DNA : rRNA Hybridization Studies of Chromobacterium fluviatile

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(Received 5 March 1981; revised 8 July 1981)

Hybrids were prepared between 14C-labelled rRNA from each of nine species of Gram-negative bacteria and the DNA of Chromobacterium fluviatile. Two parameters were determined for each hybrid – the T_mel, which is the temperature at which 50% of the hybrid was denatured, and the percentage of rRNA bound under defined stringent conditions. The former gives a measure of the stability of the duplex once formed and the latter probably reflects the amount of the genome involved in the coding of rRNA. These parameters were used as a measure of the relatedness of C.fluviatile to each of the other nine species. The results suggest that C. fluviatile is more closely related to C. violaceum than to any other species tested. The taxonomic relationship of C. fluviatile to other Gram-negative bacteria was assessed by incorporating the results into a database already published by De Ley et al. (1978), and the use of principal components analysis was explored as an alternative way of presenting such data. This analysis confirmed the isolated position of C. fluviatile but, until further isolates are studied, it seems best to retain the species in the genus Chromobacterium.

INTRODUCTION

Chromobacterium fluviatile was first isolated from samples of water during a study of purple-pigmented bacteria associated with the River Wey (Moss et al., 1978). Although it has many of the phenotypic characters of the genus Chromobacterium, as described by Sneath (1974), C. fluviatile has DNA with a GC content of 50–52 mol %, which is very much lower than that of species previously accepted in the genus (63–72 mol %). Several authors have expressed doubts about the oxidative psychrophilic species, C. lividum, and the fermentative mesophilic species, C. violaceum, being congeneric (Moffett & Colwell, 1968; Sneath, 1974; Snell & Lapage, 1973). On the basis of a detailed study of the hybridization of rRNA from either C. violaceum NCTC 9757 or C. lividum NCTC 9796 with the DNA of a large number of other Gram-negative bacteria, De Ley et al. (1978) proposed that strains of C. lividum be referred to a new genus, Janthinobacterium.

De Smedt & De Ley (1977) and others have pointed out that the rRNA cistrons have changed less than the rest of the bacterial genome and thus rRNA :DNA hybridization studies should reveal information about relationships between bacteria at the generic and suprageneric levels. In contrast, DNA :DNA hybridization is most useful for studying the relationship between bacteria which are already closely related, such as strains of a species (see, for example, Owen & Holmes, 1980).

The extent to which the rRNA cistrons have been conserved in prokaryotes during the course of evolution has been demonstrated by a direct comparison between the primary structures of 16S rDNA from the chloroplast of Zea mays, which by implication has a prokaryote origin, and 16S rRNA from Escherichia coli (Schwarz & Kossel, 1980). As many as 74% of the approximately 1500 bases were shown to be in homologous positions and lengths of as many as 53 consecutive identical nucleotides occurred.

On the basis of the relatedness demonstrated by rRNA :DNA hybridization, a number of taxonomic groups above the generic level become apparent and these have been conveniently,
but informally, referred to as rRNA superfamilies by De Ley (1978). Four such rRNA superfamilies have so far been recognized amongst the Gram-negative bacteria: one contains the Enterobacteriaceae and Vibrionaceae; a second contains genera such as Agrobacterium, Rhizobium, Phyllobacterium, Acetobacter, Gluconobacter and Zymomonas; a third group contains pseudomonads related to Pseudomonas fluorescens, xanthomonads, Azotobacter and Azomonas; and a fourth rRNA superfamily includes C. violaceum, Janthinobacterium lividum, Pseudomonas acidovorans, Pseudomonas solanacearum and Alcaligenes. The organism isolated from germinating seeds of Psychotria nairobiensis and Ardisia crispa, which was considered to occur in the leaf nodules of these plants, and was originally identified as a strain of C. lividum (Bettelheim et al., 1968), was shown to belong to the second rather than the fourth superfamily.

Usually the data obtained for $T_{meo}$ and the percentage of rRNA bound has been presented as scatter diagrams of the two parameters for each reference strain investigated (see, for example, De Ley et al., 1978). Principal components analysis is a method of generating low-dimensional representation of multivariate data. The technique linearly transforms the original variables, with the minimum mathematical loss of information, into a set of derived variables or principal components. The principal components, which have the property of being uncorrelated, can then be used as axes on which the data can be plotted and its structure visualized in two or three dimensions.

The present study was carried out to establish whether Chromobacterium fluviatile belongs to one of the rRNA superfamilies recognized by De Ley (1978), and how it is related to Chromobacterium violaceum and Janthinobacterium lividum. The opportunity was also taken to illustrate the use of multivariate analysis in the presentation of such data and to show how this approach may be followed to explore the taxonomic position of individual strains with reference to an existing database using, in this instance, the extensive collection of information obtained by De Ley et al. (1978) using labelled rRNA from the type strains of C. violaceum and J. lividum.

**METHODS**

**Bacterial strains.** $^{14}$C-labelled rRNA of the following strains was made available by Professor De Ley: *Chromobacterium violaceum* NCTC 9757, *Janthinobacterium lividum* NCTC 9796, *Alcaligenes faecalis* NCIB 8156, *Alcaligenes denitrificans* ATCC 15173, *Pseudomonas acidovorans* ATCC 15568, *Pseudomonas fluorescens* ATCC 13525, *Alteromonas marinopraesens* ATCC 143393, *Vibrio parahaemolyticus* ATCC 17802, and *Gluconobacter oxydans* subsp. *oxydans* NCIB 9013. The preparation of these materials is described by De Ley & De Smedt (1975).

DNA was prepared from the type strain of *Chromobacterium fluviatile* NCTC 11159 using the methods of Marmur (1961) and Kirby et al. (1967) as described by De Ley et al. (1970). The GC content of this material was measured using the $T_{m}$ method of Marmur & Doty (1962).

**DNA : rRNA hybridization.** Single-stranded DNA was obtained by denaturing a solution of DNA at 105 °C for 10 min and fixed on Sartorius membrane filters (SM11309) using the method described by De Ley & Tytgat (1970). Membranes were dried overnight at room temperature, followed by 2 h in a vacuum desiccator at 80 °C. Discs containing about 50 µg DNA were cut out and used to determine the thermal stability of DNA : rRNA hybrids with each of the nine preparations of $^{14}$C-labelled rRNA using the methods of De Ley & De Smedt (1975) and De Smedt & De Ley (1977). For each experiment two parameters were determined – the temperature at which half of the DNA : rRNA hybrid was denatured ($T_{meo}$), and the ‘% rRNA binding’ (De Ley et al., 1978). The ‘% rRNA binding’ was calculated as the weight (µg) of $^{14}$C-labelled rRNA bound under stringent conditions (De Smedt & De Ley, 1977) per 100 µg of DNA fixed on the membrane. Chemical determination of DNA was made on a filter disc subjected to a simulated hybridization process in the absence of $^{14}$C-labelled rRNA. The fixed DNA remaining was released from the filter by the method of Meys & Schilperoort (1971) and determined by the diphenylamine method (Burton, 1956).

**Numerical analysis.** Data for the single strain of *Chromobacterium fluviatile* used in this study, together with those for 83 strains of a wide range of Gram-negative species examined by De Ley et al. (1978), were subjected to principal components analysis. Five parameters associated directly with the properties of their DNA (the $T_{meo}$ and
RESULTS AND DISCUSSION

The GC content of the preparation of DNA from *C. fluviatile* NCTC 11159 was 51.0 mol % \( (T_m) \), confirming the values previously reported by Moss et al. (1978). The values for \( T_{m(e)} \) and % rRNA binding of the DNA of *C. fluviatile* with \(^{14}\)C-labelled rRNA from nine strains of Gram-negative bacteria are shown in Table 1. The highest value for \( T_{m(e)} \) was obtained with the rRNA of *C. violaceum*. The \( T_{m(e)} \) of the homologous DNA :rRNA hybrid of *C. violaceum* has been reported to be 80 °C (De Ley et al., 1978) and the rather low value for the heterologous hybrid between *C. fluviatile* and *C. violaceum* (71 °C) indicates that it may still be necessary to remove *C. fluviatile* from the genus *Chromobacterium* after further study. In a study of the genus *Agrobacterium*, De Smedt & De Ley (1977) reported values for \( T_{m(e)} \) of 81 °C for the homologous DNA :rRNA hybrid of *Agrobacterium tumefaciens*, 79–81 °C for heterologous hybrids within the same species and 76–78 °C for heterologous hybrids with other congeneric species.

De Ley and his colleagues present their data as scatter diagrams, or similarity maps, by plotting \( T_{m(e)} \) against % rRNA binding for each strain using a single reference strain for each map. A simplified version of such a similarity map, based on a more detailed map in De Ley et al. (1978) with the addition of *C. fluviatile*, is shown in Fig. 1. In this figure *C. fluviatile* appears to be more closely related to *C. violaceum* than is *J. lividum* and the latter is as distinct from *C. violaceum* as are *Pseudomonas testosteroni* and *Comomonas percolans*. Although only one strain of *C. fluviatile* was used, it seems likely from their phenotypic relatedness that if the DNA from several strains had been available they would have formed a small diameter cluster similar to those of other species. A detailed discussion of the significance and interpretation of these similarity maps is given by De Ley et al. (1978), but it should be noted that the distance between two points on such maps is not linearly related to taxonomic distance. Points tend to lie about the diagonal axis of the map and taxonomic distances become more compressed towards the origin of the map.

The principal components analysis of the results that could be presented by two such similarity maps (i.e. those based on *J. lividum* and *C. violaceum* as reference strains) with the additional data for the GC content of the DNA makes it possible to visualize most of this information in two dimensions (Fig. 2) using the first two principal components (or eigenvectors) which represent 84.8 % of the original data. The third principal component contributes a further 9.8 %. This reduction from five dimensions to three derived, uncorrelated dimensions implies that there was some significant correlation in the original data. Indeed, some of the variables showed significant correlation (Table 2), in particular the two sets of % rRNA binding values, probably reflecting the relatedness of the two reference strains. All of

Table 1. Hybridization of DNA from *Chromobacterium fluviatile* and \(^{14}\)C-labelled rRNA from reference strains

<table>
<thead>
<tr>
<th>Source of (^{14})C-labelled rRNA</th>
<th>( T_{m(e)} )</th>
<th>% RNA binding</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chromobacterium violaceum</em> NCTC 9757</td>
<td>71·1, 70·8</td>
<td>0·21, 0·22</td>
</tr>
<tr>
<td><em>Janthinobacterium lividum</em> NCTC 9796</td>
<td>67·6, 68·4</td>
<td>0·16, 0·20</td>
</tr>
<tr>
<td><em>Alcaligenes faecalis</em> NCIB 8156</td>
<td>65·5</td>
<td>0·16</td>
</tr>
<tr>
<td><em>Alcaligenes denitrificans</em> ATCC 15173</td>
<td>65·8</td>
<td>0·20</td>
</tr>
<tr>
<td><em>Pseudomonas acidovorans</em> ATCC 15568</td>
<td>67·5</td>
<td>0·16</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> ATCC 13525</td>
<td>62·7</td>
<td>0·17</td>
</tr>
<tr>
<td><em>Alteromonas marinopraesens</em> ATCC 143393</td>
<td>61·8</td>
<td>0·16</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em> ATCC 17802</td>
<td>62·2</td>
<td>0·11</td>
</tr>
<tr>
<td><em>Gluconobacter oxydans</em> NCIB 9013</td>
<td>59·3</td>
<td>0·16</td>
</tr>
</tbody>
</table>
Fig. 1. Similarity map of hybrids between ³H-labelled rRNA of *Chromobacterium violaceum* NCTC 9757 and DNA from a variety of bacteria. Key: ▲, *C. violaceum*; x, *C. fluviatile*; ○, *J. lividum*; ▽, *Pseudomonas testosteroni* and *Conomonas percolans*; ■, *Pseudomonas acidovorans*, *Pseudomonas solanacearum*, H₂-oxidizing pseudomonads, *Alcaligenes* and *Bordetella*; Δ, *Vibrionaceae*; ■, *Enterobacteriaceae* and *Pseudomonas fluorescens*; □, agrobacteria, rhizobia and related genera including 'Chromobacterium folium'; ▼, *Cytophaga*.

Fig. 2. Map of eigenvectors 1 and 2 from a principal components analysis of five characters of 84 strains (key as in Fig. 1).

the five original variables contribute to the first principal component; the second principal component is mainly derived from the mol % GC and the % rRNA binding for both reference strains; and the third principal component contains information from all of the $T_{m(e)}$ and the % rRNA binding measurements but not the mol % GC data. Figure 2 can be described in the following general terms: values for mol % GC and $T_{m(e)}$ increase from the bottom left to the top right of the diagram, whereas the % rRNA binding values tend to increase from the top left to the bottom right. The position of *C. fluviatile* in Fig. 2 would indicate that it is taxonomically distinct from both *C. violaceum* and *J. lividum*, but this figure also gives the impression that these two species are very closely related; however, Fig. 3 shows the result of plotting data using the first and third principal components as axes, and it can be seen that
DNA : rRNA hybridization of chromobacteria

Table 2. Correlation coefficients between the variables used in the principal components analysis

<table>
<thead>
<tr>
<th>Variable*</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.46</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-0.24</td>
<td>0.45</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.42</td>
<td>0.64</td>
<td>-</td>
<td>0.43</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>0.23</td>
<td>-</td>
<td>0.19</td>
<td>0.85</td>
<td>0.57</td>
</tr>
</tbody>
</table>

P < 0.001 \( r = 0.36 \)


they are clearly separated. It is of interest to note that Figs. 2 and 3 show a clear separation of Cytophaga which is compressed into unrelated groups close to the origin of Fig. 1.

Principal components analysis provides a way of plotting taxonomic data generated by hybridization studies in two or three dimensions as opposed to scatter diagrams of \( T_{meq} \) versus % rRNA binding for each reference strain studied. Multivariate analysis of data generated by nucleic acid hybridization studies using a variety of reference strains provides us, therefore, with another approach to the study of bacterial taxonomy.

With the separation of Janthinobacterium from Chromobacterium (De Ley et al., 1978) the latter genus is left with two fermentative, Gram-negative, violacein-producing species sharing many other phenotypic characters but differing in their response to temperature and with a large difference in the GC content of their DNA. Although the difference in GC content is greater than that normally accepted for species within a genus it seems unwise to establish a further monotypic genus of purple-pigmented bacteria for C. fluviatile at this time and it is proposed that it be retained in the genus Chromobacterium for the time being.

We thank Professor De Ley for his encouragement and for permission to use the results of his group as the database for our analysis. We also thank Professor De Ley for the provision of materials and hospitality, P. Segers for technical assistance, R. Tytgat for assistance in the determination of the GC composition, and the University of Surrey for financial assistance during the visit of M. O. Moss to the Laboratory of Microbiology and Microbial Genetics of the State University of Ghent.

REFERENCES


