ley (1970).

Measurement of DNA Renaturation Rates

The equipment for the determination of the renaturation rates was the same as used for the melting profiles of DNA. The four cuvettes, which could be sealed completely, contained respectively 2 X SSC as a blank, DNA solution of a strain A, the same volume of a DNA solution of a strain B, and a mixture of equal volumes of DNA of A and B. DNA solutions of strains A and B had the same weight concentrations. The DNA was denatured in the cuvettes at 100 °C for 10 min. The reassociation reactions were started by quickly cooling the solutions to the optimal renaturation temperature (TOR); \( \text{TOR} = 0.51 \times \% (G + C) + 47.0 \) (Gillis et al., 1970). The absorbance at 260 nm was measured over 30 min every 15 sec and plotted. The renaturation rates \( v' \) were determined as decrease in absorbancy/min (\( \frac{\Delta A}{t} \)), and the degree of binding (\( \% D \)) was calculated according to the formula given by De Ley et al. (1970):

\[
\% D = 100 \times \left( 4v'_M - v'_A - v'_B \right) / 2 \left( v'_A \times v'_B \right).
\]

Hybridization on Membrane Filters

DNA/DNA hybridization on membrane filters was accomplished as described by Meyer and Schleifer (1978) in 6XSSC at 60 °C under optimal conditions (25 °C below \( T_M \)). The melting point of the DNA was lowered by the addition of formamide (McConaughy et al., 1969).
Molecular Weight Determination

Molecular weight determination of the DNA preparations was carried out by horizontal 1.5% agarose (Biorad, standard low-m #1) gel electrophoresis in a 0.04 M Tris-acetate buffer pH 8.0, using as reference restriction fragments (Eco RI and Hind III) of Lambda DNA (Boehringer, Mannheim).

Results and Discussion

The renaturation of single stranded DNA fragments obeys a second order reaction pattern under ideal conditions, and is influenced by several parameters such as fragment length and source of DNA, temperature, ionic strength of the buffer and DNA concentration (Marmur and Doty, 1961; Subirana and Doty, 1966; Wetmur and Davidson, 1968). These factors may greatly influence the renaturation rate itself. The degree of binding, however, is calculated by comparing the renaturation rate of a mixture (\(v'M\)) with the rates of its two components (\(v'\)A and \(v'\)B). Because all three reactions proceed under the same conditions, the degree of binding might be influenced less by some of these factors. Moreover, the mixture might to some extent compensate for different conditions of the two components such as fragment length or DNA concentration. With all this in view, the influence of several parameters on the degree of binding was examined.

Effect of Temperature

To avoid any errors due to differences in samples or filling of the cuvettes, the same DNA solutions were denatured and allowed to renature 10-15 times at different temperatures. As a control, the same samples were measured at the same temperature up to 15 times. There was virtually no change in the degree of binding, but a gradual decrease in renaturation rates totalling about 8%. This is probably due to strand breaks caused by the high temperatures employed for denaturing the DNA. An example of the dependence of the renaturation rate and the degree of binding upon temperature is given in Fig. 1. While on the whole there is a strong dependence of the renaturation rate on temperature, there is a range of about 15°C where the degree of binding is almost independent of temperature.

Effect of Concentration of DNA Solutions

The calculation of the degree of binding according to the equation: \(\% D = 100 \times \frac{(4v'M - v'A - v'B)/2 \sqrt{(v'A \times v'B)}}{v'A \times v'B}\) (de Ley et al., 1970) is based on the assumption that renaturation strictly follows a second order reaction pattern. Thus the renaturation rate \(v'\) should be proportional to the square of the DNA concentration: \(v' = k' \times c^2\), where \(c\) is the DNA concentration and \(k'\) is an apparent reaction rate constant. With a concentration of \(\frac{c}{2^2}\), \(v'\) should decrease to a quarter. The mixture \(\left(\frac{c_A}{2A} + \frac{c_B}{2B}\right)\) of two completely heterologous DNA solutions \((c_A\) and \(c_B)\) should therefore renature half as fast as the components alone, \(\left(\frac{c_A + c_B}{2}\right)\) resulting in a degree of binding of 0%. Since we rarely obtained values below 30% at the DNA con-
Fig. 1. The effect of temperature on the renaturation rate and the degree of binding. $T_{OR}$ is the temperature of optimal renaturation (Gillis et al., 1970).

○, *Peptococcus variabilis* DSM 20369; △, *P. anaerobius* ATCC 15794; □, mixture; ●, degree of binding (%D).

It is obvious that with higher DNA concentrations both the deviation from the second order reaction and the deviation from the theoretical degree of binding increase. Determination of the degree of binding of two heterologous DNA’s, however, give somewhat lower values at high DNA concentrations, and somewhat higher
Table 1. Effect of DNA concentration

<table>
<thead>
<tr>
<th>Concentration of DNA (µg/ml)</th>
<th>( v'_c )</th>
<th>Renaturation rates ( (\times 10^{-9}) )</th>
<th>Deviation from the theoretical degree of binding (%D)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( v'_c/2 ) (theoretically)</td>
<td>( v'_c/2 ) (measured)</td>
</tr>
<tr>
<td>134.4*</td>
<td>6.45</td>
<td>1.61</td>
<td>2.71</td>
</tr>
<tr>
<td>90.4*</td>
<td>5.69</td>
<td>1.42</td>
<td>2.16</td>
</tr>
<tr>
<td>45.2*</td>
<td>2.16</td>
<td>0.54</td>
<td>0.69</td>
</tr>
<tr>
<td>33.6*</td>
<td>0.80</td>
<td>0.20</td>
<td>0.21</td>
</tr>
<tr>
<td>22.6*</td>
<td>0.69</td>
<td>0.1725</td>
<td>0.175</td>
</tr>
<tr>
<td>16.8*</td>
<td>0.21</td>
<td>0.0525</td>
<td>0.045</td>
</tr>
</tbody>
</table>

The renaturation rates \( v' \left( \frac{\Delta A}{t} \right) \) of *E. coli* K 12 (a) and *Peptococcus asagharolyticus* DSM 20364 (b) measured at various DNA concentrations. Simultaneously the corresponding rates at \( c/2 \) were determined. \( v'_c/2 \) should theoretically be a quarter of \( v'_c \) as postulated by a second order reaction pattern. The deviation from the theoretical degree of binding was calculated according to: 

\[
%D = \frac{4v'_M - v'_A - v'_B}{2v'_A \cdot v'_B} \times 100
\]

\( v'_M = 2 \times v'_c/2 \) and \( v'_A = v'_B = v'_c \).

values at low concentrations. A possible explanation may be a steric hindrance of the reassociation due to the heterologous DNA, which results in a lower renaturation rate at high concentrations. At low DNA concentrations unspecific base pairing may contribute to a higher renaturation rate than theoretically expected. Since the renaturation rates cannot be determined exactly at very low concentrations we suggest 30–40 µg/ml as the optimal DNA concentration. Under these conditions the background does not exceed 25–30% D.

**Effect of Incubation Time**

The effect of incubation time on the degree of binding is shown in Fig. 2. With closely related strains, the degree of binding is nearly independent of incubation time, whereas remotely related organisms show increasing values of %D with time. Because renaturation follows (nearly) second order kinetics only for a limited time (Gillis et al., 1970), we always calculated the decrease in absorbancy over 20 minutes, beginning 10 min after the start of the reaction.

**Effect of Fragment Length and Different DNA Preparations**

We examined the degree of binding between all possible combinations of three different DNA preparations of two strains. For each strain two of the DNA solutions (I, III) came from high molecular weight DNA preparations sheared to a fragment length of 235,000–800,000 daltons, the main fraction being 460,000 ± 100,000 daltons. The remaining two solutions (II) came from low molecular weight DNA
Fig. 2. The effect of incubation time on the degree of binding of closely and remotely related bacteria.

○, *Peptostreptococcus magnus* DSM 20367 X *P. micros* DSM 20468; △, *Peptococcus anaerobius* ATCC 14955 X *P. variabilis* DSM 20362; □, Hare Group IX NCTC 9811 X *P. micros* DSM 20468; ¶, *Psc. anaerobius* DSM 20357 X *P. prevotii* DSM 20338.

preparations sheared to a fragment length of 190.000–650.000 daltons (main fraction: 370.000 ± 100.000). The results are given in Table 2. The lower molecular weight of preparation II has no obvious effect on the degree of binding, although the renaturation rates are slower (values not shown) [Marmur and Doty, 1961]. The homologous reactions with different DNA preparations gave values of 95 ± 2% D standard deviation.

Table 2. Effect of fragment length and different DNA preparations on the degree of binding

<table>
<thead>
<tr>
<th>Strain</th>
<th>DNA</th>
<th>Degree of binding (%D)*</th>
<th>Molecular weightb (daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NCTC 9801</td>
<td>I II III</td>
<td>II III</td>
</tr>
<tr>
<td>NCTC 9801</td>
<td>I</td>
<td>95 95</td>
<td>78 77 76</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>93 93</td>
<td>76 72 77</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>- 74</td>
<td>74 77</td>
</tr>
<tr>
<td>NCTC 9814</td>
<td>I</td>
<td>- 95</td>
<td>95 94</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>- 97</td>
<td>- 97</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Mean values.

b Main fraction (± 100000).
Comparison with the Membrane Filter Method

Hybridization data obtained with the membrane filter technique at optimal conditions (see Material and Methods) were compared with the renaturation method data (Fig. 3). The agreement is excellent above 30 %D. Below 25–30 %D no definite statement can be made with the optical method on the degree of relatedness.

Discussion

The various parameters examined had quite different effects on the renaturation rate and the degree of binding respectively. While the renaturation rate itself was strongly affected by different conditions, the degree of binding changed only slightly. A characteristic example is the dependence on temperature as shown in Fig. 1. The renaturation rate exhibits a clear maximum at TO, The bell-shaped dependence on temperature, as reported by other authors (Wetmur and Davidson, 1968) could only be confirmed for the low temperature side. On this side the temperature span between minimal (freezing point) and maximal (TO) reaction rate is 60–80°C, depending on the G + C content of the DNA. On the high temperature side there is a span of only about 25°C, because the reaction rate becomes zero at TM. This results in a steeper decrease of the curve at temperatures above TO. On the other hand slight deviations from TO, especially to lower temperatures, do not markedly influence the degree of binding. The situation is quite similar with different fragment lengths. The renaturation rate decreases with smaller fragments, while the degree of binding is unaffected, at least within the range of 300,000–550,000 daltons (Table 2). Much longer fragments should be avoided as explained by de Ley et al. (1970) and much smaller fragments give too low renaturation rates.

A disadvantage of the optical method is the high background, reaching 25–30 %D, instead of about 10 %D using the membrane filter technique. We tried to get better
resolution in this range, but the only effective measure so far found is to employ
the lowest possible DNA concentrations. A further disadvantage is that only two
DNA's can be compared at the same time. Apart from this, the optical method
offers some important advantages over the membrane filter technique:

a) No labelled DNA is required; this saves costs and time, and allows the determi-
nation of relatedness also among obligate cell parasites, such as chlamydiae and
rickettsiae (Myers and Wisseman, 1980), whose DNA cannot easily be labelled
in vivo.

b) There is no requirement for high molecular weight DNA, which is sometimes
difficult to prepare, especially with Gram-positive cocci.

c) The determination of the degree of binding between two organisms takes only
about 40 min.

d) The method is simple and the reproducibility is excellent. In our hands the stan-
dard deviation was $\pm 2.4 \% D$ from over 100 measurements. We never obtained
values above 100 %D, even with different DNA preparations of the same organ-
ism. This indicates low systematic and statistical error.

On the whole DNA/DNA hybridization by the optical method is a rapid and
precise procedure for determining close phylogenetic relationships among bacteria.

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