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# Deoxyribonucleic Acid Homologies Among Species of the Genus *Neisseria*

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Eleven aerobic species of *Neisseria*, a *Mima* sp., and a *Herellea* sp. were tested for deoxyribonucleic acid (DNA) homology in direct hybridization experiments. DNA labeled with either $^{14}C$ or $^{32}P$ was prepared from five species of *Neisseria*. Unlabeled DNA from the various microorganisms was immobilized on membrane filters, which, after pretreatment, were incubated with labeled DNA (4,000 counts per min per filter) for 14 hr at 67 C. The measure of relatedness was expressed as the relative percentage of direct binding compared to that obtained with homologous DNA. All serological types of *N. meningitidis*, including the newly proposed types, were homologous to the standard strain of *N. meningitidis* with one possible exception, type Z. The genus *Neisseria* is heterogeneous in nature, forming at least three distinct groups: first, *N. meningitidis* and *N. gonorrhoeae;* second, *N. perflava*, *N. subflava*, *N. sicca*, *N. flavescens*, and *N. flava;* third, *N. catarrhalis* and *N. caviae*. *Mima* and *Herellea* species show no significant homology with the *Neisseria*.

Nucleic acid hybridization has been used to study genetic similarities in several groups of bacteria: the enterobacteria (11), streptococci (14), mycoplasmas (12, 14, 15), pasteurellae (16), the myxobacters (J. L. Johnson and E. J. Ordal, Bacteriol. Proc., p. 34, 1966), and the psittacosis group of agents (4).

The genetic relationships among the gram-negative cocci have not been studied adequately, but they are assumed to be close (5). Several investigators (1, 2) have demonstrated intergeneric transformation between the *Neisseria* and the *Mima* or *Moraxella*. Transformation is also possible between all the species of *Neisseria*, except *N. gonorrhoeae*, *N. catarrhalis*, and *N. caviae*.

The frequent isolations of organisms resembling *N. meningitidis* which do not fit into established serological groups has raised the question of the true identity of these strains. In the absence of any serological confirmation, a few cultural reactions alone have been used for their tentative classification. If these organisms are to be truly identified as new serological types of the meningococcus, a more precise identification is desirable.

In the present study, deoxyribonucleic acid (DNA) hybridization was applied to the study of relationships among members of the accepted serological groups of *N. meningitidis*, the "untypable" strains of *N. meningitidis*, and among other members of the genus *Neisseria* as well as the prototype species of *Mima* and *Herellea*.

## MATERIALS AND METHODS

*Organisms.* The source and serological type of each strain used in this study are listed in Table 1. The procedures used in the identification and the methods of cultivating these strains have been described earlier (7).

*Media and cultural techniques.* The microorganisms were grown in Trypticase Soy Broth (BBL), Frantz modified broth (18), or on Meuller-Hinton agar. Broth cultures were grown on a reciprocal shaker at 37 C, and agar cultures were incubated in a moist incubator (37 C) with an atmosphere of 10% $CO_2$ in air.

*Enzymes and radioisotopes.* Ribonuclease and Pronase were purchased from Calbiochem. The Pronase was "self-digested" for 2 hr at 37 C prior to use. Adenine-8-$^{14}C$ was purchased from Calbiochem. $^{32}P$, in the form of $Na_2HPO_4$, was purchased from the New England Nuclear Corp., Boston, Mass.

*DNA extraction.* Cells were harvested from broth by centrifugation, washed in 150 ml of saline-ethylenediaminetetraacetic acid (saline-EDTA), consisting of 0.15 M NaCl and 0.1 M EDTA, *p*H 8.0 (9), and resuspended in 30 to 50 ml of saline-EDTA. The cells were lysed by overnight incubation at 37 C with 0.5% sodium deoxycholate or 1% sodium lauryl sulfate and 0.25% Pronase. An equal volume of liquified phenol was then added, and the material was gently mixed and allowed to stand at room temperature with periodic gentle shaking. After 2 to 3 hr, the mixture was centrifuged, the aqueous layer was removed, and the phenol extraction was repeated. After the second extraction, the DNA was dialyzed overnight at 4 C in SSC (0.15 M NaCl, 0.015 M sodium citrate, *p*H 7.2) and

TABLE 1. *Identity and source of strains used in homology studies*

| Species | Strain | Source[a] | Serological type |
|---|---|---|---|
| *Neisseria meningitidis* | 1027A | NIH | A |
| | CL4 | Case isolate | A |
| | SD72 | Carrier isolate | B |
| | SD6 | Case isolate | C |
| | 236 | Case isolate | C |
| | M158D | NIH | D |
| | Bo | WRAIR | Untypable |
| | 29EUR | WRAIR | Untypable |
| | X | Slaterus (17) | X |
| | Y | Slaterus (17) | Y |
| | Z | Slaterus (17) | Z |
| *N. gonorrhoeae* | 116 | WRAIR | |
| *N. flava* | | NIH | |
| *N. caviae* | | Pelczar (13) | |
| *N. catarrhalis* | | NIH | |
| *N. subflava* | | NIH | |
| *N. flavescens* | | NIH | |
| *N. perflava* | | NIH | |
| *N. sicca* | | NIH | |
| *Mima* sp. | 9957 | ATTC | |
| *Herellea* sp. | 9955 | ATTC | |
| *Escherichia coli* | B | NIH | |

[a] NIH, National Institutes of Health; WRAIR, Walter Reed Army Institute of Research; ATCC, American Type Culture Collection.

treated with 40 μg of ribonuclease per ml for 2 hr at 37 C. Two additional phenol extractions were performed, and the DNA was "spooled" by the addition of two volumes of cold ethyl alcohol. This step was introduced prior to the final dialysis. Each preparation was analyzed spectrophotometrically, and the DNA concentrations were determined on the basis of absorbance at 260 mμ.

*Production of labeled DNA.* Both $^{32}P$ and $^{14}C$ were used to label DNA in this study. Adenine-*8*-$^{14}C$ was incorporated into the bacterial DNA as described earlier (9). $^{32}P$ labeling was accomplished by growing the bacteria for 24 to 30 hr in Frantz broth containing 5 μc (per ml) of $Na_2H^{32}PO_4$. When radioactive phosphate was added, the phosphate in the medium was reduced from 0.050 to 0.025 M.

*Immobilization of DNA on nitrocellulose filters.* The DNA was denatured at a concentration of 500 μg/ml by boiling for 5 min in SSC and quick cooling. Concentrated SSC was then added to the denatured DNA solution to bring the concentration to 6 × SSC, and the material was passed through a Millipore HA filter (0.45-μ average pore diameter) at an approximate speed of 5 ml per min. (The filters were presoaked and washed in 6 × SSC.) After application of the DNA, the filters were dried overnight in a desiccator and then placed under vacuum at 80 C for 2 hr (3).

Two different sizes of Millipore filters were used. In the direct binding experiments, duplicate or triplicate 22-mm pads were used, and the DNA was applied at a concentration of 100 μg/ml. For the competition experiments, the DNA (75 μg/ml) was applied to a 90-mm filter, and 11-mm circles were punched from the larger filter.

*Preincubation of the DNA-filters.* The preincubation medium of Denhart (3) was used in this study. It consisted of 0.02% each of Ficoll (Pharmacia), polyvinylpyrrolidone (Oxford Laboratories), and bovine albumin (Armour fraction V) in 3 × SSC. Preincubation was carried out at 67 C for 6 hr.

*Formation and detection of the hybrids.* In the direct binding experiments, labeled DNA purified as described earlier was sheared by passage through a French pressure cell at 10,000 psi (11). The sheared DNA was denatured by boiling in 0.1 × SSC for 5 min and quick cooling. A suitable amount (approximately 3,000 to 5,000 counts/min) was added to the preincubated DNA-filters and incubated for 16 hr at 67 C. The filters were then carefully washed with 50 ml of SSC on each side. After washing, the radioactivity on the filters was determined in a Packard Tri-Carb scintillation counter. Relationships between strains are expressed as the percentage of direct binding as compared to that obtained in the homologous reaction.

In the competition experiments, the preincubated DNA-filters were mixed with the sheared radioactive DNA as described for the direct binding experiments. However, included in the incubation mixtures were various concentrations of sheared unlabeled homologous or heterologous DNA (6). Relationships are expressed as the relative depression of binding of the labeled DNA as compared to that obtained in a homologous reaction.

## RESULTS

*Characterization of the DNA hybridization reaction on membrane filters.* When high-molecular-weight single-stranded DNA (from 10 to 500 μg) was added to 22-mm membrane filters, the efficiency of binding steadily decreased. Maximal binding was reached between 400 and 500 μg (Fig. 1). When $^{14}$C-labeled DNA fragments (0.3 μg per filter) were allowed to hybridize with increasing amounts of unlabled homologous DNA on filters, the percentage of bound radioactivity increased (Fig. 1) to a maximum of 70% at 500 μg of unlabeled DNA. The experiments to be described were carried out with membrane filters containing 500 μg of unlabeled DNA.

*Relatedness among various strains of N. meningitidis.* Representative strains of all the accepted serological groups (A, B, C, and D) were compared for their degree of homology (Table 2). Also included were representative strains which do not fall within the accepted serological grouping (BO, 29EUR, X, Y, and Z). With one exception, the degree of hybridization obtained with all the strains was indistinguishable from that obtained with the homologous strain. The one exception, strain Z, hybridized the SD6 strain of *N. meningitidis* to a lesser degree. These results were confirmed in a competition experiment (Fig. 2). The degree of relatedness between strains SD6 and Z is the same as between SD6 and *N. gonorrhoeae*.

*Relatedness between N. meningitidis and other species of the genus Neisseria.* The degree of relatedness among *Neisseria* species was determined by the use of 5 preparations of labeled DNA fragments and 13 preparations of unlabeled DNA (Table 3). In contrast to the findings shown in Table 2, the hybridization between different species was always lower than

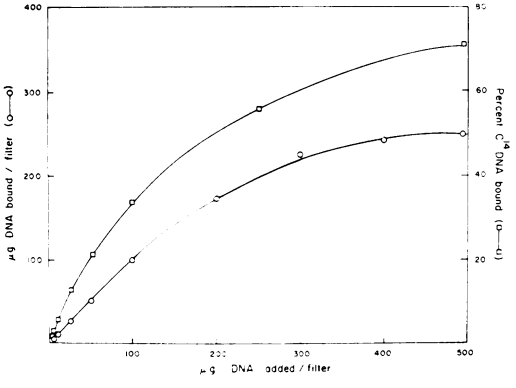


FIG. 1. *Ability of membrane filters to bind single-stranded DNA and the binding of $^{14}$C-labeled DNA fragments to these filters. High-molecular-weight labeled DNA was denatured and applied to membrane filters, the bound isotope being used to measure the amount of DNA remaining on the pad. $^{14}$C-labeled DNA fragments (0.3 μg, 10,000 counts per min per μg) were incubated with filter pads containing various amounts of unlabeled DNA, and the percentage bound was plotted against the amount of DNA applied to the filter.*

TABLE 2. *Reaction of $^{14}$C-labeled DNA fragments from Neisseria meningitidis SD6 with N. meningitidis DNA from various strains*[a]

| Source of immobilized DNA | Serotype | Labeled DNA bound | Relatedness |
|---|---|---|---|
| | | % | % |
| 1027A | A | 73 | 102 |
| CL4 | A | 74 | 104 |
| SD72 | B | 70 | 98 |
| SD6 | C | 71 | 100 |
| 236 | C | 74 | 104 |
| M158D | D | 74 | 104 |
| Bo | | 71 | 100 |
| 29EUR | | 72 | 101 |
| X | | 74 | 104 |
| Y | | 69 | 97 |
| Z | | 63 | 89 |
| Monkey kidney (MK2) | | <0.1 | <0.1 |
| None | | <0.1 | <0.1 |

[a] Membrane filters containing 500 μg of DNA were incubated with 0.3 μg of DNA fragments (10,000 counts per min per μg) in preincubation medium at 67 C for 16 hr.

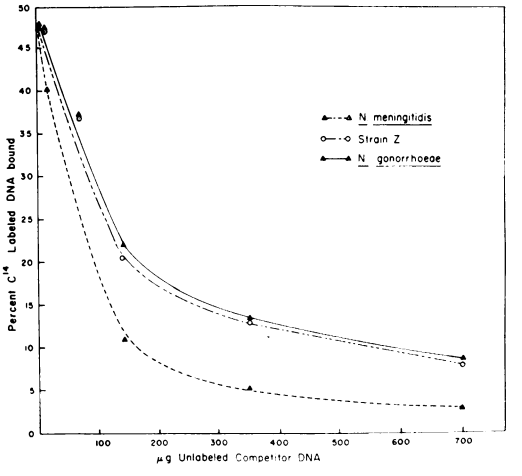


FIG. 2. *Competition between DNA fragments in the reaction with Neisseria meningitidis DNA. $^{14}$C-labeled DNA fragments (0.3 μg, 10,000 counts per min per μg) were incubated with 45 μg of N. meningitidis DNA in the presence of various quantities of unlabeled DNA fragments from N. gonorrhoeae or strain Z. The reaction was carried out in a total volume of 1.5 ml on 11-mm circles cut from a 90-mm filter pad containing 3 mg of N. meningitidis DNA.*

TABLE 3. *Reaction of $^{14}C$-labeled DNA fragments from five Neisseria species with DNA from various sources*

| Source of immobilized DNA | Source of labeled DNA fragments | | | | |
|---|---|---|---|---|---|
| | *N. meningitidis* | *N. sulflava* | *N. flavescens* | *N. flava* | *N. catarrhalis* |
| *N. meningitidis* (SD6) | 100 | 45 | 43 | 31 | 19 |
| *N. gonorrhoeae* | 80 | | 58 | | 26 |
| *N. perflava* | 55 | 75 | 42 | 40 | 11 |
| *N. subflava* | 48 | 100 | 47 | 45 | 18 |
| *N. sicca* | 45 | 60 | 33 | 45 | 10 |
| *N. flavenscens* | 42 | 47 | 100 | 48 | 28 |
| *N. flava* | 30 | 50 | 47 | 100 | 12 |
| *N. catarrhalis* | 15 | 17 | 27 | 19 | 100 |
| *N. caviae* | 10 | 15 | 12 | 15 | 33 |
| *Mima* sp. | 5 | 5 | 11 | 5 | 10 |
| *Herellea* sp. | 5 | 5 | 9 | 5 | 15 |
| *Escherichia coli* | 3 | | 2 | | 2 |
| Monkey kidney (MK2) | <0.1 | | <0.1 | | <0.1 |
| None | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |

that obtained in the homologous system. *N. meningitidis* and *N. gonorrhoeae* were closely related. Most of the "nonpathogenic" species were less related to *N. meningitidis* (30 to 60%). *N. catarrhalis* and *N. caviae* showed little homology with *N. meningitidis* or with the other *Neisseria* species. Reciprocal hybridization experiments were used to confirm these results.

*Mima* and *Herellea* species were unrelated to the *Neisseria*, as was *Escherichia coli*.

## DISCUSSION

The determination of DNA homology among bacterial strains, based on hybridization of DNA on membrane filters, proved to be an effective and highly reproducible procedure.

It is clear that virtually complete DNA homology exists not only among strains of serological groups A, B, C, and D of *N. meningitidis* but also among those strains that have not been satisfactorily classified by existing serological criteria. These results indicate that "untypable" strains represent different serotypes of the same species, rather than different species of microorganisms. Strain Z may be sufficiently different from the other strains of *N. meningitidis* to represent a different species. The results illustrated in Fig. 2, however, do not require that strain Z be identical to *N. gonorrhoeae*, because the regions of each strain that are homologous to *N. meningitidis* need not be completely identical.

The generally accepted view has been that the genus *Neisseria* represents a very closely related group of species and that *Mima*, *Herellea*, and *Moraxella*, although different genera, are closely allied to *Neisseria* (1, 5). This view is supported only be a few transformation experiments (1, 2, 8) and by a few cultural characteristics (5).

The results of this study indicate that the genus *Neisseria* is a heterogeneous group which consists of at least three main subgroups: subgroup 1, *N. meningitidis* and *N. gonorrhoeae*; subgroup 2, *N. perflava*, *N. subflava*, *N. sicca*, *N. flavescens*, and *N. flava*; subgroup 3, *N. catarrhalis* and *N. caviae*.

The third subgroup can be considered significantly different enough from the other *Neisseria* to question their proper classification as *Neisseria*. Further work with a number of other microorganisms of the *Moraxella* group may clarify the proper position of subgroup 3.

The results of this study clearly indicate that the *Mima* and *Herellea* strains used in these studies have virtually no homology with the *Neisseria*. The amount of homology found probably corresponds to very little more than the regions for ribosomal and transfer ribonucleic acid.

The apparent discrepancy between the results obtained in this investigation and the indication of close relatedness among the above species as shown by the ease of genetic transformation among these same species may be attributed to the following factors: (i) the markers transferred were antibiotic resistance markers, which may be similar in a number of microorganisms; (ii) the genetic material transferred might have been episomal DNA which was not truly integrated in the bacterial chromosome.

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