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Taxonomy of the Neisseriae: Deoxyribonucleic Acid Base Composition, Interspecific Transformation, and Deoxyribonucleic Acid Hybridization

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Deoxyribonucleic acid (DNA) base composition, intergenic transformation efficiency, and DNA hybridization were used to determine the relatedness of a variety of established or proposed species of *Neisseria* and *Branhamella*. These studies indicated that these bacteria form three genetic groupings. Group I, comprised of *N. meningitidis, N. gonorrhoeae, N. subflava, N. flava, N. perflava, N. sicca, N. mucosa, N. cinerea, N. flavescens, N. lactamica, N. elongata, N. canis*, and *N. denitrificans*, was characterized by DNA base compositions ranging between 49.3 and 55.6 mol% guanine plus cytosine. Group II, comprised of *N. cuniculi, N. caviae*, and *N. ovis*, was characterized by DNA base compositions ranging between 45.3 and 47.3 mol% guanine plus cytosine. Group III, comprised of one species, *B. catarrhalis*, was characterized by DNA base compositions between 41 and 42 mol% guanine plus cytosine. Transformation and DNA hybridization results revealed that members of each group, with few exceptions, exhibited high DNA homology with other members of the same group but most often distinctly lower levels of homology with members of a different group. These data suggest that *N. ovis, N. caviae*, and *N. cuniculi* may be significantly different from other neisseriae and from branhamellae to warrant their separation in a distinct genus.

The deoxyribonucleic acid (DNA) base compositions of several *Neisseria* and *Branhamella* species have been reported to range between 40 and 53 mol% guanine plus cytosine (G+C) (5, 6, 8, 18, 19, 26, 30, 31, 34). Because different methods have been used, there have been variations in the G+C values reported, and not all *Neisseria* species have been examined.

Transformation has played an important role in revealing taxonomic relationships of certain species of *Haemophilus* (20, 21), *Streptococcus* (28, 29), *Bacillus* (27), and *Neisseria* (8; B. W. Catlin, Bacteriol. Proc., p. 74, 1960, and p. 90, 1961). The studies on *Neisseria* supported the separation of *N. catarrhalis* into a new genus, *Branhamella*, and the incorporation of *N. flava, N. perflava*, and *N. subflava* into a single species (*N. subflava*). The absence of transformation between selected *Neisseria* species and the positive transformation between asaccharolytic neisseriae and moraxellae have also supported changes in the family *Neisseriaceae* (1, 2).

The results of nucleic acid hybridizations performed by Kingsbury (17) revealed that the genus *Neisseria* was a heterogeneous group comprised of at least three distinct subgroups and questioned the proper classification of *N. catarrhalis* and *N. caviae* as members of the genus *Neisseria*. On the basis of nucleic acid hybridization studies, Bøvre reported that *N. ovis, N. caviae*, and *B. catarrhalis* showed distinct degrees of relatedness to several *Moraxella* species (3, 4). This supported the inclusion of *Moraxella* in the family *Neisseriaceae*.

The purpose of this study was to use DNA base composition determinations, transformation, and DNA hybridization studies to clarify the taxonomic positions of recognized or proposed species of *Neisseria* and *Branhamella*. Special attention was focused on evaluating the potential of transformation as a toxonomic tool for determining the relatedness of members of these genera.

MATERIALS AND METHODS

The bacterial strains examined are listed in Table 1. Stock cultures were preserved by freeze-drying. Working cultures were maintained at −70°C in Trypticase soy broth (BBL Microbiology Systems) supplemented with 6% lactose. Cultures were routinely passaged on GC agar consisting of GC medium base (Difco Laboratories) and 1% (vol/vol) chemically defined supplements (36) and were incubated at 36°C for 18 h (5% CO_2, humidity).

Preparation of DNA for base composition determinations. A modification of the procedure originally described by Marmur (25) was followed for the extrac-

TABLE 1. List of microorganisms used in this study

Laboratory strain no.	Species	Source[a]
M1803	*B. catarrhalis*	ATCC 25238[b] (1)
M1835	*B. catarrhalis*	Human nasopharynx (2)
M637	*N. canis*	ATCC 14678[b] (1)
M597	*N. caviae*	ATCC 14659[b] (1)
M601	*N. cinerea*	ATCC 14685[b] (1)
M635	*N. cuniculi*	ATCC 14688[b] (1)
CPH21	*N. cuniculi*	Alice Reyn (3)
M598	*N. denitrificans*	ATCC 14686[b] (1)
M1558	*N. elongata*	ATCC 25295[b] (1)
M953	*N. flava*	ATCC 14221 (1)
F62	*N. gonorrhoeae*	D. Kellogg (4)
M1589	*N. gonorrhoeae*	ATCC 27631 (1)
M1802	*N. lactamica*	ATCC 23970[b] (1)
M893	*N. lactamica*	CDC A2894 (5)
M1723	*N. lactamica*	G. Taunay (6)
Ne15	*N. meningitidis*	B. W. Catlin (7)
M628	*N. meningitidis*	ATCC 13077[b] (1)
M630	*N. meningitidis*	ATCC 13102 (1)
M1801	*N. mucosa*	ATCC 19696[b] (1)
M599	*N. mucosa*	ATCC 19693 (1)
CPH12	*N. mucosa*	Alice Reyn (3)
M1770	*N. ovis*	ATCC 19575 (1)
M1804	*N. perflava*	ATCC 10555[a] (1)
Ne16	*N. perflava*	B. W. Catlin (7)
M1827	*N. perflava*	Human nasopharynx (8)
M472	*N. sicca*	NRL 30016 (9)
M600	*N. subflava*	ATCC 19243 (1)

[a] (1) American Type Culture Collection, Rockville, Md. (2) Public Health Laboratory, San Francisco, Calif. (3) Ulrich Berger, Hygiene-Institut der Universität, Heidelberg, Germany. (4) Thomas Maier, Public Health Laboratory, San Luis Obispo, Calif. (5) Communicable Disease Centers, Atlanta, Ga. (6) G. Taunay, Sao Paulo, Brazil. (7) Medical College of Wisconsin, Milwaukee, Wis. (8) Our own laboratory. (9) *Neisseria* Reference Laboratory, U.S. Public Health Service Hospital, Seattle, Wash.

tion of DNA. In this modification, the DNA was extracted from cells grown in GC broth (prepared by using the formula of GC agar with the omission of starch and agar) or in a GC biphasic medium (1 part GC broth overlaid on 4 parts GC agar) and incubated at 36°C for 18 h (air) on a rotary shaker (200 rpm; New Brunswick Scientific Co., New Brunswick, N.J.). The procedure was further modified to include additional ribonuclease and protease treatment of the crude DNA as follows. (i) Ribonucleic acid in the cell lysate, after treatment with lauryl sulfate, was digested at 37°C for 60 min by the addition of 50 μg of ribonuclease (Sigma Chemical Co.) per ml, which was preheated at 80°C for 10 min to destroy deoxyribonuclease, and (ii) excess protein in the cell lysate was digested at 37°C for 45 min by the addition of 2 mg of protease (Sigma) per ml. The crude DNA was then collected by precipitation with 2 volumes of chilled 100% ethanol. The DNA extraction then followed Marmur's procedure at the step in which the crude DNA is treated with sodium perchlorate. The purified DNA was stored in standard saline citrate (SSC: 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0) at 4°C at a concentration between 0.5 and 1.5 mg per ml in the presence of a few drops of chloroform. The DNA could be held in this manner for at least 6 months before performance of melting-point studies. Immediately before determinations of thermal melting point (T_m), the DNA was reprecipitated in 100% ethanol, washed progressively in 70, 80, 90, and 95% ethanol, and dissolved in 0.1 × SSC.

Before the T_m determinations were made, the purity of the DNA was checked on eight representative samples. The DNA concentration was determined by the diphenylamine reaction (7), with calf thymus DNA (Sigma) as a standard. Protein determinations on the DNA samples were performed by the method described by Lowry et al. (22). All samples contained less than 2% protein. To confirm purity, the hyperchromicities of all DNA samples were measured after thermal denaturation.

Determination of the DNA base composition. The T_m determinations were made with a Beckman DU spectrophotometer equipped with a Gilford thermoprogrammer 2527 and thermal cuvettes. DNA was diluted to approximately 20 μg/ml in one lot of 0.1 × SSC buffer. The temperature of the DNA was raised at a rate of 0.25°C per min, and the absorbance at 260 nm was recorded on a Moseley X-Y recorder (Hewlett Packard Co.). A buffer blank was run in parallel with each DNA sample to correct for optical density changes due to solvent expansion. The T_m was determined as the temperature at the midpoint of the absorbance rise. The equation used to relate T_m to moles percent G+C was that used by Snell and Lapage (34):

$$\text{mol\% G+C} = \text{mol\% G+C of reference strain} + \text{slope of equation} \times (T_m \text{ of unknown} - T_m \text{ of reference strain})$$

For comparison, the T_m determinations were adjusted to the equivalent values in SSC by means of the correction described by Mandel and Marmur (24), where T_m (1 × SSC) = T_m (0.1 × SSC) + 15.4°C. The value 2.44, determined both by Marmur and Doty (25) and by De Ley (9), was used for the slope of the equation. *Escherichia coli* B was chosen as the reference organism. The value 50.0, previously derived by T_m determination (26), was the value used for the moles percent G+C of the reference strain. The value 91.3 was used as the T_m of the reference strain and was derived by averaging 17 T_m determinations of *E. coli* B DNA (Sigma) under test conditions. Substituting these values in the equation gives:

$$\text{mol\% G+C} = 50.0 + 2.44 \times (T_m \text{ unknown} - 91.3)$$

The average T_m from a minimum of three separate determinations was used as the T_m of the unknown. To ensure repoducibility of results and to correct for minor differences in technique, chemical supplies, or other test conditions, the DNA from the reference strain, *E. coli* B, was run in parallel with each sample. For the unknown T_m to be considered valid, the T_m of the reference DNA had to be in good correlation (±0.5%) with its predetermined value. To control for any significant variation in T_m caused by the DNA extraction procedure, the DNA from *E. coli* ATCC 25922 was extracted in parallel with those of the test organisms, and its T_m was compared with that of the commercially prepared *E. coli* B DNA (Sigma). The

T_m for the DNA extracted from *E. coli* ATCC 25922 was 91.3°C and was identical to that determined for the DNA of *E. coli* B (Sigma).

Selection of streptomycin-resistant mutants. A heavy suspension of organisms containing approximately 10^9 colony-forming units per ml was swabbed liberally in a confluent manner on GC agar containing 1,000 μg of streptomycin (streptomycin sulfate, USP, Eli Lilly Co.) per ml. The plates were incubated for 75 h (36°C, 5% CO_2). Isolated colonies were picked and subcultured three times on plain GC agar and GC agar containing 1,000 μg of streptomycin per ml to confirm that a stable streptomycin-resistant, nondependent mutant had been selected.

Preparation of DNA for transformation experiments. DNA was prepared from the streptomycin-resistant organisms by the technique described by Maier, Zubrzycki, and Coyle (23). The procedure was modified slightly to include a 15-min incubation of the cell lysate with ribonuclease A (Sigma), 50 μg/ml (37°C, water bath), prior to its treatment with protease (Sigma). The final concentration of DNA was quantitated by the diphenylamine reaction (7).

Standard transformation procedure. Transformation procedures were performed in a manner slightly modified from that described by Maier, Zubrzycki, and Coyle (23). Eighteen-hour-old cultures of the recipient organisms were suspended at a concentration of approximately 3×10^8 colony-forming units per ml in GC broth containing 2 mM $MgCl_2$ and were exposed to the action of a Vortex mixer to minimize cell clumping. Virulent-type colonies were selected for studies evaluating *N. gonorrhoeae* as a recipient organism. To a tube containing 1.8 ml of the organism suspension was added 0.2 ml of DNA solution to make a final concentration of 1 to 2 μg/ml. This concentration of DNA was considered to be optimal on the basis of DNA dilution experiments performed on representative organisms. The mixture was incubated in a water bath at 37°C for 30 min. Deoxyribonuclease (Sigma) was added to the mixture at a final concentration of 25 μg/ml (to destroy unbound DNA), and after 2 min the mixture was serially diluted in GC broth. Portions (0.1 ml) of the appropriate dilution were spread onto each of three plates containing 20 ml of antibiotic-free GC agar. As controls for each experiment, a suspension of cells not treated with DNA was plated from the same dilutions to determine the number of spontaneous streptomycin-resistant mutant colony-forming units. The plates were incubated for 5 to 6 h (36°C, CO_2) to allow phenotypic expression of antibiotic resistance. The agar containing the microcolonies was layered on top of 20 ml of GC agar containing 1,000 μg of streptomycin per ml. The double-layered agar plates were incubated for 72 h (36°C, 5% CO_2). At this time, the streptomycin-resistant transformants were counted, and the average count of the three plates was used for determination of all colony counts. The number of colony-forming units exposed to DNA was determined from plate counts of the same dilutions of cell suspension that were plated on plain GC agar only.

DNA hybridization. The extraction of DNA was similar to the procedures described above except that Trypticase soy broth was used instead of GC broth to grow all organisms except *N. gonorrhoeae*. The extracted DNA was diluted to an optical density of 2.0 (260 nm) in 0.1 × SSC (approximately 100 μg/ml) and was sheared at 21,000 lb/in^2 in a Ribi cell fractionator (model RF-1; Ivan Sorvall, Inc., Norwalk, Conn.). This automated, temperature-controlled French pressure cell was the most effective of several procedures tried in producing homogeneous DNA fragments. Homogeneity was determined by analytical zone centrifugation (the majority of DNA fragments were approximately 400,000 daltons).

Renaturation rates were determined for hybridization by the technique of De Ley et al. (10). The optimal renaturation rate temperature was based on the base composition of each isolate and was calculated by the formula of De Ley (11). The average was 74°C, and this temperature was used in all experiments. Renaturation rates were recorded for 30 min on a Gilford spectrophotometer (model 252) with thermal programmer (model 2527; Gilford Instruments, Oberlin, Ohio). The blank in the thermal cuvettes consisted of a guanine solution (optical density, 2.0; 260 nm), and the instrument was set at a dwell time of 4 s with a chart speed of 0.5 cm/min.

RESULTS

DNA base composition. The DNA base compositions of the 16 established or proposed species of *Neisseria* and *Branhamella* included in this study are listed in Table 2 and are compared with the values previously reported for the same species. As depicted in Fig. 1, the G+C values appear to cluster in three distinct groups. The majority of the species examined, including *N. meningitidis, N. gonorrhoeae, N. subflava, N. flava, N. perflava, N. sicca, N. mucosa, N. cinerea, N. flavescens, N. lactamica, N. elongata, N. canis*, and *N. denitrificans*, was characterized by G+C values of 49.3 to 55.6 mol%. Two strains of *B. catarrhalis* formed a second group characterized by distinctively lower values of 41 to 42 mol% G+C. The DNA base compositions of *N. cuniculi, N. ovis*, and *N. caviae* fell intermediate between those of the latter two groups, with values between 45.3 and 47.3 mol%.

Transformation. Preliminary screening of several reference strains of *Neisseria* and *Branhamella* revealed considerable variation in their competence for transformation. For purposes of this study, it was necessary to select recipient cells with high-level competence (homologous transformation efficiencies greater than 0.5%) to ensure that differences between the transformation frequency and the spontaneous mutation rate to streptomycin resistance would be great enough to reveal gradations of transformation frequencies based on the homology of heterologous DNA. As a result, five reference strains of *Neisseria* and *Branhamella* species were selected to serve as recipients for the donor DNAs from nine other reference *Neisseria* and *Branhamella* species. The results of these studies are summarized in Table 3. Significant levels of transformation between all species of *Neisseria*

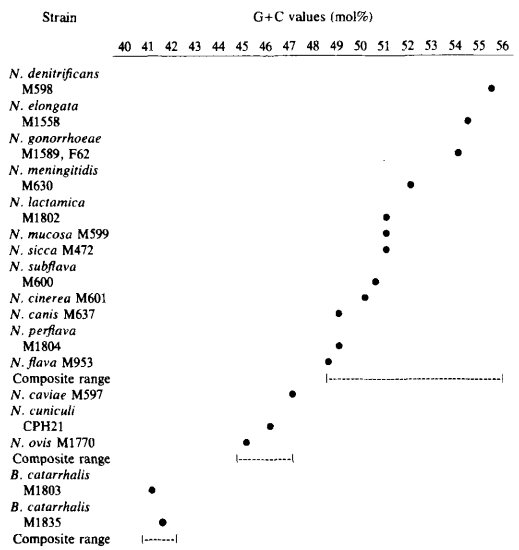

FIG. 1. Principal groupings of reference strains of *Neisseria* and *Branhamella* species according to DNA base ratios. Each dot represents the moles percent G+C calculated from the average of a minimum of three T_m determinations.

examined were revealed. Both *N. meningitidis* and *N. gonorrhoeae* were transformed by each other's DNA at significantly higher frequencies than any other donor DNA, indicating a close genetic relationship. *N. gonorrhoeae* and *N. meningitidis* were transformed by the DNA of *N. sicca*, *N. flava*, *N. perflava*, and *N. subflava* at intermediate frequencies. DNA from *N. lactamica* transformed *N. perflava* at high frequencies and *N. gonorrhoeae* and *N. meningitidis* at intermediate to high levels, although *N. lactamica*, as a recipient, was not transformed by any heterologous DNA at significant levels. It is of interest to note that *N. perflava* accepted DNA from *N. sicca* at slightly higher frequencies than it accepted DNA from *N. flava* and *N. subflava*, since the latter two organisms, along with *N. perflava*, have been proposed to be included in a single species, *N. subflava*, without the inclusion of *N. sicca*. DNA from *N. mucosa* transformed several *Neisseria* species at intermediate frequencies. In contrast to the transformation occurring between members of the genus *Neisseria*, *B. catarrhalis* was not transformed by any heterologous DNA at a significant level and could not serve as a donor to transform any recipient cells of *Neisseria* species at a significant level. It has been shown that *N. caviae* and *N. ovis* contain dehydrogenase systems similar to that of *B. catarrhalis* (14, 15). Our transformation studies, however, revealed that DNA from *N. caviae*, *N. ovis*, and *N. cuniculi* transformed *B. catarrhalis* at very low levels and did not indicate a close genetic relatedness (Table 4).

DNA hybridization. Table 5 lists the results of DNA hybridization studies between selected species of *Neisseria* and *Branhamella* and confirms the close genetic relatedness occurring

TABLE 2. DNA base compositions of *Neisseria* and *Branhamella* species

Test organism	Present study			Previous reports		
	Strain[a]	T_m (avg)	G+C (mol%)	T_m	Buoyant density	Chromatography
N. denitrificans	M598	93.58 (±0.16)[b]	55.6			
N. elongata	M1558	93.21 (±0.32)	54.7		53.0 [6][c]	
N. gonorrhoeae	F62	92.64 (±0.09)	53.3			49.6 [18]
	M1589	92.67 (±0.17)	53.3			
N. meningitidis	M630	92.1 (±0.27)	52.0	51.5 [25]	50.0 [30]	50.5–51.3 [8, 18]
N. lactamica	M1802	91.98 (±0.24)	51.7			
N. mucosa	M599	91.96 (±0.28)	51.6		50.5–52.0 [29]	
N. sicca	M472	91.78 (±0.19)	51.2	49.0 [25]	51.0 [30]	51.5 [8]
N. subflava	M600	91.7 (±0.2)	51.0			50.5 [8]
N. cinerea	M601	91.66 (±0.18)	50.9		49.0 [5]	
N. canis	M637	91.15 (±0.3)	49.6			
N. perflava	M1804	91.14 (±0.19)	49.6	49.0 [25]	48.0 [30]	49.2–50.5 [8, 17]
N. flava	M953	91.0 (±0.06)	49.3			49.5 [8]
N. flavescens				49.0 [25]	46.5–47.5 [5, 30]	50.1 [8]
N. caviae	M597	90.21 (±0.17)	47.3	46.6–47.3 [33]	44.5 [5]	47.3–50.4 [17]
N. cuniculi	CPH21	90.0 (±0.11)	46.8	44.6 [33]		
N. ovis	M1770	89.36 (±0.13)	45.3	46.0–46.3 [33]	44.5–45.0 [5]	
B. catarrhalis	M1803	87.73 (±0.12)	41.3	41.0–41.9 [25, 33]	41.0–42.5 [5, 30]	40.1–45.7 [8, 18]
	M1835	87.85 (±0.09)	41.6			

[a] Strains used in this study only.
[b] Numbers in parentheses represent standard deviations.
[c] Numbers in brackets refer to references.

TABLE 3. Average ratios of interspecific to intraspecific transformation among *Neisseria* and *Branhamella* species

Recipient cells	DNA preparations from the streptomycin-resistant strains of:									
	B. catarrhalis M1803	*N. mucosa* M1801	*N. flava* M953	*N. lactamica* M1723	*N. gonorrhoeae* F62	*N. perflava* Ne16	*N. meningitidis* Ne15	*N. subflava* M600	*N. sicca* M472	Control[a]
N. gonorrhoeae F62	.00021	0.00697[b]	0.00848	0.028	1.0	0.0088	0.078	0.0092	0.0169	<0.00032
N. meningitidis Ne15	0.016	0.073	0.124	0.21	0.78	0.097	1.0	0.111	0.231	0.023
N. perflava Ne16	0.00011[b]	0.0045[b]	0.0089[b]	0.36[b]	0.0042[b]	1.0	0.0011[b]	0.014[b]	0.028	0.0002[b]
N. lactamica M1723	<0.0000329[b]	0.000039[b]	<0.000029	1.0	0.00039	0.000028	0.0002	0.00024	0.00013	0.000034
B. catarrhalis M1803	1.0	0.000033[b]	<0.00022[b]	<0.000004[b]	<0.00001[b]	<0.000055[b]	<0.0000144[b]	0.0000042[b]	0.0000057[b]	0.0000077[b]

[a] Ratio of spontaneous mutation frequency to streptomycin resistance to intraspecific transformation frequency.
[b] Based on one determination only.

between those species of *Neisseria* characterized by DNA base compositions between 49.3 and 55.6 mol% G+C (Fig. 1). With the exception of *N. denitrificans* and *N. canis*, all members of this group demonstrated at least 60% DNA binding with one another. The slightly lower DNA homologies of *N. canis* and *N. denitrificans* with the other members of this group suggest that species of animal origin other than humans are more distantly related to the human isolates. Further studies on additional strains of *N. canis* and *N. denitrificans* would be desirable before any further recommendations on their taxonomic status are made. As indicated in the transformation studies (Table 3), the hybridization data revealed a high level of homology between *N. meningitidis* and *N. gonorrhoeae* DNA (93.17% binding), and *N. lactamica* DNA demonstrated the highest degrees of binding with *N. meningitidis, N. gonorrhoeae*, and *N. perflava* DNA (79, 80, and 75% binding, respectively). Among those tested, *N. mucosa* DNA demonstrated the highest degree of homology with *N. perflava* DNA (77 to 91% binding). A high level of homology among *N. perflava, N. sicca*, and *N. flava* DNA was revealed in this study. As summarized in Table 6, the incorporation of *N. perflava, N. subflava*, and *N. flava* into a single species (*N. subflava*) without the inclusion of *N. sicca* is not supported by these data. These data, in fact, suggest that *N. perflava, N. sicca*, and *N. flava* are more closely related to one another than they are to *N. subflava*. Although the data revealed a high degree of relatedness between *N. mucosa* and *N. perflava* DNAs, *N. mucosa* DNA did not appear to have high enough homology with *N. subflava, N. flava*, or *N. sicca* DNA to be considered part of a single species.

DNAs from organisms (*N. mucosa, N. denitrificans*, and *N. canis*) representing the group of neisseriae with DNA base compositions of 49.3 and 55.6 mol% G+C demonstrated only low levels of homology with *B. catarrhalis* (16 to 35% binding). These data and the transformation data support the placement of *B. catarrhalis* in a separate genus.

DNAs from organisms (*N. ovis* and *N. cuniculi*) representing that group of gram-negative cocci with DNA base compositions of 45.3 to 47.3 mol% G+C demonstrated low degrees of homology with the DNAs of selected neisseriae representing the range of 49.3 to 55.6 mol% G+C (23 to 42% binding) and with *B. catarrhalis* (32 to 38% binding). *N. ovis* and *N. cuniculi* demonstrated significantly greater homology with each other (57.8% binding). These data and the transformation data (Table 4) suggest that *N. ovis, N. cuniculi*, and *N. caviae* represent a third genetic group, distinct from the other gram-

TABLE 4. Ratios of average interspecific to intraspecific transformation among *Branhamella* and *Neisseria* species

Recipient cells	DNA preparations from streptomycin-resistant strains of:				
	B. catarrhalis M1803	*N. caviae* M597	*N. ovis* M1770	*N. cuniculi* M635	Control[a]
B. catarrhalis M1803	1	<0.000033	0.000032	0.000026	0.000013

[a] Ratio of spontaneous mutation frequency to streptomycin resistance to intraspecific transformation frequency.

negative cocci, with genetic affinities intermediate between the neisseriae and the branhamellae.

DISCUSSION

Although much uncertainty exists over comparisons of moles percent G+C determined in different laboratories where different strains and materials have been used, this study found the precision of T_m determinations performed within this laboratory to be high. Of a minimum of three individual T_m values determined on each organism in this study, the standard deviation was less than ±0.4°C (equivalent to less than ±1 mol% G+C) on each DNA sample. The G+C values determined in this study correlate well (<2.2% difference) with those previously reported (Table 2), with the exception of those determined for two strains of *N. gonorrhoeae*. Minor differences (<2.2%) in reported G+C content could be due to differences in strains, methods, or reagents, or, in the case of T_m determinations, variations in temperature calibrations. In an attempt to minimize any variations caused by these factors, each G+C value was calculated with respect to an internal standard of *E. coli* B DNA (Sigma), which had a G+C value (50.0 mol%) in the range of the organisms investigated in this study. The reason for the large difference between the G+C value reported for *N. gonorrhoeae* by Lee et al. (19) and the values obtained for that organism in this study is unclear. The G+C value reported in their study and based on a chromatographic analysis of one strain was 49.6 mol%, whereas the values determined in this study for *N. gonorrhoeae* (strains M 1589 and F 62) were 53.3 mol%. If real, a 3.7 mol% G+C difference between these strains would indicate considerable heterogeneity within this species, which has not previously been suggested. The values listed under *N. gonorrhoeae* (F 62) in Table 2 represent the composite of two individual DNA extractions of this organism. All results demonstrated close agreement with each other. Unless additional strains of *N. gonorrhoeae* indicate differently, our study suggests that the G+C content of *N. gonorrhoeae* is higher than that previously accepted.

Preliminary screening for competent recipients for transformation studies revealed considerable variation in the levels of competence demonstrated by different species of gram-negative cocci and by different strains of a single species. In several instances, low-level competence did not appear to be related to the number of laboratory passages from the source. Varying the type and concentration of cations, in several cases, did not significantly increase the efficiency of transformation among strains demonstrating low-level competence. In addition to the variation in competence levels demonstrated by different strains, some organisms appeared to be stable in their high transformation efficiency whereas others varied from high to low levels of competence, even when experimental conditions were duplicated as closely as possible. It has previously been reported that some competent strains of *N. meningitidis* yield a proportion of incompetent variants that exhibit either decreased transformibility or a complete and permanent loss of competence (16). Competence in transformation has been correlated with pili in some species of *Neisseria* and *Moraxella* (12, 13, 35). Transformation studies with *N. gonorrhoeae* revealed that the nonpiliated T3 and T4 colonies, which emerge upon nonselective subculture of T1 and T2 colonies, have at least 1,000-fold lower competence levels than are obtained with the piliated T1 and T2 colonies (35). Thus, in the case of *N. gonorrhoeae*, by relating competence to a colonial marker, one can easily select colonies that are consistently competent for transformation. The same situation could exist with other *Neisseria* species, except that no colonial marker for competence has been identified. As a result, arbitrary selection of colonies to serve as recipients in transformation studies may result in a mixture of competent cells and incompetent cells that could cause apparent variations in competence. Additional studies addressing the factors affecting competence in other *Neisseria* species would be valuable for elucidating these problems and would enable transformation to be a more easily utilized tool for determining relationships.

In the past, most investigators have chosen

TABLE 5. DNA hybridization between selected gram-negative cocci

Test strain	Degree of DNA:DNA binding (%)[a]													
	N. gonorrhoeae F62	*N. perflava* M1804	*N. perflava* M1827	*N. mucosa* M1801	*N. mucosa* CPH12	*N. subflava* M600	*N. sicca* M472	*N. lactamica* M893	*N. flava* M953	*N. dinitrificans* M598	*N. canis* M637	*N. cuniculi* CPH21	*N. ovis* M1770	*B. catarrhalis* M1803
N. meningitidis M628	93.17	80.33b	78.00	66.84	65.18	75.69	79.40	79.04	63.35					
N. gonorrhoeae F62			74.49	69.86	65.35	73.89	73.49	80.43	61.61					
N. perflava M1804			95.73[b]	85.74[b]	76.99[b]	81.96[b]	92.54[b]	74.03[b]						
N. perflava M1827				91.15	83.02	78.30	91.06	75.38	92.61	48.18[b]	54.17[b]		32.05[b]	
N. mucosa M1801					95.32	80.11		68.40	83.07	45.64[b]	66.41[b]		42.08[b]	35.21[b]
N. mucosa CPH12						75.25	67.61	66.05	69.46					
N. subflava M600							80.43	64.28	81.63					
N. sicca M472								64.55	95.79					
N. lactamica M893									60.80	51.28[b]	45.18[b]		37.63[b]	
N. flava M953											29.49[b]	23.48[b]	25.78[b]	24.31[b]
N. denitrificans M598													16.18[b]	16.26[b]
N. canis M637													57.78[b]	32.51[b]
N. cuniculi CPH21														38.91[b]
N. ovis M1770														
B. catarrhalis M1803														

[a] Results represent the average of three separate determinations, unless otherwise indicated.
[b] Results based on one determination only.

TABLE 6. DNA hybridization between *Neisseria* species proposed to be incorporated as a single species[a]

Test strain	Degree of DNA:DNA binding (%)[b]				
	N. perflava M1804	*N. perflava* M1827	*N. subflava* M600	*N. sicca* M472	*N. flava* M953
N. perflava M1804		95.73[c]	81.96[c]	92.54[c]	
N. perflava M1827			78.30	91.06	92.61
N. subflava M600				80.43	81.63
N. sicca M472					95.79
N. flava M953					

[a] Reference 8.
[b] Results represent the average of three separate determinations unless otherwise indicated.
[c] Results based on one determination only.

high-level resistance to streptomycin as the chromosomal marker for studying transformation between members of the family *Neisseriaceae*. The relative conservation in nature of genes coding for ribosomal components and the observation that the integration and expression barriers are less likely to operate with ribosomal markers than with other markers have been previously discussed (2). Siddiqui and Goldberg (32) reported that the use of several markers in transformation experiments has increased information concerning the genetic relatedness of selected species of *Neisseria*. *N. gonorrhoeae* was observed to exhibit marker selectivity in its ability to be transformed by heterologous DNA and was often transformed by the gene for streptomycin resistance at higher frequencies than with nutritional markers. This observation suggests that the potential for use of transformation as a guide to genetic relatedness may be enhanced by the use of several chromosomal markers.

Despite the problems encountered in finding suitable recipient cells, the results of this study indicate that transformation can serve as a valuable taxonomic tool. The genetic relationships revealed in this transformation study were supported by the DNA hybridization results of this study and others (17). Because transformation, unlike DNA hybridization, is influenced not only by the homology of the DNA but also by the internal biological environment of the cell (including integration and restriction enzymes), absolute correlations between the techniques cannot be made. For instance, some recipient organisms in transformation appear much more

TABLE 7. Genetic groupings of *Neisseria* and *Branhamella* species based on DNA base composition, transformation, and DNA hybridization studies

Group	Microorganism	DNA base composition range (mol%)	Transformation with members of group			DNA hybridization with members of group		
			I	II	III	I	II	III
I	*N. gonorrhoeae* *N. meningitidis* *N. subflava* *N. flava* *N. perlava* *N. sicca* *N. mucosa* *N. cinerea*[c] *N. flavescens*[c] *N. elongata*[c] *N. lactamica*	49.3–54.7	High	TND	Very low	≥62[b]	≥42	≤35
II	*N. cuniculi* *N. ovis* *N. caviae*	45.3–47.3	TND	TND	Very low	≤42	57.78	≤39
III	*B. catarrhalis*	41–42	Very low	Very low	High	35	≤39	—

[a] Test not done.
[b] Percent binding.
[c] Data incomplete (see specific tables). Affiliation of *N. cinerea*, *N. flavescens*, and *N. elongata* with group I was based on genetic data previously reported (4, 6, 8, 16).

restrictive about the DNA by which they are transformed than others. This is quite evident when *N. lactamica* and *N. meningitidis* are compared as recipients for donor DNA (Table 3). Although DNA from *N. lactamica* transformed *N. meningitidis* at high frequencies, it was not transformed by DNA from *N. meningitidis* reciprocally at a significant level. One might assume that this strain of *N. lactamica* is more restrictive in regard to discriminating against foreign DNA than is *N. meningitidis*. Although such differences in discrimination for foreign DNA occurring in various recipient cells may cause some difficulty in the interpretation of results, this study found that the differences need not preclude the use of transformation in taxonomy.

The determination of DNA hybridization among members of the aerobic gram-negative cocci by renaturation rate proved to be an effective, reproducible procedure. In general, the degrees of relatedness between aerobic gram-negative cocci revealed by this method corroborated those previously determined by the filter-membrane technique (17). Previous DNA hybridization results (17) indicated that the neisseriae can be divided into three main subgroups, as follows: (subgroup 1) *N. meningitidis*, and *N. gonorrhoeae*; (subgroup 2) *N. perflava, N. subflava, N. sicca, N. flavescens*, and *N. flava*; and (subgroup 3) *B. catarrhalis* and *N. caviae*. The results of our study did not indicate that *N. caviae* and *B. catarrhalis* exhibit sufficient DNA homology to be considered members of the same group. In addition, this study revealed greater homology between the DNAs of *N. sicca* and *N. subflava* (*N. flava, N. subflava*, and *N. perflava*) than previously reported.

Data from the DNA base composition, transformation, and DNA hybridization studies reported here suggest that the members of the genera *Neisseria* and *Branhamella* form three natural genetic groups (Table 7). These groups are separated by variations in DNA base composition, transformation barriers, and degree of DNA hybridization occurring within and between group members. Group I, comprised of *N. meningitidis, N. gonorrhoeae, N. subflava, N. flava, N. perflava, N. sicca, N. mucosa, N. cinerea, N. flavescens, N. lactamica*, and *N. elongata*, is characterized by the following: G+C values of 49.3 to 54.7 mol%, significant levels of intergenic transformation occurring between members of this group but not with members of group 3, and high degrees of DNA hybridization (over 60% binding) with members of group 1 but significantly lower degrees of hybridization (less than 42% binding) with members of groups 2 and 3. Group 2, comprised of *N. cuniculi, N. ovis* and *N. caviae*, is characterized by the following: G+C values of 45.3 to 47.3 mol%, lack of significant transformation occurring with members of group 3, and low degrees of DNA hybridization with members of groups 1 and 3 (less than 42% binding) but significant degrees between members of group 2 (57.8% binding). Group 3, comprised of *B. catarrhalis*, is characterized by the following: G+C values of 41 to 42 mol% G+C, lack of significant transformation with members of groups 1 and 2, and low degrees of DNA hybridization with members of groups 1 and 2 (less than 39% binding). These data suggest that *N. cuniculi, N. caviae*, and *N. ovis* form a group sufficiently distinct from the other species of *Neisseria* and *Branhamella* to be considered for incorporation into a separate genus.

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