Deoxyribonucleic Acid Hybridization Studies on
*Flavobacterium meningosepticum*

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Seventeen flavobacteria, including 12 strains of *Flavobacterium meningosepticum*, were investigated to determine their genetic relationships by deoxyribonucleic acid (DNA)-DNA hybridization and guanine plus cytosine content. The percentage of binding among the strains tested ranged from 6 to 87%. There was no correlation between the serotype and DNA-duplex formation. The guanine plus cytosine content of the representative strains of the five serotypes ranged from 31 to 50%.

The significance of the flavobacteria as a cause of purulent meningitis was not recognized until the late 1950s, when epidemics caused by these organisms were first reported (1, 2). King (4), at the Center for Disease Control (CDC), used serological procedures for typing strains isolated in epidemiological studies, and he classified the causative agent as *Flavobacterium meningosepticum*.

Strains isolated over a period of years were divided into six serological types, A through F (7). Cabrera and David (2) reported that *F. meningosepticum* was an opportunistic pathogen which was transmitted to highly susceptible premature infants through contaminated water supplies. The prognosis for infants with the disease was poor, whereas in adults the infection was usually mild (1, 2, 8, 9).

The purpose of this study was to determine the relationships among the recognized serotypes of *F. meningosepticum* and some related organisms. Quantitative measurements of relatedness at the molecular level were based on (i) the base composition determined by CsCl buoyant density centrifugation, and (ii) the nucleotide sequences determined by deoxyribonucleic acid (DNA) renaturation studies.

**MATERIALS AND METHODS**

**Bacteria.** Cultures of *F. meningosepticum* and related organisms were obtained from the culture collection of the Special Bacteriological Lab., CDC. They were maintained on trypticase soy agar (TSA; BBL) and stored at 4 C. The cultures used in this study are listed in Table 1.

**Standard DNA.** Samples of DNA from *Micrococcus lysodeikticus* and tritium-labeled DNA from *F. meningosepticum* strain 14, serotype A (20,000 counts per min per µg of DNA) were prepared by J. J. Brendle and M. Rogul, Walter Reed Army Institute of Research, Washington, D.C.

**DNA extraction.** All cultures were grown on TSA for 18 h at 37 C with the exception of *F. aquatilis*, which was incubated at 30 C. Growth from 30 plates was emulsified in 350 ml of a solution containing 0.15 M NaCl and 0.1 M ethylenediaminetetraacetic acid, pH 8.0. The bacteria were lysed by adding 2 ml of 25% sodium dodecyl sulphate per 25 ml of cellular suspension. Then extraction and purification were performed by the method of Marmur (5) as modified by Rogul et al. (10). The purified DNA was redissolved in a 1:10 dilution (0.1 X) of a standard saline citrate (SSC) solution consisting of 0.15 M NaCl and 0.015 M sodium citrate and was stored at 4 C under chloroform.

**Preparation of DNA-agar complex.** The labeled DNA (40 µg/ml) was sheared in a Bronson sonifier, model W 185-D, by the method of Gross and Wayne (3). Five milliliters of a solution containing approximately 1 mg of sheared DNA/ml of 0.1 X SSC was heated at 100 C for 9 min. The denatured DNA was rapidly mixed with 5 ml of molten 6% lonomag no. 2 (Oxoid) and immediately chilled. The DNA-agar complex was sieved twice through a 30 mesh wire screen and washed by filtration with 300 ml of 2 X SSC at 55 to 60 C until the agar grains separated. The preparation was stored under chloroform at 4 C. The DNA entrapped in the agar gel was assayed by dissolving a 0.1-g sample of the complex in 0.9 ml of 5 M NaClO₄ and determining the absorbancy at 260 nm in a Beckman DC spectrophotometer against a blank of 0.1 g of 0.6% agar dissolved in 0.9 ml of NaClO₄.

**DNA duplex formation.** One to two micrograms of denatured, sheared, labeled DNA in 2 X SSC was incubated with unlabeled, entrapped DNA (1:40, wt/wt) in a sealed vial at 55 C. The DNA mixtures were incubated for 20 h to allow maximum duplex formation. The contents of each vial were transferred...
to a water-jacketed column which had a sintered glass disk inserted at the base. The unbound labeled DNA was washed out by adding 2-ml volumes of 2X SSC at 5-min intervals for 1 h at 55 C. Each of the 12 fractions was collected in a scintillation vial. The temperature was lowered to 40 C, and then, at 5-min intervals for 15 min, 2-ml volumes of 0.1 X SSC were added to each of the columns. The columns were equilibrated for 8 min, and 2-ml volumes were eluted into scintillation vials. Again, 2-ml volumes of 0.1 X SSC were added to each column, and the temperature was increased by an increment of 3 C. This procedure was repeated until the temperature of the eluate was 85 C. Three 2-ml volumes of 0.1 X SSC and a final wash of 2 ml of distilled water were eluted from each column at this temperature. The scintillation mixture consisted of 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl) benzene in 1 liter of a mixture of toluene, 95% ethanol, and Triton X-100 (3:1:1.5, vol/vol/vol).

**Determination of GC content.** The guanine plus cytosine (GC) content of the DNA extracted from the six representative serotypes was determined by CsCl buoyant density centrifugation in a Beckman model E ultracentrifuge, equipped with ultraviolet optics, by the procedure of Schildkraut et al. (11). DNA from *M. lysodeikticus* was used as the reference.

**RESULTS AND DISCUSSION**

When the homologous system (labeled and unlabeled DNA of *F. meningosepticum* strain 14, serotype A) was incubated at 50, 55, 60, and 65 C to determine the temperature for optimal renaturation, data revealed that the sharpest sigmoid curve, and thus the most specific binding, was obtained by incubation at 55 C. Further evidence of the advantage of incubating at 55 C was found in the sharp elution profile of the homologous system (Fig. 1), which indicated that this temperature favored the formation of a duplex similar to the native DNA. The Tm(e) of the reference DNA was 67 C. (Tm(e) is the temperature at which 50% of the duplexes dissociated.) The experiments were repeated at least three times. The binding percentages reported were averages of these experiments, and the maximum variation for any system was 4%. The “raw” binding percentage of the homologous system was 85%.

The elution profiles of some of the heteroduplexes are shown in Fig. 2. The majority of the duplexes dissociated at elution temperatures ranging from 49 to 58 C. In no case was the profile of a heteroduplex as sharp or as narrow as that of the homologous system. The most stable heteroduplex formed at an incubation temperature of 55 C had a Tm(e) of 64 C. This duplex was formed by the DNA of strain 753, which was agglutinated by type B antiserum but was not classified biochemically as *F. meningosepticum*. The DNA of all other strains used in this study formed heteroduplexes which were considerably less stable (6 to 47% binding) than the duplex formed by the DNA of strain 753, which demonstrated 87% binding (Table 2). The DNA of *F. aquatile*, *Proteus mirabilis*,

| Table 1. Source and serotype of the strains of flavobacteria used |
|---------------------------------------------------------------|
| **Species**     | **Strain no.** | **Source of culture** | **Serotype** |
|-----------------|----------------|-----------------------|--------------|
| *F. meningosepticum* | 14*          | Spinal fluid, premature infant, died | A            |
| *F. meningosepticum* | B2628       | Tracheal suction      | A, weak F    |
| *F. meningosepticum* | 4750        | Plastic ice cube, CDC | A, weak F    |
| *F. meningosepticum* | 422*        | Blood                 | B            |
| *F. meningosepticum* | 3375*       | Spinal fluid, premature infant, died | C            |
| *F. meningosepticum* | B249        | Blood                 | C            |
| *F. meningosepticum* | 6925*       | Ceylon, infant died   | D            |
| *F. meningosepticum* | B1294       | Sputum                | D            |
| *F. meningosepticum* | 8388*       | Not known             | E            |
| *F. meningosepticum* | B1651       | Trachea               | E            |
| *F. meningosepticum* | 8707*       | Spinal fluid          | F            |
| *F. meningosepticum* | B3201       | Urine                 | F            |
| *Flavobacterium* spp. | B753       | Throat, 2-yr-old infant | B            |
| *Flavobacterium* spp. | B2103       | Sputum, autopsy       | B            |
| *Flavobacterium* spp. | A8657       | Throat                | Not typable  |
| *Flavobacterium* spp. | B7377       | Cervical swab         | Not tested   |
| *F. aquatile*     | —*          | CDC culture collection | —            |
| *Proteus mirabilis* | —           | CDC culture collection | —            |

* — Representative strain of that particular serotype.
— —, Not applicable.
The GC content of five of the representative serotypes (Table 2) ranged from 31 to 39%, whereas the GC content of strain 422, serotype B was 50%. Only two of the strains, 3375, serotype C and 8388, serotype E showed a correlation between the GC content and relative percentage of binding. In terms of base compo-

FIG. 1. Thermal elution profiles of homologous reassociated DNA from *F. meningosepticum* strain 14, serotype A, incubated at 55°C for 20 h.

and strain B 7377 did not form stable duplexes with the reference strain.

The biochemical properties of the six representative serotypes used in this study corresponded with those observed by Olsen (7). Strain 753, which showed the highest relative percentage of binding, was not classified as *F. meningosepticum* because it failed to produce acid from mannitol and lactose, reduced NO₃⁻ and NO₂⁻, and produced a yellow pigment at 37°C. Strains of *F. meningosepticum*, which had the same biochemical characteristics and serotype as the reference, strain 14, serotype A, and strains that differed only in serotype showed a considerably lower relative percentage of binding than strain 753. No correlation between the serotype of a strain and its corresponding relative percentage of binding was observed. Two strains of *F. meningosepticum*, B 3201, serotype F and 6925, serotype D, showed a lower percentage of binding than the unclassified strains A 8657 and B 2103. Although strain B 7377 is nonfermentative, it was included because it produced indol and a yellow pigment, two distinctive biochemical properties of *F. meningosepticum* (8).

FIG. 2. Thermal elution profiles of DNA heteroduplexes formed at 55°C between labeled DNA from *F. meningosepticum* and DNA from various flavobacteria.
Flavobacterium meningosepticum could not be genetically related to the other representative serotypes. Mitchell et al. (6) and Weeks (12) have shown a need for a detailed examination of the genus Flavobacterium because of the marked phenotypic diversity among its members. Certain "flavobacteria," including the type species of the genus F. aquatile, have already been classified into other genera, as further definitions of the genus have come into general use. Our data support the viewpoint that the genus Flavobacterium contains organisms which are genetically diverse. We observed a wide range of binding 6 to 47%, within those strains classified as F. meningosepticum. Only one strain, 753, could be considered to be the same species as F. meningosepticum 14 from which the labeled DNA was prepared. Interestingly, strain 753 was not identified biochemically as F. meningosepticum. Further renaturation studies and an Adansonian analysis with a wider range of strains and features would perhaps indicate more accurately the clusters of this particular group of organisms.

In certain instances, serotyping has been a useful epidemiological tool (1) in the identification of certain strains of gram-negative bacteria incriminated in specific outbreaks of infantile meningitis. However, the results of this investigation indicate that some strains of F. meningosepticum, serotypes A through F, are not closely related and probably do not belong in the same species. Although there is no cross agglutination between the six representative serotypes, cross agglutination does occur among other strains of F. meningosepticum (Weaver, unpublished results). This finding also strongly indicates that the antigenic relationships of these strains need further investigation.

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