Thermal Stability of Interspecies *Neisseria* DNA Duplexes

By D. T. KINGSBURY,* G. R. FANNING, K. E. JOHNSON
AND D. J. BRENNER

*Department of Microbiology, Naval Medical Research Institute,
Bethesda, Md. 20014, and Division of Biochemistry, Walter Reed Army
Institute of Research, Washington, D.C. 20012, U.S.A.*

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SUMMARY

The thermal stability of interspecies DNA duplexes is markedly increased by raising the incubation temperature. When the DNA reassociation reactions are carried out at 75° in 0.12 M-phosphate buffer the thermal denaturation temperature of the reassociated product is almost identical to that of the native DNA, indicating that only DNA segments of very similar nucleotide sequence are associating. The genus *Neisseria* very clearly forms three groups based on the relatedness of their DNA to that of *N. meningitidis*; the 'pathogenic' *Neisseria* which have at least 80% of their nucleotide sequences similar; the 'non-pathogenic' *Neisseria* which share only 8 to 15%; and *N. catarrhalis* which shows no relatedness.

INTRODUCTION

The usefulness of DNA-DNA hybridization procedures in studying taxonomic relationships has been clearly demonstrated in many groups of bacteria (McCarthy & Bolton, 1963; Brenner, Martin & Hoyer, 1967; Kingsbury, 1967; Heberlein, De Ley & Tijtgat, 1967; Hoyer & McCullough, 1968; Johnson & Ordal, 1968). Recently, thermal stability has been used to investigate the extent of base pairing within reassociated interspecies polynucleotide sequences (Brenner & Cowie, 1967, 1968). The importance of incubation temperature in discriminating between distantly and closely related DNA sequences has been shown in several laboratories (Martin & Hoyer, 1966; Brenner *et al.* 1967; Brenner & Cowie, 1967, 1968; Johnson & Ordal, 1968). The experiments reported here utilized increased incubation temperature and studies of thermal stability to further assess previously determined relationships within the genus *Neisseria* (Kingsbury, 1967).

DNA relationships among the *Neisseria* are of particular interest since both pathogens and non-pathogens, as well as several tentatively assigned organisms, are included in this genus (Kingsbury & Ivler, to be published). Earlier work (Kingsbury, 1967) indicated that the *Neisseria* species form at least three distinct groups: the 'pathogenic' *Neisseria*, the 'non-pathogenic' *Neisseria* and the species *N. catarrhalis* and *N. caviae*. A similar view, based on genetic transformation studies in this genus, has been advanced by Henriksen & Bøvre (1968). The present study, using *N. meningitidis* as the reference organism, reaffirms this division of the genus *Neisseria* into three groups. Furthermore, the extensive duplex formation and the stability of reassociated nucleo-

* Present address: Department of Biology, University of California (San Diego), La Jolla, California 92037.

tide sequences shared between *N. meningitidis*, *N. gonorrhoeae* and strain z (Slaterus, 1961) are indicative of extensive conservation of nucleotide sequences in the DNA of these pathogens.

METHODS

Organisms. The source of each strain used in this study and the procedures used for identification and cultivation have been described (Kingsbury, 1966, 1967).

Enzymes and radioisotopes. Ribonuclease and Pronase were purchased from Calbiochem, Los Angeles, California, U.S.A. The Pronase, in distilled water, was self-digested by incubation for 2 hr at 37° prior to use. Ribonuclease was heated at 90° for 10 min. to inactivate any deoxyribonuclease present as a contaminant. Adenine-8-¹⁴C was purchased from Calbiochem. ³²P in the form of carrier-free H₂PO₄ was obtained from New England Nuclear Corporation, Boston, Massachusetts, U.S.A.

DNA extraction. Organisms were harvested from broth by centrifugation and washed in 150 ml of 0.15 M-NaCl + 0.1 M-EDTA, pH 8.0. Organisms were lysed and DNA prepared as previously described (Brenner & Cowie, 1968). The crude DNA preparations were repeatedly (3 to 4 times) precipitated with cold 95% ethanol and resuspended in SSC/100 (SSC = 0.15 M-NaCl + 0.015 M-sodium citrate; 3 × SSC is used to designate a threefold concentration). The DNA preparations were resuspended in a mixture of 0.1 M-NaCl, 0.05 M-EDTA and 0.5 M-tris buffer, and incubated with ribonuclease 25 µg./ml. at 37° for 60 min. Sodium lauryl sulphate was added to 0.5% and the DNA was incubated overnight at 37° with 50 µg/ml of Pronase. The concentration of sodium lauryl sulphate was increased to 1% and an equal volume of water-washed phenol was added. The mixture was shaken and centrifuged. The aqueous phase was collected and shaken twice with an equal volume of chloroform. The aqueous phase was then repeatedly (3 to 4 times) precipitated with 2 vol. of 2-ethoxyethanol. The DNA was finally resuspended in 0.12 M-PB (PB = a buffer solution consisting of equimolar Na₂HPO₄ and NaH₂PO₄, pH = 6.8).

DNA samples (except those unlabelled samples which were subsequently bound to nitrocellulose filters) were fragmented by mechanical shear at 50,000 p.s.i. to a molecular weight of approximately 2×10^5 (Britten & Kohne, 1966) and filtered through Metrical filter discs (0.45 µ pore size, Gelman Instrument Company, Ann Arbor, Michigan, U.S.A.). Labelled single-stranded DNA fragments in 0.12 M-PB were further purified by passing them through a hydroxyapatite (Bio-Gel HT, Bio-rad Laboratories, Richmond, California, U.S.A.) column at 60° and discarding the material that bound to the column. (Single-stranded DNA does not bind to the column under these conditions.)

Preparation of labelled DNA. ¹⁴C-labelled DNA was prepared as previously described (Kingsbury & Duncan, 1967). ³²P labelling was accomplished by growing the bacteria in Frantz broth containing 5 µC/ml. of carrier-free H₂PO₄ and 0.001 M-unlabelled phosphate.

Formation of DNA hybrids on nitrocellulose filters. The hybridization procedures used for nitrocellulose filter reactions have been described (Kingsbury, 1967). In the present studies only direct binding experiments were carried out. In each case 125 µg. of DNA was immobilized on a 25 mm. B-6 filter (Schleicher and Schuell Company, Keene, New Hampshire, U.S.A.) and incubated with 1 µg. of ¹⁴C-labelled DNA fragments.

Thermal elution of reassociated DNA duplexes from hydroxyapatite. A modification of the technique of Miyazawa & Thomas (1965) for fractionating double-stranded

DNA bound to hydroxyapatite was used to determine the stability of reassociated *Neisseria* DNA (Britten & Kohne, 1966). Thermally denatured ^{32}P -labelled *N. meningitidis* DNA fragments ($0.1\text{ }\mu\text{g.}$, containing 2000 to 5000 counts/min.) were incubated at 60° or 75° with an approximate 5000-fold excess of homologous or heterologous denatured, unlabelled DNA fragments in 1 ml. of 0.12 M-PB . The duration of incubation (12 hr) was chosen to insure maximal reassociation of the unlabelled DNA fragments. The 'zero-time' binding (the amount of labelled DNA bound to hydroxyapatite immediately after denaturation) was between 1 and 2%. The control values obtained for reassociation of labelled fragments with one another during the 12 hr incubation period were between 2 and 4%.

Following incubation, samples were quickly cooled and frozen until use. Each sample was subsequently thawed and passed through 10 ml. of hydroxyapatite equilibrated with 0.12 M-PB at the temperature at which the fragments had been incubated. The earlier hydroxyapatite experiments used water-jacketed columns. Later experiments employed a batch procedure for thermal elution which allowed six or more samples to be processed simultaneously. In the batch procedure reaction mixtures were added to 10 ml. of hydroxyapatite contained in test tubes in a circulating water bath. Elutions were accomplished by sedimenting the tubes in a heated centrifuge. The details of the batch procedure are given elsewhere (Brenner, Fanning, Rake & Johnson, private communication). Virtually identical results were obtained with either the column or batch procedure. Each sample was washed with 15 ml. portions of 0.12 M-PB until the eluted radioactivity (representing unreassociated DNA not bound to hydroxyapatite) was down to a background level (5–6 washes). The hydroxyapatite-bound DNA was then consecutively washed with 15 ml. portions of 0.12 M-PB at increasing 2.5° temperature increments to 100° and finally with 2–3, 15 ml. portions of 0.4 M-PB to elute any material remaining bound to the column. When the elution temperature was raised above the dissociation temperature of the reassociated nucleotide sequences, the resultant single-stranded DNA was eluted from the column.

The contents of the elution tubes were precipitated in 5% trichloroacetic acid in the presence of approximately $100\text{ }\mu\text{g.}$ of yeast ribonucleic acid carrier. The precipitates were collected on membrane filters, dried, and placed in counting vials. Scintillation fluid was added (15 ml. per sample) and the samples were counted in either a Packard (Packard Instrument Company, Downers Gove, Illinois, U.S.A.) or a Nuclear Chicago (Nuclear-Chicago Corporation, Des Plaines, Illinois, U.S.A.) liquid scintillation spectrometer. Percentage reassociation was determined by dividing the counts present in the thermal elution by the total counts.

RESULTS

Effect of temperature on DNA–DNA reassociation. The effect of different incubation temperatures on interspecies DNA reassociation is shown in Table 1. When the incubation temperature was raised from 60° to 75° , the reassociation of homologous *Neisseria meningitidis* DNA dropped some 5–8% (the term reassociation is applied to both the homologous and heterologous reactions although the interspecies strands had never before been associated). The relative ability of *N. meningitidis* DNA fragments to reassociate with DNA from *N. gonorrhoeae* or strain z was only slightly diminished at the more stringent 75° incubation temperature. (Relative binding is used to compare the interspecies reactions with the homologous reaction under the identical

incubation conditions. In every case, the homologous *N. meningitidis* reaction is arbitrarily designated 100%.) Relatedness between *N. meningitidis* and *N. flava* was diminished more than twofold and between *N. meningitidis* and *N. sicca* was diminished more than fivefold when the criterion was changed to the high temperature of incubation. There was very little relatedness between *N. meningitidis* and *N. catarrhalis* at 60°. At 75°, no reaction was detectible between the DNA of these organisms.

As the ionic strength was raised, the effective temperature of incubation was lowered. Sodium ion concentration was similar in 0.12 M-PB and SSC. The three-fold increase in ionic strength from 0.12 M-PB to 3 × SSC resulted in a 5 to 6° decrease in effective incubation temperature (Marmur & Doty, 1962). Therefore, when the incubation is done at 67° in 3 × SSC, the extent of the reaction should be similar to that occurring at 60° in PB. The data presented in the last column of Table 1 show this to be true.

Table 1. *Relatedness of the DNA extracted from Neisseria species*

Source of unlabelled DNA	Relative relatedness to labelled <i>N. meningitidis</i> DNA*				
	60° Incubation (0.12 M-PB)		75° Incubation (0.12 M-PB)		67° Incubation (3 × SSC)
	%	Tm†	%	Tm	%
<i>N. meningitidis</i>	(100)‡	87	(100)	88	(100)
<i>N. gonorrhoeae</i>	78	85	76	86	80
Strain z	88	87	81	87	89
<i>N. sicca</i>	45	77	8	86	45
<i>N. flava</i>	35	75	15	86	30
<i>N. catarrhalis</i>	10	79	0	—	15
<i>E. coli</i>	0	—	0	—	0

* Average of 2 to 4 experimental values. The 60° and 75° incubations in 0.12 M-PB were carried out in free solution and assayed on hydroxyapatite. The reactions at 67° in 3 × SSC utilized unlabelled DNA immobilized on 25 mm. nitrocellulose filters.

† Tm = temperature at which 50% of the DNA is denatured.

‡ Homologous *N. meningitidis* reassociation was arbitrarily designated 100%. Relatedness is expressed as % relative to the homologous DNA reaction. The homologous reaction at 60° in PB gave approximately 85% reassociation; at 75° in PB, 75–80%; and 67° in 3 × SSC, approximately 60%.

Little or no difference was observable in the thermal elution midpoint (Tm) obtained from reaction products obtained at 60° or 75° involving homologous *Neisseria meningitidis* DNA fragments. Similarly, thermal elution mid-points from reactions involving the closely related DNA of *N. meningitidis*, *N. gonorrhoeae* and strain z were close to identical at both incubation temperatures. Alternatively, the marked decreases in duplex formation in *N. meningitidis*–*N. sicca* and *N. meningitidis*–*N. flava* reactions at 75° was accompanied by marked increases in the corresponding elution mid-points.

Relationships between Neisseria meningitidis and the other Neisseria species. It is clear from Table 1 that these *Neisseria* species fall into three main groups on the basis of DNA relatedness. First are the species normally associated with disease, *N. meningitidis*, *N. gonorrhoeae*, and strain z. The second group contains species generally considered 'non-pathogenic' represented by *N. sicca* and *N. flava*. The third group has little relationship to the other *Neisseria* tested and is represented in this study by *N. catarrhalis*.

The specificity of the DNA reassociation reaction was clearly increased at the higher incubation temperature. Figure 1 shows thermal elution profiles of interspecies DNA

Fig. 1 (A). Thermal elution profiles of reassociated DNA duplexes formed between pathogenic *Neisseria* at 60°. 0.1 µg samples of single-stranded, ^{32}P -labelled *Neisseria meningitidis* DNA fragments were incubated with approximately 500 µg. of unlabelled single-stranded DNA from *N. meningitidis*, *N. gonorrhoeae* or strain Z. (B). Thermal elution profiles of reassociated DNA duplexes formed between pathogenic *Neisseria* at 75°. Reactions are identical to those in (A) except for incubation temperature.

Fig. 2. (A) Thermal elution profiles of reassociated DNA duplexes formed between *Neisseria meningitidis* and non-pathogenic *Neisseria* species at 60°. 0.1 µg. samples of single-stranded, ^{32}P -labelled *N. meningitidis* DNA fragments were incubated with approximately 500 µg. of unlabelled, single-stranded DNA from *N. flava*, *N. sicca* and *N. catarrhalis*. (B) Thermal elution profiles of reassociated DNA duplexes formed between *Neisseria meningitidis* and non-pathogenic *Neisseria* species at 75°. Reactions are identical to those in (A) except for incubation temperature. No observable reaction took place between *N. meningitidis* and *N. catarrhalis* at 75° (see Table 1).

duplexes from group 1 when the incubations were carried out at 60° (Fig. 1*a*) and 75° (Fig. 1*b*) in 0.12 M-PB. The profiles are sharp and tend towards a Gaussian distribution at both incubation temperatures. In both cases, the T_m of these interspecies DNA duplexes was within one or two degrees of that exhibited by a homologous *N. meningitidis* reaction. (T_m = temperature of which 50% of the DNA is denatured.)

In contrast, Fig. 2 shows thermal elution profiles obtained from reactions between *N. meningitidis* and members of groups 2 and 3. The profiles from the 60° (Fig. 2*a*) incubation were very broad and the T_m was depressed as much as 13° below that of reassociated *N. meningitidis* DNA. The greater specificity of the duplexes formed at 75° (Fig. 2*b*) is shown by the comparative sharpness and the greatly increased thermal stability of profiles obtained from reactions carried out at the higher temperature. Where no interspecies polynucleotide sequences exist with a stability approaching that of *N. meningitidis* DNA, as is apparently the case with *N. catarrhalis*, no DNA duplexes were detectable by the more selective criterion.

To insure reaction specificity, a control DNA from *Escherichia coli* was included in each of these experiments. Although its base composition is similar to that of *N. meningitidis*, 50 mole % guanine + cytosine (G + C), no relationship was detected between the DNA of *N. meningitidis* and *E. coli*, at any of the three incubation temperatures used.

DISCUSSION

The optimal temperature for reassociation of denatured DNA free in solution is some 25° below its T_m (Marmur & Doty, 1962). The T_m for *Neisseria meningitidis* DNA is about 88° in 0.12 M-PB and about 93° in 3 × SSC. Therefore, reactions carried out at 60° in 0.12 M-PB and at 67° in 3 × SSC closely approximate optimal conditions for reassociation. A 75° incubation temperature for reactions in 0.12 M-PB constitutes a highly stringent criterion for reassociation in the *Neisseria*, as evidenced by a 5–8% decrease in homologous *N. meningitidis* DNA duplex formation. The use of stringent incubation conditions allows the formation of only those interspecies DNA duplexes that exhibit a high degree of thermal stability. It seems likely that these stable interspecies duplexes reflect extensive base sequence similarity between organisms. Similarly, duplexes that exhibit a T_m 10° to 25° below the T_m of homologous *N. meningitidis* DNA (Fig. 2) and which are not formed at 75° may be assumed to contain a significant proportion of unpaired nucleotide bases.

An alternative explanation for decreased stability in interspecies DNA duplexes is that the related sequences contain a preferentially high percentage of adenine (A) + thymine (T) base pairs. This explanation is considered unlikely because bacterial DNA usually shows a sharp and unimodal thermal melting transition indicative of a Gaussian distribution of G + C centred around the mean base composition. In the *N. meningitidis*–*N. sicca* reaction (Table 1), and in several interspecies enterobacterial DNA reactions (Brenner & Cowie, 1968; D. J. Brenner & S. Falkow, unpublished observations), at least 75% of the duplexes (corresponding to up to 30% of the genome) formed at 60° are lost in a 75° reaction. It is doubtful whether bacterial DNA with a mean 50 mole % G + C contains such a large fraction of A + T-rich sequences.

This argument does not rule out a combination of unpaired bases and preferentially high A + T containing duplexes as causative factors in reducing the T_m . It has been reported (Brenner & Cowie, 1968) that at least half of the diminished stability in

Escherichia coli-*Salmonella typhimurium* duplexes is attributable to unpaired bases. Preliminary results using labelled *E. coli* DNA fractionated according to base composition and reacted with *S. typhimurium* DNA give no indication of increased interspecies reaction when labelled A+T-rich fragments are used (D. J. Brenner, unpublished observation). This question remains to be answered directly for the *Neisseria* reactions.

The existence of three subgroups within the genus *Neisseria*, based on DNA relatedness, as suggested earlier (Kingsbury, 1967), is firmly supported by the present data on interspecies duplex stability. The pathogenic species tested are very closely related and form stable interspecies hybrids. Reciprocal experiments using labelled strain z DNA and unlabelled *N. meningitidis* also give an approximate value of 90% relative relatedness (67° , $3 \times \text{SSC}$) (D. T. Kingsbury, unpublished observation). The coincidence of the reciprocal values rules out the possibility that strain z is a deletion mutant of *N. meningitidis*. Hoyer & McCullough (1968) noted even higher relatedness in *Brucella* species. It is tempting to speculate that the apparent conservation of nucleotide sequences seen in both of these pathogenic genera is the result of selection pressures resulting from obligate host-parasite interaction.

The degree of relatedness among the bulk of non-pathogenic *Neisseria* species is not nearly as high as that of the pathogens (Kingsbury, 1967). The stability patterns in non-pathogenic interspecies duplexes remain to be investigated. The low degree of relatedness and lack of stable interspecies duplexes between *Neisseria catarrhalis* and *N. meningitidis* is not surprising in view of the low G+C of *N. catarrhalis*. Catlin & Cunningham (1961) found *N. catarrhalis* strains with G+C percentages ranging from 40.7 to 44.6. These authors suggested that the *N. catarrhalis* strains formed a group unto themselves.

The recent work of Henriksen & Bøvre (1968), suggesting that *Neisseria meningitidis*, *N. gonorrhoeae*, *N. sicca* and *N. flava* may be variants of the same species, is not supported by DNA reassociation data.

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