Genetic Relationships among *Neisseria* Species Assessed by Comparative Enzyme Electrophoresis

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The electrophoretic mobilities of 12 enzymes from 19 *Neisseria* species (including 6 strains of *N. perflava*), *Gemella haemolysans*, *Escherichia coli* and *Branhamella catarrhalis* were characterized by polyacrylamide slab gel electrophoresis. All strains and species tested exhibited qualitatively different zymogram patterns. Species and strain relationships were quantified by pairwise comparisons of all 12 enzyme systems to obtain similarity indices; these data were subjected to numerical clustering methods to obtain groups and a phenogram. The electrophoretic classification compared favourably with those obtained by other criteria. In addition, the quantitative clustering data indicated that *N. ovis* and *N. caviae* are sufficiently different from the other *Neisseria* species to warrant their separation into a distinct group. These two species also lacked the characteristic NADPH-diaphorase zymogram pattern found in all the other *Neisseria* species. Intra-species similarity indices were generally greater than the inter-species index values. However, certain species such as *N. meningitidis* and *N. gonorrhoeae* had similarity index values in the range of inter-strain index values.

INTRODUCTION

The determination of genetic and evolutionary relationships among bacterial species offers insight into the adaption of bacteria to specific environments. The family *Neisseriaceae* has attracted considerable attention in this regard because its member species inhabit a broad range of animal hosts, and several species are of medical importance. Relationships among the *Neisseriaceae* have been studied using a variety of approaches, including DNA hybridization, DNA base composition, immunological comparison of proteins, biochemical profiling, and interspecific transformations (for recent reviews, see Bøvre, 1980; Vedros, 1981). Although these studies have led to a redefinition of the taxonomic structure of the family, there still remain many unanswered questions.

In this report, we describe the application of comparative enzyme electrophoresis to the analysis of species and strain relationships within the genus *Neisseria*. Enzyme electrophoresis has been widely used in studies of genetic relationships among eukaryotes but has been little used for this purpose with bacteria. The method provides an indirect but convenient approach to genomic comparison (Avise, 1974; Wilson *et al.*, 1977; Ayala, 1978). The underlying rationale is that the proportion of enzymes exhibiting electrophoretic mobility differences reflects the extent of amino acid substitution separating lineages derived from a common ancestor: the greater the
Table 1. List of strains used in this study

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<td>Gemella haemolysans</td>
<td>ATCC 10379</td>
<td>Formerly N. haemolysans (Berger, 1960)</td>
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</table>

* Sources: ATCC, American Type Culture Collection, Rockville, Md., USA; Berger, Ulrich Berger, Hygiene-Institut der Universität, Heidelberg, FRG; Reyn, Alice Reyn, Staten Serum Institute, Copenhagen, Denmark; Kellog, Douglas Kellog, Centers for Disease Control, Atlanta, Ga., USA; Vedros, Neylan A. Vedros, University of California, Berkeley, Calif., USA; Hoke, Carolyn Hoke, Kaiser Permanente Hospital, San Francisco, Calif., USA.

number of substitution differences, the greater the proportion of enzymes showing mobility differences. The characterization of specific enzymes (as opposed to general protein patterns) allows homologous genetic loci to be compared; this, in turn, is a condition for valid genetic similarities to be established (Wilson et al., 1977). Accordingly, in comparing any two species, measurement of the proportions of electrophoretically similar and dissimilar enzymes provides a quantitative index of genetic relatedness (Avise & Aquadro, 1982). Obviously, the greater the number of enzymes compared, the more comprehensive will be the picture of the genomic relationship.

Comparative enzyme electrophoresis has been used previously in bacterial systematics, but most of the studies have examined relatively few enzymes with the objective of using zymogram patterns as phenetic characters. In this study, we have developed quantitative aspects of comparative enzyme electrophoresis and applied population genetic methods to analyse our data.

**METHODS**

*Micro-organisms and growth conditions.* The species and strains examined are listed in Table 1. In addition to the *Neisseria* and presumed *Neisseria* species, *Escherichia coli*, *Branhamella catarrhalis* and *Gemella haemolysans* were included as unrelated species. Freeze-dried culture stocks were rehydrated in Trypticase soy broth (TSB), inoculated on GC agar or Mueller–Hinton agar plates, and incubated for 18 h at 36 °C (5% carbon dioxide, 100% humidity). Harvested cells were inoculated into TSB and incubated for 18 h on a rotary shaker (New Brunswick) at 36 °C (air, 200 r.p.m.). Cultures of gonococci were inoculated into GC broth which had been added to an inert, non-toxic bottom layer of solidified resin (Giard & Vedros, 1981). Media were obtained from BBL.
Genetic relationships among neisseriae

Preparation of extracts. The cells of all cultures were harvested by centrifugation (7000 g, 20 min) and washed twice with Hanks' balanced salt solution at pH 7.2. The pellets were resuspended in 10 vols 0.1 M-Tris/HCl buffer at pH 7.5. The cells were then disrupted in a Ribi cell fractionator (model RF-1, Ivan-Sorvall, Norwalk, Conn., USA) at 30000 lbf in^-2 (207 MPa). To stabilize enzyme activity, sucrose was added to the crude cell homogenate to a final concentration of 20% (w/v). The sucrose, by making the solution denser, also facilitated loading of the specimen into wells during electrophoresis. Samples (100 μl) were frozen until use.

Polyacrylamide gel electrophoresis. Electrophoresis was done on vertical gel slabs (14 × 12 × 0.1 cm) using the discontinuous buffer system described by Ornstein (1964). A 10% separation gel with 5% stacking gel was used for all the enzymes studied except glutamate dehydrogenase and isocitrate dehydrogenase, for which a straight 5% separation gel was used without a stacking gel. A cell fractionate of N. perflava (strain M1804) was used in each gel as a reference standard. Bromophenol blue was used as a tracking dye. Electrophoresis was done at a constant current of 30 mA per gel slab at 4 °C until the tracking dye reached the bottom of the gel; 3 h was a typical run time. After electrophoresis, the gel slabs were removed, washed in distilled water, and stained to visualize the enzymes.

Enzyme staining procedures. Specific staining procedures for the enzymes were as described in Harris & Hopkinson (1976) and Shaw & Prasad (1970). After staining, the gels were dried under a vacuum and the relative mobilities of the enzyme bands measured (ratio of the distance that the enzyme had moved from the origin to the distance moved by the tracking dye). The N. perflava (strain M1804) reference sample in each gel facilitated comparison and normalization of mobilities of samples run on separate gels.

Biochemicals used in staining the gels were obtained from Sigma.

Numerical taxonomic methods. Electrophoretic banding patterns for each enzyme were compared pairwise by counting bands with identical and dissimilar mobilities. Relationships between sample pairs were assessed for each enzyme using Jaccard's similarity coefficient (Sj), defined as $S_j = (\text{no. of shared bands})/(\text{total no. of bands possible}).$

An average similarity index ($S_j$) was generated for each sample pair by summing the individual $S_j$ value for all enzymes and dividing by the total number of enzymes.

To compare the results of this study with those of other studies, $S_j$ values were transformed to distance ($D_j$) indices using the formula

$$D_j = -\ln S_j.$$ 

The distance measure is similar to Nei's (1972) distance coefficient $D = -\ln I$, where $I$ is an index of genetic identity, and, like $S_j$, assumes values from 0 to 1. Because $I$ and various measures of similarity (of which $S_j$ is one) are strongly correlated, and exhibit similar absolute values in a given species comparison, genetic distances can be closely approximated using this general equation (Nei, 1975; Avise & Aquadro, 1982).

The various species were clustered using the UPGMA method on the $S_j$ data matrix (Sneath & Sokal, 1973). Genetic distance measures, $D_j$, could not be used for clustering because non-finite $D_j$ values (where $S_j = 0$) cannot be accommodated in the clustering program. The $D_j$ values shown in the phenogram (Fig. 5) were converted from $S_j$ values after clustering.

RESULTS

Survey of enzymes

Our initial electrophoretic survey of enzymes in Neisseria included 25 enzymes and used acrylamide, starch, and agar gel electrophoresis. Of the enzymes surveyed, 12 were selected for further comparative study: superoxide dismutase (SOD; EC 1.15.1.1), isocitrate dehydrogenase, NADP+ dependent (IDH; EC 1.11.1.42), NADH-diaphorase (DIA-II) NADPH-diaphorase (DIA-I), glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49), 'malic enzyme' (ME; EC 1.1.1.40), glutamate dehydrogenase, NAD dependent (GDH-I; EC 1.4.1.2), glutamate dehydrogenase, NADP dependent (GDH-II; EC 1.4.1.4), leucine aminopeptidase (LAP; EC 3.4.11.1), glutamate-oxaloacetate transaminase (GOT; EC 2.6.1.1), adenylate kinase (AK; EC 2.7.4.3), and peroxidase (PO; EC 1.11.1.17). These 12 enzymes were found in most of the species and strains studied, whereas the other 13 enzymes were either present at very low activity levels in all the species or were absent in many of the strains studied. In addition, the above 12 enzymes were easy to stain for activity and could be run on acrylamide slab gels; such gels gave the best resolution. More fundamentally, the 12 enzymes are essential metabolic enzymes whose genes are presumed to be transmitted vertically on the bacterial chromosome; hence, they can be considered stable characters appropriate for a genetic study of this sort.
Species variation in enzyme patterns

Ten of the 12 enzymes studied exhibited simple one- or two-band electrophoretic patterns, as illustrated schematically in Fig. 1. The exceptions were the two diaphorases, the complex multiple-band patterns of which are illustrated photographically in Figs 2 and 3. As noted in the zymograms, none of the species tested were identical to any other in all enzyme systems.

Strains of B. catarrhalis, E. coli and G. haemolyticus were included in the study to provide external reference points. B. catarrhalis is related to Neisseria at the family level (Reyn, 1974) whereas E. coli and G. haemolyticus are genetically unrelated to any Neisseria species. The enzyme mobility patterns given by these two species were markedly distinct from each other and from those of the Neisseria species.
Genetic relationships among neisseriae

Fig. 2. NADH-diaphorase pattern of various species in the study: 1, G. haemolysans; 2, N. caviae; 3, N. animalis; 4, N. cuniculi; 5, N. cinerea (strain M601); 6, N. cinerea (strain M1901); 7, N. perflava (strain M1804); 8, N. subflava; 9, E. coli; 10, N. gonorrhoeae; 11, N. ovis; 12, N. meningitidis; 13, B. catarrhalis; 14, N. canis; 15, N. elongata; 16, N. mucosa 'var. heidelbergensis'.

All the 'true' Neisseria species show characteristic bands: a major band whose migration varies slightly from species to species, and two cathodal minor bands whose migration is fairly constant from species to species.
Species differences were more pronounced for some enzymes than for others: the patterns for DIA-I, DIA-II, ME, SOD, IDH and GDH-II varied considerably from species to species. In contrast, the patterns of AK, G6PD and LAP varied little among the Neisseria species: half the species, for example, exhibited a common AK electromorph. The enzyme patterns of N. ovis and N. caviae were, in general, quite distinct from those of the other Neisseria species. Certain species, e.g. N. meningitidis and N. gonorrhoeae, exhibited strong resemblance in their electrophoretic patterns. These points are better summarized in the phenogram generated from the electrophoretic data.

Several of the zymograms suggested group relationships among the Neisseria species, e.g. most of these species showed three distinct DIA-I bands (Fig. 3) with a major band and two cathodal minor bands. The mobility of the major band varied only within a very narrow range. The two minor bands stood out against the background of the other bands. This pattern was noted for all Neisseria species except N. ovis and N. caviae; the three unrelated species, G. haemolysans, B. catarrhalis and E. coli, also did not have this characteristic pattern. N. caviae and N. ovis also differed from other Neisseria species in having no detectable G6PD activity.
Genetic relationships among *Neisseria* 

The degree of variability in enzyme mobility between species prompted investigation of variability between strains within a species. Six strains of *N. perflava* were analysed; the resultant zymograms are illustrated in Fig. 4. For each enzyme the variability between strains of *N. perflava* was less than that between *N. perflava* and other species.

**Numerical analysis of electrophoretic data**

Similarity indices were computed for each species pair by band matching as described in Methods. These data were transformed by numerical clustering to yield the phenogram shown in Fig. 5. Five very tight clusters are apparent: (a) *N. gonorrhoeae* and *N. meningitidis*; (b) *N. cuniculi* and *N. cinerea* (strain M601); (c) *N. cinerea* (strain M1901) and *N. denitrificans*; (d) *N. canis* and *N. animalis*; and (e) *N. sicca, N. macacae* and *N. mucosa* ('var. heidelbergensis'). Two species, *N. ovis* and *N. caviae*, clustered outside the main *Neisseria* groups and are as distinct from it as are the unrelated species, *B. catarrhalis, E. coli* and *G. haemolysans*.

The inter-strain similarity indices for the *N. perflava* strains are shown in Table 2. The interspecies index values tend to be lower than the inter-strain values. The distributions of both sets of similarity index values are shown in Fig. 6. The range of inter-species index values is 0 to 0.486 (mean 0.137) (excluding *N. ovis, N. caviae, G. haemolysans, B. catarrhalis* and *E. coli*); 90% of the values are below 0.27. In contrast, the inter-strain values range from 0.161 to 0.958 (mean 0.479); 90% of the inter-strain index values are above 0.4. (The corresponding mean genetic distance between species is 2; 90% of the $D_j$ values exceed 1.3. The mean genetic distance between strains is 0.75; 90% of the $D_j$ values are less than 1.0.)
Fig. 6. Distribution of similarity indices. (a) Inter-species similarity indices. The histogram distinguishes the indices for pairs of 'true' Neisseria species and for pairs involving outlying species. ■, N. ovis, N. caviae, G. haemolysans, B. catarrhalis, E. coli; □, all other Neisseria species. (b) Inter-strain similarity indices for N. perflava (■; Table 2) and for N. gonorrhoeae (□).

Table 2. Similarity coefficients between six N. perflava strains

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DISCUSSION

A principal objective of this study was to evaluate the utility of comparative enzyme electrophoresis for the study of bacterial systematics and evolution. Relationships within the Neisseria group have been the subject of a variety of genetic analyses, which provide a context against which to compare the results of the enzyme electrophoresis analyses reported here.
The electrophoretic data cluster most of the *Neisseria* species into a core group. Within this core group, clusters occur at several levels. Several species cluster very tightly, for example, *N. gonorrhoeae, N. meningitidis, N. cinerea* (strain M601) and *N. cuniculi*. It is interesting that both *N. gonorrhoeae* and *N. meningitidis* are primary pathogens, and that *N. cinerea* is increasingly noted as a possible pathogen (Knapp et al., 1983). *N. canis* and *N. animalis* also form a tight cluster despite being rather distant from the other species.

Two species, *N. ovis* and *N. caviae*, fall outside this core group. By the electrophoretic criteria, they are as distant from the other species in the core group as are the three non-*Neisseria* species, *G. haemolysans, B. catarrhalis* and *E. coli*. The separation of *N. ovis* and *N. caviae* from the rest of the genus *Neisseria* was previously recognized by Holten (1974a, b), who suggested that they be placed in a separate group, 'false *Neisseria*'. Holten characterized 'false' *Neisseria* along with *B. catarrhalis* as lacking G6PD activity; this possibly reflects basic differences in the glucose metabolism of 'true' and 'false' *Neisseria*.

It is noteworthy that all the 'true' *Neisseria* species had a characteristic DIA-I zymogram pattern, whereas *N. ovis* and *N. caviae*, as well as the non-*Neisseria* species, *E. coli*, *G. haemolysans* and *B. catarrhalis*, did not. We propose that this pattern may be another character by which 'true' *Neisseria* species are characterized, and by which newly discovered isolates can be classified. For example, *N. maccaha* isolated from the oropharynx of the rhesus monkey (*Macaca mulatta*) has this DIA-I pattern. In a separate study, this strain was shown to be related to *N. mucosa* and *N. sicca* (Vedros et al., 1983).

In sum, these electrophoretic groupings are in general agreement with taxonomic schemes obtained from biochemical analyses of phenetic characters (Hoke & Vedros, 1982a), from genome level comparison (Hoke & Vedros, 1982a, b; Kingsbury, 1967); and from immunological distance measures (Holten, 1974b). The only significant disagreement involves *N. cuniculi*. Some workers have placed this species with *N. caviae* and *N. ovis*, in the 'false' *Neisseria* group (Hoke et al., 1982b). However, in the present study, we found *N. cuniculi* to possess G6PD activity as well as the characteristic DIA-I pattern, and to cluster with *N. caviae, N. meningitidis* and *N. gonorrhoeae*, which supports the placement of *N. cuniculi* within the 'true' *Neisseria* group.

At the quantitative level, the distance measures obtained by comparative enzyme electrophoresis correlate well with quantitative DNA hybridization. A comparison of percentage DNA binding (Kingsbury, 1967) with electrophoretic distance $D_J (D_J = -\ln S_J)$ for eight bacteria, using *N. meningitidis* as the reference species in both cases, gave a correlation coefficient of 0.87.

The enzyme electrophoretic data provide a quantitative picture of the differences between species and strains. Similarity index values between strains of the same species were typically greater than 0.4, whereas the between-species values were typically below 0.25. By this criterion, certain species were similar enough to be classified as a single species, e.g. *N. meningitidis* and *N. gonorrhoeae*. Also, some species are genetically more homogeneous than others; *N. gonorrhoeae* strains, for example, are more homogeneous than *N. perflava* strains (unpublished). These similarity index values are comparable with those derived by Baptist et al. (1969) for the *Enterobacteriaceae*: inter-species similarity indices of about 0.25, and intra-species strain similarities of 0.5 or above. Two very closely related species, *Shigella sonnei* and *E. coli* had similarities equivalent to strain similarities.

Since enzyme electrophoresis has been extensively used in the study of vertebrate systematics and evolution, it is of interest to compare bacterial and vertebrate similarity scales. Our electrophoretic study, and that of Baptist et al. (1969), indicate that strain-level differences in bacteria are comparable to species-level differences in vertebrates (Avise & Aquadro, 1982). This comparison poses interesting evolutionary questions regarding the age of bacterial species and the rate of bacterial evolution.

This study shows that enzyme electrophoresis is a useful tool for differentiating closely related bacterial strains and species. For example, on the electrophoretic scale, *N. gonorrhoeae* and *N. meningitidis* have a similarity index of 0.416 on the scale of 0 to 1; more than half the scale is thus available for differentiation. In contrast, the DNAs of *N. gonorrhoeae* and *N. meningitidis*
typically hybridize at 80–95% (Hoke & Vedros, 1982b; Kingsbury, 1967) which means that, at
most, only 20% of the DNA hybridization scale is available for differentiation. Similarly, using
immunological distance measures (ID), N. gonorrhoeae and N. meningitidis differ by 0–10 ID
units on a scale of 0 to 200, which means that only 10% or less of the scale is available for
differentiation. The potential for strain differentiation is also well illustrated with N. perflava
strains, which are very difficult to differentiate phenetically or by DNA hybridization, but
easily differentiated by enzyme electrophoresis. Studies are in progress using comparative
electrophoresis to study strain differences within the two pathogenic species, N. gonorrhoeae and N. meningitidis, and to assess genetic homogeneity within these respective species (Chun et al., 1984).

For more distantly related species, enzyme electrophoresis may be less definitive. Random
similarities can begin to appear at $S_i$ values as high as 0·17 (e.g. E. coli and N. macacae).
However, even with low similarity values, one can use the electrophoretic data to obtain a
meaningful classification by using the total data matrix: this is illustrated in the present study.

A major advantage of enzyme electrophoresis is the simplicity of the technique. A large
number of comparisons can be made simultaneously and the data analysed numerically. The
data can be stored for reference and cross-compared in later studies: migration rates can be
compared between different gels if a standard marker strain is

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malate dehydrogenase in genus Neisseria. Acta patho-
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