Deoxyribonucleic Acid Reassociation in the Classification of Flavobacteria

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(Received 18 June 1975)

SUMMARY

DNA-DNA reassociation studies showed that *Flavobacterium meningosepticum* strains had a genetic relatedness of 91 to 100 % with inter-strain duplexes having high thermal stabilities. The only exception, strain NCTC10016, had an average relatedness of only 43 % to other strains of *F. meningosepticum*. The apparent divergence in DNA base sequence of this strain was reflected in the structural differences of some enzymes. There was a gradation of DNA relatedness among the *Flavobacterium* group II-b strains, but three strains were sufficiently related to constitute a species. Low levels of genetic relatedness were confirmed between *F. meningosepticum* and strains of *Flavobacterium* group II-b, group II-f, *F. aquatile*, *F. breve*, *F. heparinum*, *F. pectinovorum*, *F. odoratum* and *Moraxella saccharolytica*. All strains had base compositions in the range 32 to 46 % guanine plus cytosine. The genome sizes of representative strains of *F. meningosepticum* and *Flavobacterium* group II-b were 2.50×10^9 to 3.52×10^9 daltons, whereas the group II-f strains had smaller genomes of 1.64×10^9 to 1.81×10^9 daltons. The taxonomic implications of these findings are discussed.

INTRODUCTION

The genetic relatedness of bacteria can be estimated from the extent of DNA reassociation. The degree of base sequence divergence within related DNAs can then be calculated from the thermal stability of the DNA-DNA duplexes formed. We have used these approaches to investigate the genetic relatedness of flavobacteria difficult to classify by conventional bacteriological methods (Hendrie, Mitchell & Shewan, 1968).

Flavobacterium meningosepticum, a species that is occasionally pathogenic for man (e.g. Lapage & Owen, 1973), is similar in phenotypic characteristics and in DNA base compositions to strains of King's group II-b (Owen & Lapage, 1974), and to Weaver's unclassified group II-f (Owen & Snell, 1973). Although *F. meningosepticum* is a biochemically homogeneous species (King, 1959), Sottile, Baldwin & Weaver (1973) have shown by DNA-DNA reassociation studies that strains are genotypically heterogeneous. However, although *F. meningosepticum* has six serotypes (A to F), these workers did not find any correlation between serotype and the extent of DNA relatedness.

We now report the results of DNA-DNA reassociation studies on further strains of *F. meningosepticum*, and on other miscellaneous flavobacteria with DNA base compositions in the range 32 to 46 % guanine plus cytosine (% GC). Several described species and some strains of uncertain taxonomic position have % GC values in this range (De Ley & Van Muylem, 1963; Mitchell, Hendrie & Shewan, 1969; Ferrari & Cavazzoni, 1972; McMeekin, Stewart & Murray, 1972; Weeks, 1974). *Moraxella saccharolytica* (Flamm, 1956) was

also studied because Henriksen (1973) considered that this organism should be classified as *F. meningosepticum*.

The DNA-DNA duplexes were thermally denatured to assess the extent of base-pair imperfections within the renatured molecules, and the genome sizes of certain strains determined.

METHODS

Organisms and cultivation. The twenty-six strains used are listed in Table 1. *Flavobacterium aquatile* NCIB8694 was incubated without aeration for 5 days at 22 °C in 200 ml of a medium containing (per litre distilled water): 2 g tryptone (Difco); 0.5 g yeast extract (Difco); 0.5 g K₂HPO₄; 5 ml of 50 % (w/v) glucose (sterilized separately). The other strains were grown on 150 ml nutrient agar in Roux bottles or in 200 ml nutrient broth No. 2 medium (Oxoid) with aeration in an orbital incubator (Gallenkamp), and were cultured overnight at 37 °C, except for *F. heparinum* NCIB9290 and *F. pectinovorum* NCIB9059 which were grown at 30 °C.

To incorporate the radioactive label in their DNA, three strains (*F. meningosepticum* NCTC10016 and NCTC10585, and *Flavobacterium* group II-b NCTC10795) were grown in a medium containing: 10 ml M9 salts solution (prepared at tenfold concentration: Na₂HPO₄ 60 g; KH₂PO₄ 30 g; NaCl, 5 g; NH₄Cl, 10 g; distilled water, 1 l); 1 ml 0.1 M-MgSO₄; 1 ml 0.01 M-CaCl₂; 1 g tryptone (Difco); 2.5 ml 40 % (w/v) maltose and 200 µg thiamine (both sterilized separately); 85 ml distilled water. The strains were grown overnight at 37 °C in 5 ml medium, subcultured into 10 ml of fresh medium, and then incubated at 37 °C with aeration. Deoxyadenosine (2.5 mg) and [³H]methyl thymidine (50 µCi; The Radiochemical Centre, Amersham, Buckinghamshire) of specific activity 215 mCi/mg were added early in the exponential phase of growth, and again after 30 min and 1 h.

DNA extraction and purification. The bacteria were harvested, washed and suspended in 0.15 M-NaCl and 0.1 M-EDTA buffer pH 8.0 (5 g wet weight in 50 ml buffer), and lysed at 60 °C with sodium dodecyl sulphate (SDS) at a final concentration of 2 % (w/v). The DNA was extracted and purified by the method of Marmur (1961), supplemented by treatment with 50 µg self-digested pronase (Koch-Light)/ml for 1 h at 37 °C, and with phenol (Brenner *et al.*, 1969). The DNA from *M. saccharolytica* NCTC10753 was purified by gel permeation chromatography on a Sepharose 4B column equilibrated with buffered 2 M-NaCl (Zadrazil, Satava & Sormova, 1973), because repeated attempts to obtain fibrous DNA precipitates by the Marmur procedure with this strain were unsuccessful.

The DNA samples were finally precipitated twice with 95 % (v/v) ethanol, then with either iso-propanol or 2-ethoxyethanol. Samples were dissolved in 0.01 × SSC (standard saline citrate: 0.15 M-NaCl and 0.015 M-sodium citrate) and stored at 4 °C over chloroform. Before use, the DNA was reprecipitated with 95 % (v/v) ethanol and dissolved in 0.06 M-sodium phosphate buffer (equal molar concentrations of Na₂HPO₄ and NaH₂PO₄) at a concentration of 300 to 350 µg/ml. The DNA purity was assessed from the 280:260 nm extinction ratio (Marmur, 1961).

In the preparation of ³H-labelled DNA, cultures in the late-exponential phase of growth were harvested by centrifuging, washed with 0.15 M-NaCl and 0.1 M-EDTA buffer at pH 8.0, and lysed with SDS at a final concentration of 0.1 % (w/v). The suspensions were heated at 60 °C for 2 to 3 min to assist lysis, and the lysates sonicated (MSE ultrasonic disintegrator, 100 W model) for 30 s at 22 °C to reduce their viscosity. The DNA was purified by CsCl density gradient centrifugation at 90000g (18 °C, 60 h) in a fixed angle rotor in a MSE Super-speed 65 centrifuge. At the end of the run each tube was pierced and eight drop fractions

Table 1. *Bacterial strains used and their DNA base compositions*

Species and strain designations*	Base composition† (% GC)
NCTC10016 <i>Flavobacterium meningosepticum</i> serotype A (ATCC13253; strain 14)	37·6 (1), 38·3 (2), 35·5 (3)
NCTC10585 <i>F. meningosepticum</i> serotype B (ATCC13254; strain 422)	36·8 (1), 36·4 (2), 50·0 (3)
NCTC10586 <i>F. meningosepticum</i> serotype C (ATCC13255; strain 3375)	37·2 (1), 36·4 (2), 37·5 (3)
NCTC10587 <i>F. meningosepticum</i> serotype D (strain 6925)	36·8 (1), 38·0 (3)
NCTC10588 <i>F. meningosepticum</i> serotype E (strain 8388)	36·1 (1), 39·0 (3)
NCTC10589 <i>F. meningosepticum</i> serotype F (strain 8707)	37·0 (1), 31·6 (3)
F123 <i>F. meningosepticum</i> serotype A	37·4 (4)
F134 <i>F. meningosepticum</i> serotype B	37·4 (4)
NCTC10795 <i>Flavobacterium</i> group II-b	38·5 (1)
NCTC10796 <i>Flavobacterium</i> group II-b	39·4
NCTC10797 <i>Flavobacterium</i> group II-b	36·1
F48 <i>Flavobacterium</i> group II-b	36·2 (1)
F93 <i>Flavobacterium</i> group II-b	37·0 (1)
F101 <i>Flavobacterium</i> group II-b	37·9 (1)
F129 <i>Flavobacterium</i> group II-b	36·0 (1)
F131 <i>Flavobacterium</i> group II-b	38·9
F139 <i>Flavobacterium</i> group II-b	38·8
NCTC10798 Unnamed group II-f	37·8 (5)
NCTC10799 Unnamed group II-f	37·2 (5)
NCTC10800 Unnamed group II-f	37·2 (5)
NCIB8694 <i>F. aquatile</i>	32·0 (6)
NCIB9059 <i>F. pectinovorum</i>	34·9, 34·2 (7)
NCIB9290 <i>F. heparinum</i>	44·7, 45·6 (7)
ATCC14234 <i>F. breve</i>	33·7, 26·0 (6)
ATCC4651 <i>F. odoratum</i>	36·7
NCTC10753 <i>Moraxella saccharolytica</i>	38·3 (8)
NCTC10537 <i>Escherichia coli</i> B (DNA control strain)	51·5

* Abbreviations: NCTC, National Collection of Type Cultures, London; NCIB, National Collection of Industrial Bacteria, Aberdeen; ATCC, American Type Culture Collection, Rockville, U.S.A.; F, laboratory designation of field isolate.

† For values cited in the literature, references are given in parentheses: (1) Owen & Lapage (1974); (2) Mitchell, Hendrie & Shewan (1969); (3) Sottile, Baldwin & Weaver (1973); (4) Lapage & Owen (1973); (5) Owen & Snell (1973); (6) Weeks (1974); (7) Perry (1973); (8) Snell, Hill & Lapage (1972).

were collected. The fractions that contained DNA, identified by the ^3H -label, were pooled and dialysed for 1 h at 4 °C against distilled water, then overnight at 4 °C against 0·14 M-sodium phosphate buffer.

Shearing of DNA and molecular weight estimation. The solutions of DNA, at a concentration of 300 to 400 µg/ml in 0·06 M-sodium phosphate buffer, were sonicated (MSE ultrasonic disintegrator) in 3 ml volumes for two periods of 1 min at room temperature. Molecular weights of sheared DNA were determined from band sedimentation through 1 M-NaCl and 0·05 M- Na_2HPO_4 , pH 7, in a MSE Centriscan analytical ultracentrifuge. The sedimentation coefficients were measured at 60000 g and 20 °C, corrected to $s_{20,w}^\circ$ at pH 7, and the molecular weights calculated according to Studier (1965).

DNA reassociation. About 150 to 200 µg of unlabelled, sheared DNA in 0·06 M-sodium phosphate buffer were mixed with 0·05 µg of sheared ^3H -labelled DNA. The ratio of the concentrations of unlabelled to labelled DNA was approximately 3000:1. The DNA mixture was denatured at 95 to 100 °C for 15 min and the buffer concentration adjusted to 0·28 M with 0·5 M-sodium phosphate buffer. The reassociation reaction was allowed to proceed to 100 c_0t for the unlabelled DNA in a 20 h period of incubation, where c_0t is the acronym for the product of the initial DNA concentration (c_0) and the incubation time (t) (Britten &

Kohne, 1966). Chemical units are mols/l. The incubation temperatures selected are described in Results. The methods described below refer to an incubation temperature of 65 °C.

Duplex fractionation and binding assay. The hydroxyapatite (HA) procedure of Brenner *et al.* (1969) was used to fractionate the unbound single stranded DNA fragments from the reassociated DNA duplexes. Eight samples were run simultaneously. The DNA mixtures were adjusted to 0.14 M-sodium phosphate buffer and each was added to 10 ml suspension of hydroxyapatite (Bio-Gel HT; Bio-Rad Laboratories), mixed well, and equilibrated at 65 °C for 10 min. The volume was increased to 20 ml and the mixture again thoroughly mixed. The samples were centrifuged at 60 to 65 °C and the aqueous supernatants collected. The samples were then washed twice with 20 ml portions of 0.14 M-sodium phosphate buffer at 65 °C to remove all the unbound single strand DNA. The three supernatants from each sample were pooled.

Samples not required for thermal analysis were washed three times with 20 ml portions of 0.4 M-sodium phosphate buffer at 65 °C to elute the double stranded DNA from the HA. The supernatants were collected by centrifuging and pooled.

Yeast RNA (200 µg) was added as carrier to each of the pooled supernatants, and the DNA precipitated with 5 % (w/v) trichloroacetic acid (TCA) at 4 °C. The nucleic acid precipitates were collected by suction filtration on Whatman GF/B discs (2.5 cm diameter) and washed with 2.5 % (w/v) TCA followed by 95 % (v/v) ethanol. The discs were dried for 30 min in an hot air oven and suspended in 5 ml scintillation fluid, containing: 2,5-diphenyl-oxazole, 4 g; 1,4 bis-[2-(4 methyl-5-phenyloxazolyl)]-benzene, 0.5 g; toluene, 1 l. They were then counted repeatedly in a Packard Tri-Carb model 2002 liquid scintillation spectrometer.

The observed homologous binding for each [³H]DNA was arbitrarily designated 100 % and the reassociation values of the heterologous DNA reactions were determined relative to the binding values of the homologous DNA.

Thermal stability of duplexes. The DNA duplexes bound to the HA, after removal of the single strand DNA fragments in 0.14 M-sodium phosphate buffer, were analysed by thermal denaturation. Samples were washed with 20 ml volumes of 0.14 M-sodium phosphate buffer at temperature increments of 5 °C over the range 65 to 95 °C in a water bath, being equilibrated at each temperature for 10 min with mixing at 5 min intervals. The supernatants were collected by centrifuging at 65 °C to prevent reassociation of the denatured DNA fragments and possible non-specific binding to the HA. The samples were finally washed with 20 ml 0.4 M-sodium phosphate buffer and boiled to remove any DNA duplexes that had not denatured. The DNA in each sample was precipitated with 5 % (w/v) TCA and the radioactivity measured as before. The duplex melting temperature ($T_{m(e)}$) was calculated as the temperature at which 50 % of the DNA fragments had dissociated and eluted from the HA. The temperature range of melting (ΔT) and the ranges of melting on the right (high temperature) side (ΔT_r) and on the left (low temperature) side (ΔT_l) of the duplex melting temperature were calculated according to De Ley (1969). The ratio ($\Delta T_r:\Delta T_l$) is a measure of the symmetry of the duplex melting profile.

Optimum renaturation temperature. The optimum renaturation temperature (T_{or}) was calculated from the equation of Gillis, De Ley & De Cleene (1970):

$$T_{or} = 0.51 \times \% GC + 47.0.$$

Estimation of base composition. The mean base composition of the DNA was estimated from its melting temperature (T_m), which was determined by the method of Marmur & Doty (1962). Thermal denaturation (melting) was carried out in a Pye Unicam SP 1800

spectrophotometer equipped with a SP 876 series 2 temperature programme controller and heated cell block. The extinction changes at 260 nm were recorded on a Pye Unicam AR 25 linear recorder. The temperature of the DNA solution was measured with a thermistor thermometer as described by Hill (1968). The DNA mean base composition, expressed as percentage guanine and cytosine to total bases (% GC), was calculated from the T_m determined in SSC buffer using Marmur & Doty's (1962) equation:

$$\% \text{ GC} = 2.44 T_m - 169.0.$$

Spectrophotometric determination of genome size. The genome size (molecular complexity) of the DNA was estimated by the renaturation rate method of Gillis *et al.* (1970). Sonicated, denatured DNA at a concentration of 35 $\mu\text{g/ml}$ in $2 \times \text{SSC}$ buffer was renatured in a spectrophotometer (see above) for periods of 40 min at the temperature of optimum renaturation. Three separate determinations were made on each strain. The apparent renaturation rate constant (k') was calculated from the equation $k' = v/c^2$, where v is the initial renaturation rate expressed as decrease in extinction at 260 nm/min, and c represents the DNA concentration expressed as mmol nucleotide pairs/l. The concentrations of the DNA samples were assayed by the chemical method of Burton (1956) with 2-deoxy-D-ribose as the standard.

The renaturation rate constant is inversely proportional to molecular complexity (Britten & Kohne, 1966), so genome sizes were calculated relative to a reference DNA (*Escherichia coli* B) with an average molecular weight, obtained from chemical determinations, of 2.88×10^9 daltons (Gillis *et al.*, 1970).

RESULTS

Yields of labelled DNA

The radioactive label ($[^3\text{H}]$ methyl thymidine) was incorporated in the DNA of the three reference strains (NCTC10016, NCTC10585 and NCTC10795). The use of preparative ultracentrifugation in the isolation and purification of the labelled DNA proved to be economical in both time and the amount of label used. Each 10 ml of lysate yielded approximately 30 μg of highly pure labelled DNA, with a specific activity of 1.5×10^4 to 7.0×10^4 counts/min/ μg .

Size of DNA fragments

The sonication procedure yielded DNA fragments estimated from band sedimentation to have molecular weights of 2×10^5 to 3×10^5 daltons. These results are in agreement with the values for sonically-fragmented DNA estimated from electron microscopy by Grindley, Humphreys & Anderson (1973).

Effect of temperature on reassociation of homologous DNA

Kinetic studies on DNA reassociation have shown that the specificity of base pairing and the extent of renaturation of homologous DNA depend primarily on the incubation temperature, when other factors such as DNA concentration, salt concentration of buffer, incubation time and fragment size do not limit the reaction (Britten & Kohne, 1966; Wetmur & Davidson, 1968; De Ley *et al.*, 1973). There is no direct method to calculate the most suitable temperature at which to estimate DNA relatedness, so the effect of temperature on the percentage binding of a homologous DNA system was first determined. The $[^3\text{H}]$ DNA of NCTC10016 was reassociated with homologous unlabelled DNA fragments at the following renaturation temperatures; 55, 60, 65, 70 and 75 °C. The binding values ranged from 89 to 94 % and were therefore almost independent of temperature over the range selected. The

Fig. 1. Thermal denaturation curves of DNA-DNA duplexes. The sheared denatured [^3H]DNA ($0.05 \mu\text{g}$) was reassociated with excess unlabelled, denatured DNA fragments (150 to $200 \mu\text{g}$) in 0.28 M -sodium phosphate buffer at 65°C for 20 h . The duplexes were fractionated from un-reassociated DNA and thermally denatured in 0.14 M -sodium phosphate buffer as described in the text. (a) Duplexes of *F. meningosepticum* NCTC10016 [^3H]DNA with: (●) unlabelled NCTC10016 DNA and (○) unlabelled DNA from *F. meningosepticum* NCTC10585. (b) Duplexes of *F. meningosepticum* NCTC10585 [^3H]DNA with: (●) unlabelled NCTC10585 DNA and (○) unlabelled NCTC10016 DNA. (c) Duplexes of *Flavobacterium* group II-b NCTC10795 [^3H]DNA with: (●) unlabelled NCTC10795 DNA and (○) unlabelled DNA from *Flavobacterium* group II-b F101.

renaturation temperature of 65°C used in all further reassociations, corresponded to the predicted temperature of optimum renaturation for a DNA of 35.3% GC in a buffer of approximately 0.4 M -sodium ion concentration. The effect of lowering the renaturation temperature to 55°C was investigated for some strains that had low binding values at 65°C , to determine if less stringent conditions would permit reassociation of more distantly related nucleotide sequences.

Reassociation and duplex characteristics of reference DNAs

The average percentage reassociations at 65°C for the three reference [^3H]DNAs in homologous systems were: 91% for NCTC10016, 97% for NCTC10585 and 94% for NCTC10795. The homoduplexes of these three DNAs had sharp, sigmoid-shaped melting profiles (Fig. 1) and thermal stability ranges which were similar to those of the high molecular weight native DNA obtained from the same strains (Owen & Lapage, 1974). The homoduplexes of NCTC10016 formed at 55 and 65°C had identical melting profile characteristics, with less than 5% of the reassociated molecules consisting of fragments with low thermal stability. In the absence of excess unlabelled DNA, reassociation of each of the three labelled DNAs with itself was 5% or lower.

*Reassociation between [^3H]DNA and *F. meningosepticum* strains*

Table 2 lists the percentage relative binding and duplex characteristics of reassociations between the three reference [^3H]DNAs and DNA from the eight strains of *F. meningosepticum*. Labelled DNA from NCTC10585 bound at high levels (91 to 95%) with DNA from all the *F. meningosepticum* strains except NCTC10016, to which it was only 43% reassociated. The heteroduplexes of the six strains that reassociated to 90% or more had melting profiles in the same temperature range as the NCTC10585 homoduplex (Fig. 1b). Well matched base sequences in the reassociated molecules were indicated by high thermal stabilities with $\Delta T_{m(e)}$ values of 2°C or lower ($\Delta T_{m(e)}$ designates the difference between the $T_{m(e)}$ of the homoduplex and that of the heteroduplex), by the narrow temperature ranges of melting, and by the symmetrical shapes of the profiles, which were similar to those of the homoduplex

Table 2. Binding between reference [^3H]DNAs and *F. meningosepticum* DNA and characteristics of duplexes

Source of unlabelled DNA	Reference [³ H]DNA										Flavobacterium group II-b NCTC10795 Percentage relative binding at 65 °C
	F. meningosepticum NCTC10585					F. meningosepticum NCTC10016					
	Serotype	Percentage relative binding at 65 °C	$\Delta T_{m(e)}$ (°C)	ΔT (°C)	$\Delta T_r/\Delta T_i$	Percentage relative binding at:		$\Delta T_{m(e)}$ (°C)	ΔT (°C)	$\Delta T_r/\Delta T_i$	
						55 °C	65 °C				
<i>F. meningosepticum</i>											
NCTC10585	B	100	—	8.2	0.86	44	65	10.5	16.5	1.90	21
NCTC10587	D	95	0.5	8.5	0.93	NT	34	11.5	15.0	1.58	10
NCTC10589	F	93	1.0	8.9	1.23	NT	29	11.0	19.4	2.03	16
FI34	B	93	2.0	9.4	1.14	NT	35	10.5	14.5	1.50	19
NCTC10586	C	92	1.0	8.6	1.15	NT	33	8.0	19.5	1.38	22
NCTC10588	E	92	2.0	8.6	1.46	33	32	11.0	21.5	2.53	15
FI23	A	91	1.5	9.4	1.09	NT	37	11.5	12.5	1.08	16
NCTC10016	A	43	12.0	14.5	1.59	100	100	—	9.0	0.92	12

$T_{m(e)}$, thermal elution midpoint, i.e. that temperature at which 50 % of the DNA bound to the hydroxyapatite at the 55 or 65 °C incubation temperature eluted; $\Delta T_{m(e)}$, the difference in $T_{m(e)}$ between the heterologous DNA reaction and the homologous reaction. ΔT , temperature range of melting; $\Delta T_r/\Delta T_i$, symmetry of melting profile, i.e. the ratio of the range of melting on the right (high temperature) side of $T_{m(e)}$ to the range of melting on the left (low temperature) side of the $T_{m(e)}$; NT, not tested.

profiles. In contrast, the NCTC10016:NCTC10585 ^3H -heteroduplex molecules contained re-natured regions with a higher degree of base mismatching, as shown by the parabolic shape of the melting curve (Fig. 1*b*), the decreased thermal stability, the broader range of melting, and the asymmetrically shaped profile. In the reciprocal reaction between NCTC10016 [^3H]DNA and unlabelled DNA of NCTC10585, the mean binding was 65 % at 65 °C and 44 % at 55 °C. The characteristics of the heteroduplexes formed at these two temperatures were unchanged, except that the melting temperature of the duplex formed at 55 °C was approximately 1 °C lower. The reproducibility of binding between NCTC10016 and NCTC10585 was poor (± 15 %) compared with the values of ± 5 % for reactions between most other DNAs. A reason for less reproducibility could be that partially-related DNA fragments renature to varying degrees if the reassociation reaction on repetition is initiated at different positions along the fragments, with the formation of different amounts of free ends and open loops in the duplex molecules.

The relative binding between NCTC1006 [^3H]DNA and DNA of the six other *F. meningosepticum* strains ranged between 29 and 37 % (Table 2). The $\Delta T_{m(e)}$ values of 8 to 11.5 °C and the other melting profile characteristics indicated significant levels of base mismatching within the heteroduplex molecules.

The labelled DNA from NCTC10795 *Flavobacterium* group II-b was bound only at low levels (10 to 22 %) with DNA of the eight *F. meningosepticum* strains (Table 2).

The number of DNA fragments denatured at each 5 °C increment during the thermal analysis of *F. meningosepticum* duplexes is illustrated in Fig. 2. In three heteroduplexes ([^3H]DNA of NCTC10016:NCTC10585, of NCTC10016:NCTC10587 and of NCTC10585:NCTC10016) most of the label was bound in a labile, easily-denatured form. The characteristics of these duplexes suggested they probably consisted of relatively unspecific base paired regions, interspersed within regions of well matched bases.

Reassociation between [^3H]DNA and Flavobacterium group II-b strains

[^3H]DNA of NCTC10795, the selected reference strain of *Flavobacterium* group II-b, reassociated to a high level and formed stable duplexes with two other strains (F93 and F129) of group II-b but only reassociated at values ranging from 7 to 47 % with the other six strains of the group (Table 3). The heteroduplexes of the latter had lower melting temperatures and broader melting profiles (Fig. 1*c*) than the NCTC10795 homoduplex. The heteroduplexes with strains F131 and F139 (47 % reassociated) contained a higher proportion of label bound in the form of stable duplexes than did F101 and NCTC10796 (30 % reassociated), which had most of the label bound in a labile form (Fig. 3).

The levels of binding at 65 °C were low between the [^3H]DNA of the two *F. meningosepticum* reference strains and the eight *Flavobacterium* group II-b strains (Table 3). For the reaction between NCTC10795 and NCTC10016 [^3H]DNA, a less stringent renaturation temperature (55 °C) resulted in 17 % more binding. Thermal analysis of the DNA duplexes revealed that the increase in binding was due to the formation of labile molecules with thermal stabilities below 65 °C.

Reassociation of [^3H]DNA and miscellaneous flavobacteria

The three reference [^3H]DNAs had binding levels of 28 % or less at 65 °C with the strains of group II-f, *F. aquatile*, *F. heparinum*, *F. pectinovorum*, *F. breve*, *F. odoratum* and *Moraxella saccharolytica* (Table 4). The duplexes between these strains were not investigated further, as the amount of label bound was low and it was difficult to obtain reproducible melting profiles. The group II-f strain NCTC10799, *F. heparinum* and *F. odoratum* were also

Fig. 2

Fig. 3

Fig. 2. Differential denaturation profiles of *F. meningosepticum* DNA-DNA duplexes. For methods see Fig. 1. Duplexes of *F. meningosepticum* NCTC10585 [^3H]DNA and unlabelled DNA from: (a) NCTC10585 (homoduplex), (b) *F. meningosepticum* F123, and (c) *F. meningosepticum* NCTC10016. Duplexes of *F. meningosepticum* NCTC10016 [^3H]DNA and unlabelled DNA from: (d) NCTC10016 (homoduplex), (e) *F. meningosepticum* NCTC10585, and (f) *F. meningosepticum* NCTC10587.

Fig. 3. Differential denaturation profiles of the DNA-DNA duplexes of strains of *Flavobacterium* group II-b. For methods see Figs. 1. The labelled DNA in each duplex was from NCTC10795. The unlabelled DNA was from: (a) NCTC10795 (homoduplex), (b) F131, (c) F101, (d) F129, (e) F139, and (f) NCTC10796.

reassociated with NCTC10016 [^3H]DNA at 55 °C, but the levels of binding obtained at this less stringent reassociation temperature were not significantly different from the 65 °C values.

Bacterial genome sizes

Genome size estimations are summarized in Table 5. The renaturation rates were not corrected for the % GC content of the DNA as described by Gillis *et al.* (1970), as other evidence for this effect is contradictory (Wetmur & Davidson, 1968). Christiansen, Christiansen & Bak (1973) found that the renaturation rate of T4 phage DNA was influenced by the degree of glucosylation of the bases rather than by % GC content, and they concluded that a correction for the latter was unnecessary.

The three strains of *F. meningosepticum* (NCTC10016, NCTC10585 and NCTC10589) and the three strains of *Flavobacterium* group II-b (NCTC10795, F48 and F139) had genome sizes between 2.50×10^9 and 3.52×10^9 daltons. The close agreement between the values of the reciprocal binding reactions of NCTC10016, NCTC10585 and NCTC10795 provided further evidence that these strains had approximately the same molecular complexity. The genome sizes of the group II-f strains (NCTC10799 and NCTC10800) of 1.64×10^9 to 1.81×10^9 daltons, were approximately 40 % smaller than the values of the flavobacteria strains.

Table 3. Binding between reference [^3H]DNAs and *Flavobacterium* group II-b DNA and characteristics of duplexes

Source of unlabelled DNA	Reference [^3H]DNA									
	<i>Flavobacterium</i> group II-b NCTC10795					<i>F. meningosepticum</i> NCTC10016				
	Percentage relative binding at 65 °C	$\Delta T_{m(e)}$ (°C)	ΔT (°C)	$\Delta T_r/\Delta T_i$	Percentage relative binding at 55 °C	Percentage relative binding at 65 °C	$\Delta T_{m(e)}$ at 55 °C (°C)	ΔT (°C)	$\Delta T_r/\Delta T_i$	<i>F. meningosepticum</i> NCTC10585 Percentage relative binding at 65 °C
<i>Flavobacterium</i> group II-b										
NCTC10795	100	—	8.8	0.91	27	10	18.3	24.7	2.29	24
F93	100	0	8.7	0.85	NT	15	NT	—	—	0
F129	99	0	8.8	0.91	NT	9	NT	—	—	19
F131	46	8.5	14.8	1.39	NT	25	NT	—	—	18
F139	47	10.5	17.3	1.54	NT	8	NT	—	—	0
F101	30	12.0	17.0	1.83	NT	11	NT	—	—	18
NCTC10796	28	10.4	13.8	2.00	NT	19	NT	—	—	23
NCTC10797	19	NT	—	—	NT	7	NT	—	—	16
F48	7	NT	—	—	NT	1	NT	—	—	25

For abbreviations, see Table 2.

Table 4. Binding between reference [³H]DNAs and miscellaneous flavobacteria

Source of unlabelled DNA	Relative binding of reference [³ H]DNA (%)			
	<i>F. meningosepticum</i> NCTC10016 at		<i>F. meningosepticum</i> NCTC10585 at 65 °C	<i>Flavobacterium</i> group II-b NCTC10795 at 65 °C
	65 °C	55 °C		
NCTC10798 II-f group	17	NT	4	6
NCTC10799 II-f group	6	7	7	18
NCTC10800 II-f group	16	NT	21	25
NCIB8694 <i>F. aquatile</i>	3	NT	0	12
ATCC14234 <i>F. breve</i>	7	NT	0	2
NCIB9290 <i>F. heparinum</i>	25	20	28	18
NCIB9059 <i>F. pectinovorum</i>	14	NT	4	7
ATCC4651 <i>F. odoratum</i>	3	0	20	13
NCTC10753 <i>M. saccharolytica</i>	11	NT	19	1

NT, not tested.

Table 5. Spectrophotometric estimates of genome sizes

Strain	Optimum renaturation temperature* (°C)	10 ⁻⁹ × Genome size† and s.d. (daltons)	Genome size relative to NCTC10585 (%)
<i>Flavobacterium meningosepticum</i>			
NCTC10585	65.9	2.85 ± 0.10 (2)	100
NCTC10016	65.9	2.50 ± 0.14 (4)	88
NCTC10589	65.9	2.85 ± 0.12 (3)	100
<i>Flavobacterium</i> group II-b			
NCTC10795	66.6	3.52 ± 0.25 (3)	124
F48	65.7	3.07 ± 0.38 (3)	108
F139	66.8	2.85 ± 0.06 (3)	100
<i>Flavobacterium</i> group II-f			
NCTC10799	65.9	1.81 ± 0.08 (3)	64
NCTC10800	65.9	1.64 ± 0.06 (3)	58

* Calculated from the formula $T_{or} = 0.51 \times \%GC + 47.0$ (Gillis *et al.*, 1970).† The genome size of a prokaryotic organism is the amount of chromosomal DNA it contains, expressed in molecular weight units (daltons). The apparent reaction rate constant (k') was determined in 2 × SSC buffer as described in the text. Genome size was calculated from the formula:

$$\text{Genome size} = \frac{k' (E. coli B) \times 2.88 \times 10^9}{k' (\text{unknown})}.$$

Numbers in parentheses are the numbers of determinations.

DISCUSSION

In previous studies (King, 1959; Owen & Lapage, 1974), *F. meningosepticum* formed a homogeneous species with respect to phenotypic characteristics and DNA base composition but contained strains divisible into six serological types. The close relationship among the strains used, except for NCTC10016, is confirmed by the DNA-DNA reassociation results with labelled DNA from *F. meningosepticum* NCTC10585. The seven strains previously classified as *F. meningosepticum* on biochemical and serological grounds formed a highly interrelated genotypic group. Between strains, the standard deviation of $\Delta T_{m(e)}$ was less than 1.5 °C. Sottile *et al.* (1973) also found that NCTC10016 (King's strain 14) was apparently

genetically atypical of *F. meningosepticum*. They used the DNA-agar method, with reassociations carried out in $2 \times \text{SSC}$ buffer at 55°C (a non-exacting reassociation temperature); and, with the same strains as investigated here, obtained an average of 26 % relative binding over a range of 6 to 47 % binding for labelled DNA from King's strain 14. The small differences in results between laboratories can be attributed to the variations in reassociation techniques and conditions used. However, there are significant differences between the base composition values estimated for some strains (see Table 1), particularly the value of 50 % GC reported by Sottile *et al.* (1973) for NCTC10585 (King's strain 422). This value was estimated from measurement of its buoyant density in CsCl, whereas estimates from melting temperature were appreciably lower at 36.4 to 36.8 % GC (Mitchell *et al.*, 1969; Owen & Lapage, 1974). The presence of glycosylated or unusual bases, such as substituted cytosines, decreases the buoyant density of DNA in CsCl (Szybalski, 1968) and may also influence the reassociation rate of DNA (Christiansen *et al.*, 1973). However, there is no evidence from our reassociation results on NCTC10585 to suggest that this strain contains a significant amount of unusual bases in its DNA.

The DNA sequences common to NCTC10016 and the other strains of *F. meningosepticum* (except for NCTC10585 which had 10 % higher relatedness) constitute on average 33 % of the genome, or 1.3×10^6 base pairs, as the genome size of NCTC10016 is 2.50×10^9 daltons. The factor used for the calculation of base sequence or evolutionary divergence from DNA-DNA duplex melting profiles is a decrease in $T_{m(e)}$ of 1.6°C per 1.0 % unpaired or mismatched bases (McCarthy & Farquhar, 1972); therefore within the reassociated regions of the NCTC10016 heteroduplexes there were approximately 6.6 % or 8.6×10^4 mismatched base pairs. The apparent evolutionary divergence in the DNA base sequence of NCTC10016 is not reflected in the phenotypic data, as this strain showed no significant biochemical differences from others in the species (King, 1959). However, comparative zymograms (Snell, unpublished results) indicate that NCTC10016 has structural differences in several protein molecules. The catalase, malate dehydrogenase and glutamate dehydrogenase bands of NCTC10016 had marked differences in electrophoretic mobilities from those of five other *F. meningosepticum* strains. The results on NCTC10016 could, alternatively, be attributed to evolutionary convergence, when the high level of phenotypic similarity would be consistent with a lower level of genetic relatedness. The possibility cannot be overlooked, however, that the unexpectedly low reassociation levels of NCTC10016 in heterologous *F. meningosepticum* DNA reactions is due to some structural modification of the bases, as discussed above, or to the presence of closely bound nucleoprotein, which may also reduce the rate of DNA reassociation.

Within *Flavobacterium* group II-b the strains show a gradation of DNA relatedness which is reflected in the heterogeneity of their biochemical characteristics (Owen & Lapage, 1974). The high level of genetic relatedness of three strains (NCTC10795, F93 and F129) provides evidence for at least one distinct species within group II-b. The other strains had a greater divergence in base sequence and the possibility of correlations between the level of DNA relatedness within the group and specific biochemical characteristics is currently under investigation.

In earlier studies on the unnamed group II-f (Owen & Snell, 1973), we concluded that insufficient phenotypic evidence was available to classify the strains either in *Flavobacterium* or in *Moraxella*. Even if the smaller size of the group II-f genome is taken into account, the average relatedness of this group to *F. meningosepticum* is still low (about 20 to 30 %). Similar genome size does not imply a taxonomic relationship, but it is of interest that the group II-f genome sizes of 1.64×10^9 to 1.81×10^9 daltons, correspond closely to the

1.20×10^9 to 1.70×10^9 dalton size range for species of moraxellas (Snell, unpublished results). *Moraxella saccharolytica*, an organism isolated from a case of meningitis in a child (Flamm, 1956), was considered by H. Lautrop (personal communication to S. D. Henriksen, 1973) to belong to *F. meningosepticum*. However, we found insufficient DNA relatedness between this organism and strains of *F. meningosepticum* to support this proposal. The problems encountered in the preparation of DNA from this strain also suggest it is different from the DNA of *F. meningosepticum*. Unfortunately, insufficient DNA was available for its genome size to be estimated.

We have found that DNA-DNA reassociation is a valuable technique for elucidating the relatedness of strains within a species, where high (70 % or greater) levels of binding are to be expected (Brenner, 1973; Johnson, 1973). However, since it was difficult to assign the strains showing lower levels of reassociation to any particular taxonomic rank, no firm conclusions could be drawn about the evolutionary relatedness among the various species and strains of flavobacteria investigated. Bonner *et al.* (1973) found that base sequence divergence has a significant effect upon the rate of reassociation, so reaction conditions optimized for the homologous DNA may not be appropriate for the heterologous DNA reactions. In such cases, it is important that further studies be carried out to determine the experimental conditions that will ensure the reaction is essentially complete, otherwise the extent of relatedness may be under-estimated.

We thank Dr G. O. Humphreys of the Enteric Reference Laboratory for his assistance in preparing the labelled DNA and for his many useful suggestions. We also thank Dr S. P. Lapage, Curator of the National Collection of Type Cultures, for his support and advice, and the Public Health Laboratory Service which made this work possible.

REFERENCES

- BONNER, T. I., BRENNER, D. J., NEUFELD, B. R. & BRITTEN, R. J. (1973). Reduction in the rate of DNA reassociation by sequence divergence. *Journal of Molecular Biology* **81**, 123-135.
- BRENNER, D. J. (1973). Deoxyribonucleic acid reassociation in the taxonomy of enteric bacteria. *International Journal of Systematic Bacteriology* **23**, 298-307.
- BRENNER, D. J., FANNING, G. R., RAKE, A. V. & JOHNSON, K. E. (1969). Batch procedure for thermal elution of DNA from hydroxyapatite. *Analytical Biochemistry* **28**, 447-459.
- BRITTEN, R. J. & KOHNE, D. E. (1966). Nucleotide sequence repetition in DNA. *Yearbook of the Carnegie Institution, Washington* **65**, 78-106.
- BURTON, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochemical Journal* **62**, 315-323.
- CHRISTIANSEN, C., CHRISTIANSEN, G. & BAK, A. L. (1973). The influence of glucosylation on the renaturation rate of T4 phage DNA. *Biochemical and Biophysical Research Communications* **52**, 1426-1433.
- DE LEY, J. (1969). Compositional nucleotide distribution and the theoretical prediction of homology in bacterial DNA. *Journal of Theoretical Biology* **22**, 89-116.
- DE LEY, J., TIJTGAT, R., DE SMEDT, J. & MICHELS, M. (1973). Thermal stability of DNA:DNA hybrids within the genus *Agrobacterium*. *Journal of General Microbiology* **78**, 241-252.
- DE LEY, J. & VAN MUYLEM, J. (1963). Some applications of deoxyribonucleic acid base composition in bacterial taxonomy. *Antonie van Leeuwenhoek* **29**, 344-358.
- FERRARI, A. & CAVAZZONI, V. (1972). Composizione in basi dell'acido desossiribonucleico di ceppi di flavobatteri. *Annali di Microbiologia ed Enzimologia* **22**, 137-143.
- FLAMM, H. (1956). *Moraxella saccharolytica* (sp.n) aus dem Liquor eines Kindes mit Meningitis. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene Abteilung (I)* **168**, 261-267.
- GILLIS, M., DE LEY, J. & DE CLEENE, M. (1970). The determination of molecular weight of bacterial genome DNA from renaturation rates. *European Journal of Biochemistry* **12**, 143-153.
- GRINDLEY, N. D. F., HUMPHREYS, G. O. & ANDERSON, E. S. (1973). Molecular studies of R factor compatibility groups. *Journal of Bacteriology* **115**, 387-398.
- HENDRIE, M. S., MITCHELL, T. G. & SHEWAN, J. M. (1968). The identification of yellow pigmented rods. In *Identification Methods for Microbiologists*, B, pp. 67-78. Edited by B. M. Gibbs and D. A. Shapton. London: Academic Press.

- HENRIKSEN, S. D. (1973). *Moraxella*, *Acinetobacter*, and the *Mimeae*. *Bacteriological Reviews* **37**, 522-561.
- HILL, L. R. (1968). The determination of deoxyribonucleic acid base compositions and its application to bacterial taxonomy. In *Identification Methods for Microbiologists, B*, pp. 177-186. Edited by B. M. Gibbs and D. A. Shapton. London: Academic Press.
- JOHNSON, J. L. (1973). Use of nucleic-acid homologies in the taxonomy of anaerobic bacteria. *International Journal of Systematic Bacteriology* **23**, 308-315.
- KING, E. O. (1959). Studies on a group of previously unclassified bacteria associated with meningitis in infants. *American Journal of Clinical Pathology* **31**, 241-247.
- LAPAGE, S. P. & OWEN, R. J. (1973). *Flavobacterium meningosepticum* from cases of meningitis in Botswana and England. *Journal of Clinical Pathology* **26**, 747-749.
- MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *Journal of Molecular Biology* **3**, 208-218.
- MARMUR, J. & DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *Journal of Molecular Biology* **5**, 109-118.
- MCCARTHY, B. J. & FARQUHAR, M. N. (1972). The rate of change of DNA in evolution. *Brookhaven Symposia in Biology* **23**, 1-41.
- MCMECKIN, T. A., STEWART, D. B. & MURRAY, J. G. (1972). The Adansonian taxonomy and the deoxyribonucleic acid base composition of some Gram negative, yellow pigmented rods. *Journal of Applied Bacteriology* **35**, 129-137.
- MITCHELL, T. G., HENDRIE, M. S. & SHEWAN, J. M. (1969). The taxonomy, differentiation and identification of *Cytophaga* species. *Journal of Applied Bacteriology* **32**, 40-50.
- OWEN, R. J. & LAPAGE, S. P. (1974). A comparison of strains of King's group IIb of *Flavobacterium* (with *Flavobacterium meningosepticum*). *Antonie van Leeuwenhoek* **40**, 255-264.
- OWEN, R. J. & SNELL, J. J. S. (1973). Comparison of group IIc with *Flavobacterium* and *Moraxella*. *Antonie van Leeuwenhoek* **39**, 473-480.
- PERRY, L. B. (1973). Gliding motility in some non-spreading flexi-bacteria. *Journal of Applied Bacteriology* **36**, 227-232.
- SNELL, J. J. S., HILL, L. R. & LAPAGE, S. P. (1972). Identification and characterization of *Moraxella phenylpyruvica*. *Journal of Clinical Pathology* **25**, 959-965.
- SOTTILE, M. I., BALDWIN, J. N. & WEAVER, R. E. (1973). Deoxyribonucleic acid hybridization studies on *Flavobacterium meningosepticum*. *Applied Microbiology* **26**, 535-539.
- STUDIER, F. W. (1965). Sedimentation studies of the size and shape of DNA. *Journal of Molecular Biology* **11**, 373-390.
- SZYBALSKI, W. (1968). Use of cesium sulfate for equilibrium density gradient centrifugation. In *Methods in Enzymology*, vol. 12B, *Nucleic Acids*, pp. 330-360. Edited by L. Grossman and K. Moldave. London: Academic Press.
- WEEKS, O. B. (1974). Genus *Flavobacterium* Bergey et al. 1923. In *Bergey's Manual of Determinative Bacteriology*, 8th edn, pp. 357-364. Edited by R. E. Buchanan and N. E. Gibbons. Baltimore: Williams and Wilkins.
- WETMUR, J. G. & DAVIDSON, N. (1968). Kinetics of renaturation of DNA. *Journal of Molecular Biology* **31**, 349-370.
- ZADRAZIL, S., SATAVA, J. & SORMOVA, Z. (1973). Isolation procedure for bacterial DNA based on gel permeation chromatography on a sepharose column. *Journal of Chromatography* **91**, 451-458.