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Five Structural Classes of Major Outer Membrane Proteins in *Neisseria meningitidis*

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Group B *Neisseria meningitidis* is thus far subdivided into 15 protein serotypes based on antigenically different major outer membrane proteins. Most serotypes have three or four major proteins in their outer membranes. Comparative structural analysis by chymotryptic ^{125}I-peptide mapping was performed on these major proteins from the prototype strains as well as from six non-serotypable strains. The major outer membrane proteins from each of the serotypes were first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the Laemmli system. Individual proteins within the gel slices were radioiodinated and digested with chymotrypsin, and then their ^{125}I-peptides were separated by electrophoresis and chromatography on cellulose thin-layer plates. The peptide maps obtained by autoradiography were categorized into five different structural classes which correlated with the apparent molecular weights of proteins, i.e., 46 ± 1K, 41 ± 1K, 38 ± 1K, 33 ± 1K, and 28 ± 1K. Each of the major outer membrane proteins within a strain had a distinctly different chymotryptic peptide map, indicating significant differences in the primary structure of these proteins. In contrast, outer membrane proteins of the same or very similar molecular weight from different serotype strains had similar, occasionally identical peptide maps, indicating a high degree of structural homology. The unique peptides from proteins of the same structural classes were often hydrophilic, whereas common peptides were often hydrophobic, suggesting that the serotype determinants reside within the variable hydrophilic regions of major outer membrane proteins.

Neisseria meningitidis is divided into eight serogroups (A, B, C, X, Y, Z, W135, and 29E), based on the immunological specificities of their capsular polysaccharides (1). Meningococcal strains can be further subdivided into protein serotypes (4, 5) and lipopolysaccharide immunotypes (12). For example, group B *N. meningitidis* is classified into 15 protein serotypes (5) and 8 lipopolysaccharide immunotypes (12). The antigens of protein serotypes are outer membrane proteins (OMPs), and the immunological reactivities of some serotypes have been associated with certain major OMPs (6, 7, 14, 19).

Actively growing *N. meningitidis* cells produce outer membrane evaginations or blebs, which are released into the culture medium as membrane vesicles (2). These blebs can also be removed from the organism by mild salt extraction (6). The isolated outer membrane vesicles contain both the protein and lipopolysaccharide antigens and have therefore been referred to as serotype antigens (6). The outer membrane vesicles, i.e., serotype antigen, contain three or four major proteins when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and these protein species are indistinguishable from the major proteins of the outer membranes isolated from spheroplasts (6).

The technique of radioiodination of proteins within polyacrylamide gel slices followed by peptide mapping of the labeled proteins has been used in the structural analyses of viral coat proteins (3) and gonococcal outer membrane proteins (15, 16). Our previous study showed that ^{125}I-peptide mapping could be used to differentiate between the 41,000-dalton major OMPs of meningococcal serotypes 2 and 11, even though these two proteins had nearly identical amino acid compositions (17). In the present study, comparative structural analysis by chymotryptic ^{125}I-peptide mapping was performed on the major OMPs from each of the prototype strains as well as from six nonserotypable strains of group B *N. meningitidis* to investigate differences and similarities among these proteins. Over 60 proteins were mapped, and the major OMPs could be categorized into five structural classes according to their apparent molecular weights (MWs), i.e., 46,000 ± 1,000 (46 ± 1K). 41 ± 1K, 38 ± 1K, 33 ± 1K, and 28 ± 1K. Most

of the major OMPs of the same or very similar MW from different serotype strains are structurally closely related.

MATERIALS AND METHODS

Strains, growth conditions, and preparation of outer membrane vesicles. The prototype strains for 13 serotypes of group B *N. meningitidis* were M1080, serotype 1 (T-1); M986 (T-2); M981 (T-4); M992 (T-5); M990 (T-6); M978 (T-8); M982 (T-9); M1011 (T-10); M136 (T-11); S3032 (T-12); BC4 (T-13); S3446 (T-14); and 335H (T-15). These strains have been characterized (5). Six non-serotypable strains of group B *N. meningitidis* used in this study were 8-9, 8-59, 17-7, 17-129, B-7, and BB-114. The growth conditions in tryptic soy broth (Difco Laboratories, Detroit, Mich.) have been described (7). Briefly, a log-phase culture from brain heart infusion agar (Difco) containing 1% horse serum was inoculated into 200 ml of the tryptic soy broth in a 500-ml Wheaton bottle (Wheaton Scientific, Millville, N.J.). The organisms were grown overnight at 150 rpm on a gyratory shaker at 37°C and harvested by centrifugation at 12,000 × *g* for 10 min. Outer membrane vesicles, i.e., serotype antigens, were extracted from unwashed wet cells as described (17).

SDS-PAGE. The Laemmli SDS-PAGE system (10) incorporating 4 M urea in a 10% separating gel (17) was used in the slab gel apparatus of Maizel (11). The acrylamide-to-bisacrylamide ratio was 37.5:1. The samples, containing 1 to 2 mg of protein per ml, were mixed with an equal volume of the sample digestion buffer (17) and heated in a 100°C water bath for 5 min. Samples of 20 μl were then applied to 1.2-mm-thick, 6-mm-wide sample wells and subjected to electrophoresis at 20 mA per slab gel until the bromophenol blue migrated 9 to 10 cm. The proteins in the gel were fixed and stained overnight in 40% methanol–7.5% acetic acid containing 0.025% Coomassie blue R-250. The gel was then destained in 10% methanol–7.5% acetic acid containing Dowex 1 and Dowex 50 ion-exchange resins.

^{125}I-peptide mapping of OMPs on cellulose thin-layer plates. ^{125}I-peptide maps of proteins were obtained by a modification of described methods (3, 17). Briefly, OMPs were separated on SDS-PAGE and stained with Coomassie blue. The individual protein bands were sliced from the gel with a razor blade and placed in polypropylene tubes (12 by 75 mm). Each gel slice was soaked in 10% methanol overnight to remove SDS and then dried. The proteins within the gel slices were radioiodinated by the chloramine T method by rehydrating the gel slice in 20 μl of 0.5 M sodium phosphate (pH 7.5) containing 100 μCi of Na^{125}I and 5 μg of chloramine T (3). Each iodinated gel slice was placed in a well (17.8 by 16 mm) of a tissue culture plate (Costar, Cambridge, Mass.) containing about 3 g of Dowex 1-X8 (Bio-Rad, chloride form) in 10% methanol for 1 day to remove unbound ^{125}I$^-$. The gel slice was again dried, and the protein in the slice was digested with 50 μg of either α-chymotrypsin or trypsin in 0.5 ml of fresh 50 mM ammonium bicarbonate at 37°C overnight. The soluble digestion mixture plus methylene green was applied to cellulose-coated thin-layer glass plates (10 by 10 cm; EM Laboratories, Elmsford, N.Y.). The plates were subjected to high-voltage electrophoresis (1,000 V) until the tracking dye reached the opposite edges of the plates (about 20 min). The plates were air dried and then chromatographed in a second solvent for 1 h. The electrophoretic buffer was acetic acid-pyridine-water (10:1:200) at pH 3.7, and the chromatographic solvent was *n*-butanol–pyridine–acetic acid–water (65:50:10:40). ^{125}I-peptides on the thin-layer plates were detected by radioautography using Kodak PR-2 X-ray film. For comparison of the peptide maps of two proteins, the overall peptide patterns are more informative than the exact locations of individual peptides since small variations in the locations of peptides may occur due to slight variations in electrophoresis or chromatography. The unique and common peptides of the two proteins were identified by using three maps, i.e., two separated individual peptide maps and one combined map of the mixture of these two samples.

RESULTS

Analysis of protein species in outer membrane vesicles from different serotypes on SDS-PAGE. The protein patterns of outer membranes from serotypes 1 through 15 on Laemmli SDS-PAGE are shown in Fig. 1. The protein patterns were similar to those reported with Weber-Osborn SDS-PAGE (18), except that serotype 1 in Fig. 1 had six major protein bands on Laemmli gel (the fast-moving thick band contained two closely spaced protein species which could be resolved with a smaller amount of protein), compared to four protein bands in the Weber-Osborn system (7).

Chymotryptic and tryptic ^{125}I-peptide maps of major OMPs of strain M986. The four major OMPs of the serotype 2 strain M986 are structurally different as revealed by two-dimensional chymotryptic and tryptic peptide maps. The chymotryptic ^{125}I-peptide maps of the proteins having apparent MWs of 46K, 41K, 32K, and 28K are shown in Fig. 2. Each of the four different MW proteins had a different chymotryptic map, indicating differences in their primary structure. These four proteins also had very different tryptic ^{125}I-peptide maps (maps not shown). As expected, for the same protein the chymotryptic and tryptic maps were different. Although the tryptic map of the 41K protein could resolve as many discrete peptide spots as the chymotryptic map, the tryptic maps of the 46K, 32K, and 28K proteins showed fewer discrete spots. Chymotryptic ^{125}I-peptide mapping was therefore chosen for comparative structural analysis of major OMPs from all of the serotypes.

Comparative structural analysis of major OMPs of serotypes 1 and 2 by chymotryptic ^{125}I-peptide mapping. M986 (serotype 2) and M1080 (serotype 1) strains were chosen for ini-

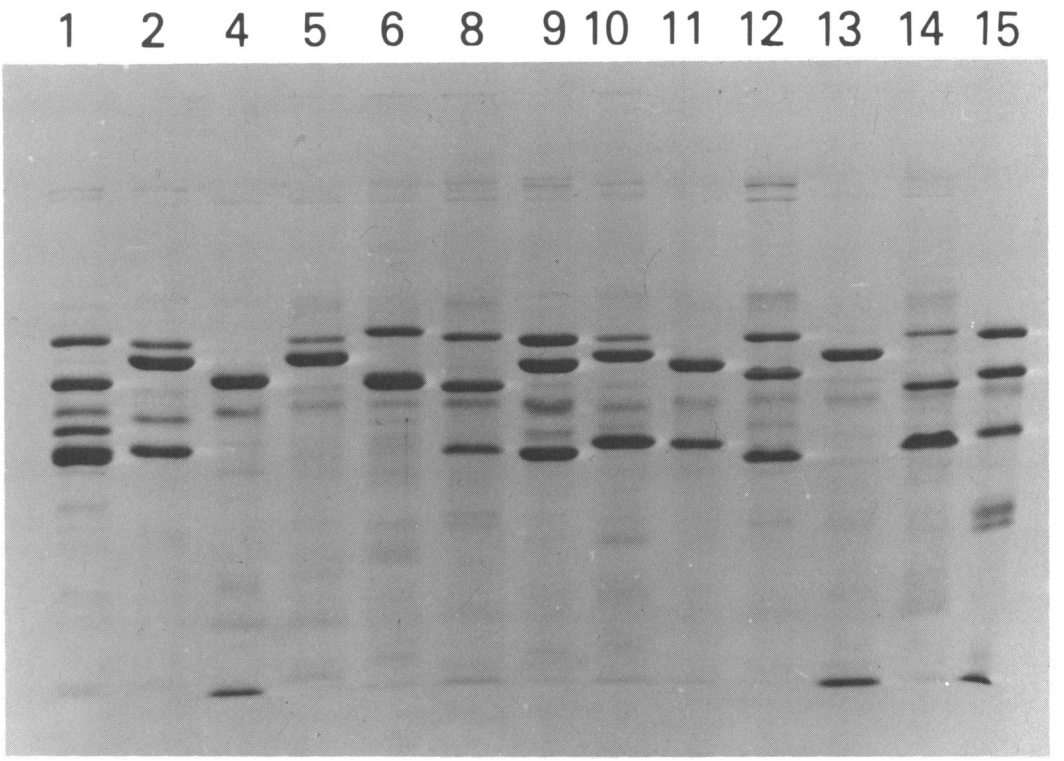

FIG. 1. *Protein patterns of outer membranes from different serotype strains on Laemmli SDS-PAGE using a 10% gel containing 4 M urea. The numbers on the top, 1 through 15, are protein serotypes. The missing serotypes, 3 and 7, were minor antigens that cross-react with serotypes 8 and 2, respectively (5). The prototype strains, with the serotypes in parentheses, are: M1080 (T-1), M986 (T-2), M981 (T-4), M992 (T-5), M990 (T-6), M978 (T-8), M982 (T-9), M1011 (T-10), M136 (T-11), S3032 (T-12), BC4 (T-13), S3446 (T-14), and 355H (T-15). The apparent MWs of the four major OMPs of M986, lane 2, were found to be 46K, 41K, 32K, and 28K, from top to bottom (6), and the major protein of M981, lane 3, was 38K.*

tial comparison of their major OMPs because the SDS-PAGE patterns of their outer membranes were different. The peptide maps of M1080 major OMPs are shown in Fig. 3. Four different peptide maps were observed for the six major OMPs of M1080. The proteins whose apparent MWs were 47K, 38K, and 33K had distinctly different maps. Three other proteins (30K, 28K, and 27K) had another type of map, but each of the three proteins had two to three unique peptides. This observation indicated that the latter three proteins in M1080 were structurally related, and that 27K and 28K proteins could be two proteolytic cleavage products of the 30K protein. However, the extraction of M1080 outer membrane vesicles from cells in the presence of 20 mM benzamidine, a protease inhibitor, did not alter the protein pattern of the outer membrane on SDS-PAGE.

Comparison of the peptide maps of the OMPs of M1080 and M986 indicated that proteins of the same or very similar apparent MW from these two strains had very similar but not identical peptide maps, suggesting that proteins of the same or very similar MW in these two strains are closely related in their primary structure.

Chymotryptic ^{125}I-peptide mapping of 46 ± 1K, 41 ± 1K, 38 ± 1K, and 28 ± 1K major OMPs from prototype strains and non-serotypable strains. To investigate whether the similarity in structure of the major OMPs of the same or very similar MW found for M986 and M1080 is common to other strains of *N. meningitidis*, comparative structural analysis by chymotryptic ^{125}I-peptide mapping was performed on the major OMPs from all prototype strains as well as from six nontypable group B strains. The major proteins of the outer membrane from different strains were grouped according to their apparent MWs and mapped. Six randomly selected ^{125}I-peptide maps from each of the 46 ± 1K, 41 ± 1K, 38 ± 1K, and 28 ± 1K proteins are

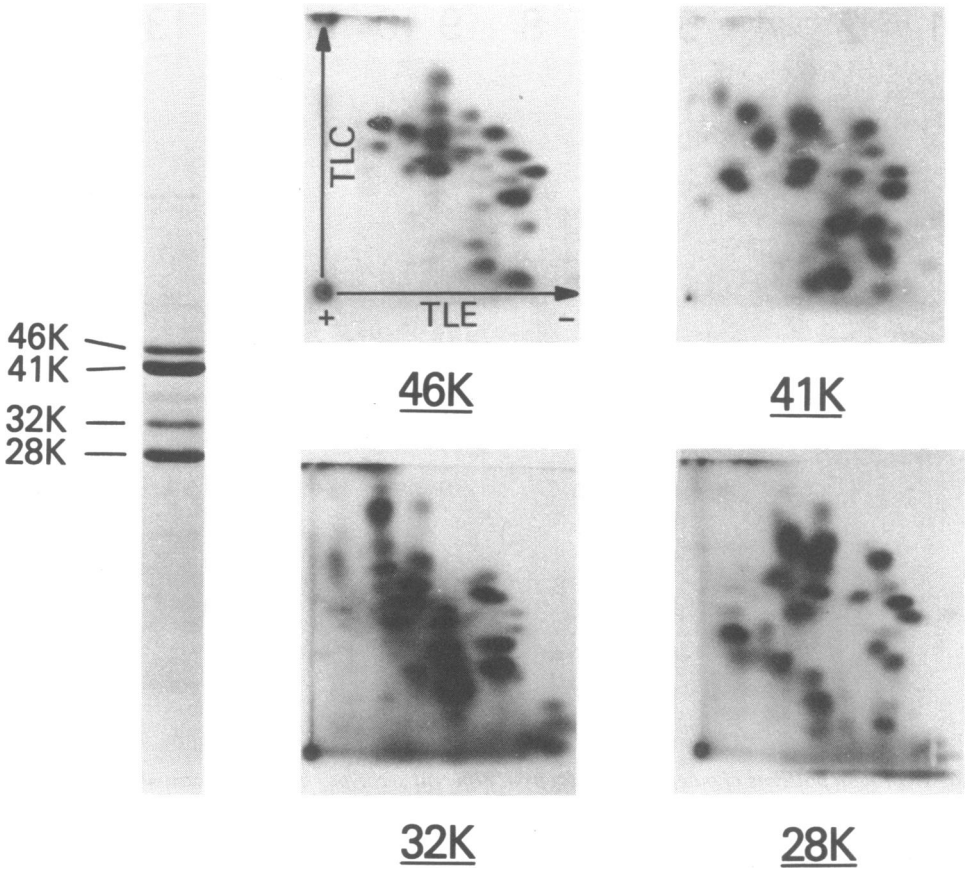

FIG. 2. *Chymotryptic ^{125}I-peptide maps of M986 (serotype 2) major OMPs. The SDS-PAGE pattern on the left shows four major OMPs of M986 with their apparent MWs indicated. The code under each peptide map is the protein from which the map is derived. The orientation of peptide maps is shown on the 46K protein map; TLE, thin-layer electrophoresis, anode (+) at left and cathode (−) at right; TLC, thin-layer chromatography (ascending).*

presented in Fig. 4, 5, 6, and 7, respectively. In each figure more than half of the major ^{125}I-peptide spots in the different maps were common, as shown in the first peptide map of the figure. In general, proteins of the same or very similar MW from different strains had similar, occasionally identical peptide maps. The one exception found was the major protein of M990, which had an apparent MW of 38K in Laemmli SDS-PAGE (see Fig. 1) but had a peptide map associated with 41 ± 1K proteins (map not shown). The MWs of membrane proteins are generally determined by Weber-Osborn SDS-PAGE because certain proteins may give anomalous MW in the Laemmli system (13). The M990 major protein had the greatest difference in the MW values on these two SDS-PAGE systems and had a MW of 41K in the Weber-Osborn system (Fig. 1, lane 7 in reference 7). Some other meningococcal OMPs such as M992 46K and M978 46K also had a small difference (about 1,000 daltons) in their apparent MWs on these two SDS-PAGE systems.

The ^{125}I-peptide maps of the 32K protein from M986 and the 33K protein from M1080 were similar, as shown in Fig. 2 and Fig. 3, respectively. Three additional 33K proteins from strains M981, M982, and M136 were examined, and their maps were similar to those of M986 and M1080 (peptide maps not shown).

Based upon the peptide maps of over 60 proteins from all serotypes, these major OMPs could be grouped into five structural classes. The proteins in each class had the same or very similar MWs, and the apparent MWs for these five classes of proteins are: class 1, 46 ± 1K; class

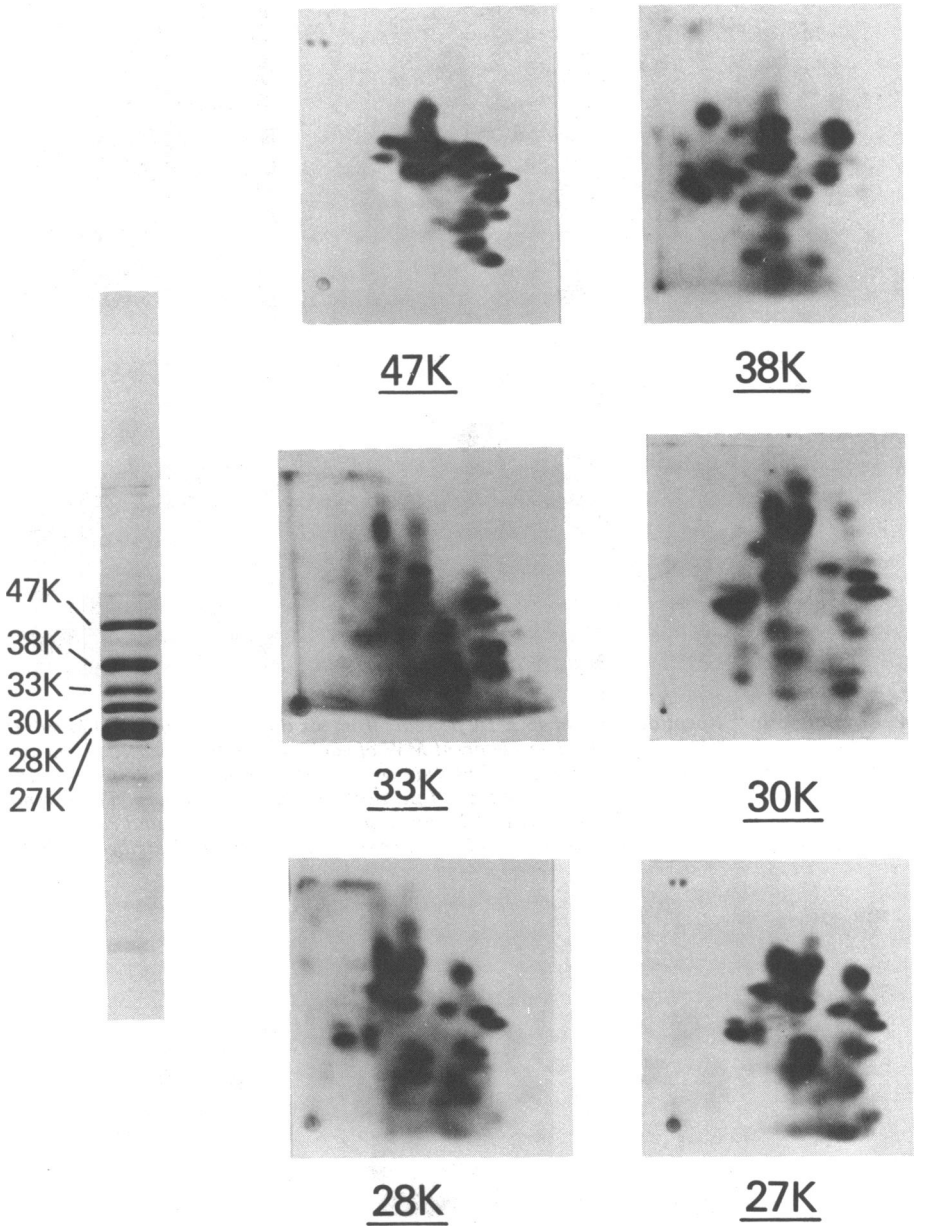

FIG. 3. *Chymotryptic ^{125}I-peptide maps of M1080 (serotype 1) major OMPs. The SDS-PAGE pattern on the left shows major OMPs of M1080 with their apparent MWs indicated. The code under each peptide map is the protein from which the map is derived. Other conditions were as described for Fig. 2.*

2, 41 ± 1K; class 3, 38 ± 1K; class 4, 33 ± 1K, and class 5, 28 ± 1K. A summary of the peptide mapping results is presented in Fig. 8.

For a direct comparison of unique and common peptides among proteins of the same or very similar MWs, a composite peptide map was drawn for three representative proteins from classes 1, 2, 3, and 5 (Fig. 9). The peptide map could be divided into halves, the upper half containing hydrophobic peptides and lower half containing hydrophilic peptides (17). The hydrophobic peptides in the upper halves of the maps from proteins of each class were mostly common peptides. This was most obvious for the class 5

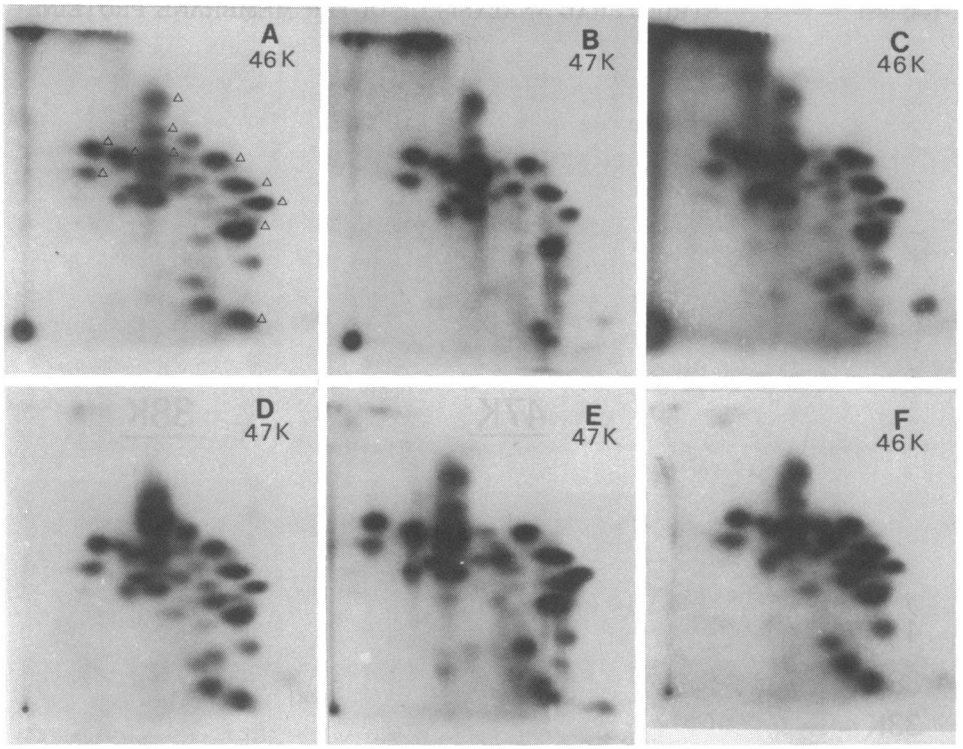

FIG. 4. *Chymotryptic ^{125}I-peptide maps of 46 ± 1K proteins from six different strains: (A) M986; (B) M990; (C) M992; (D) S3032; (E) S3446; (F) 355H. The apparent MW of each protein is indicated at the upper right corner of the map. Most of the common major ^{125}I-peptide spots in this group of proteins are indicated by triangles either on the right of or directly under the ^{125}I-peptide spots of the first map. The extended black lines at the top left corners of M986, M990, and M992 maps were caused by background radioactivity due to incomplete removal of unbound ^{125}I from these samples. Other conditions were as described for Fig. 2.*

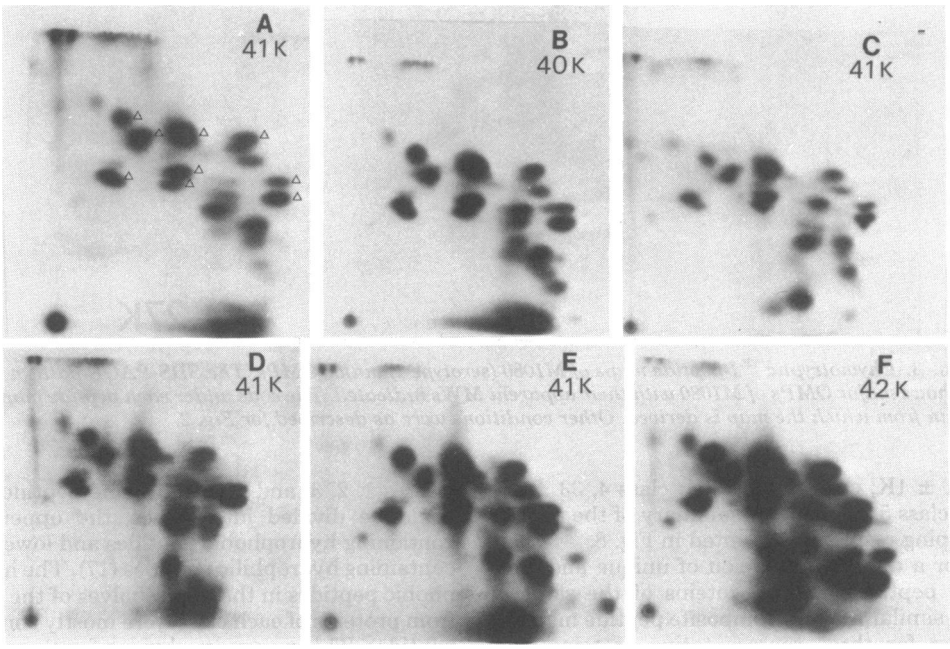

FIG. 5. *Chymotryptic ^{125}I-peptide maps of 41 ± 1K proteins from six different strains: (A) M992; (B) M982; (C) M1011; (D) BC4; (E) 17-219(SP-11); (F) 8-59(SP-13). Conditions were as described for Fig. 2 and 4.*

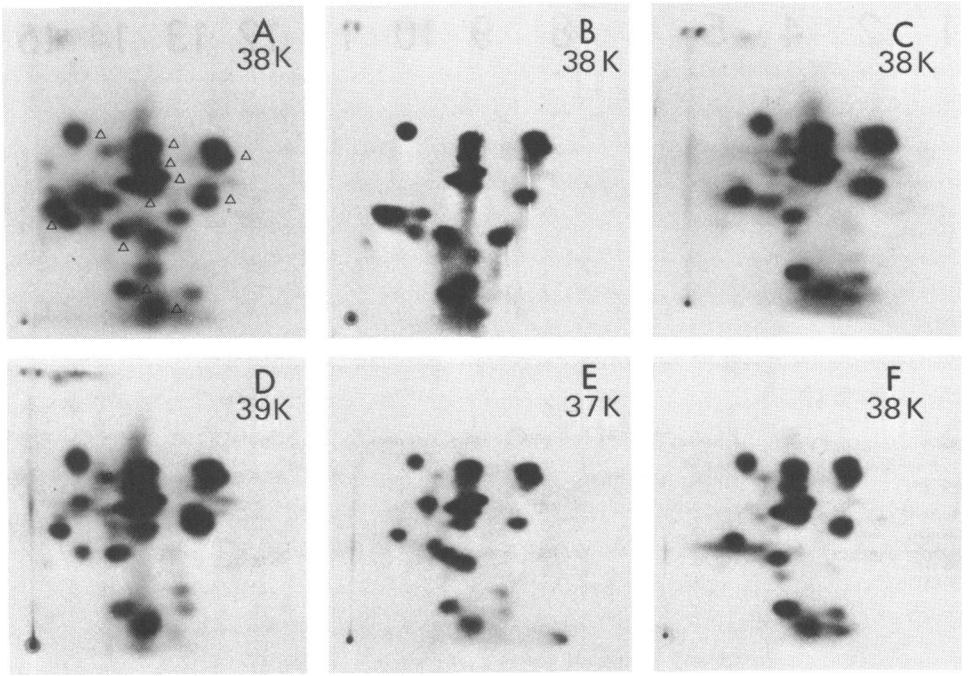

FIG. 6. *Chymotryptic ^{125}I-peptide maps of 38 ± 1K proteins from six different strains: (A) M1080; (B) M981; (C) M978; (D) 355H; (E) 8-9(SP-13); (F) BB-114. Conditions were as described for Fig. 2 and 4.*

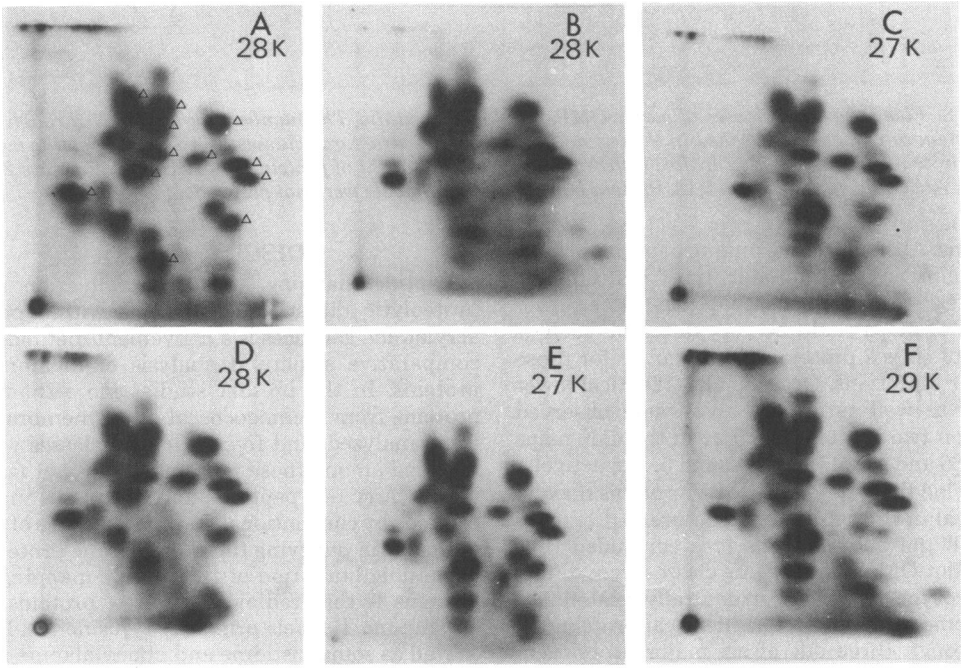

FIG. 7. *Chymotryptic ^{125}I-peptide maps of 28 ± 1K proteins from six different strains: (A) M986; (B) M978; (C) M982; (D) M136; (E) S3032; (F) S3446. Conditions were as described for Fig. 2 and 4.*

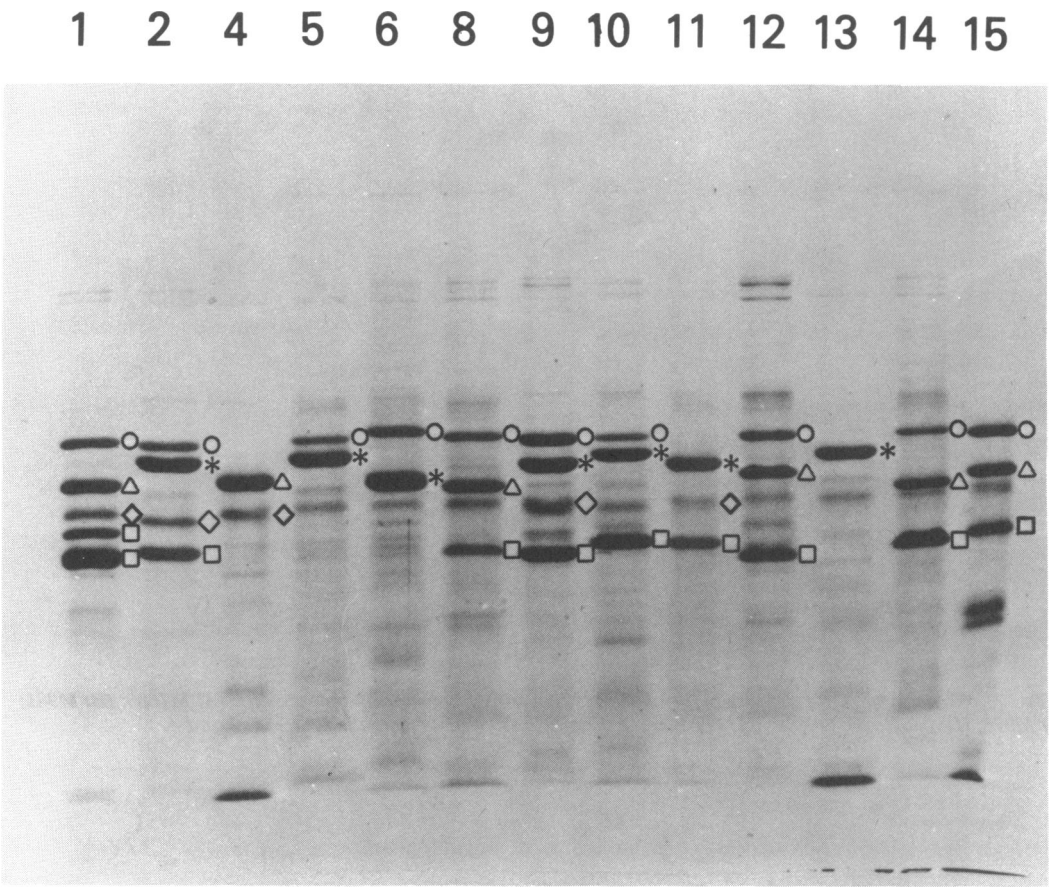

FIG. 8. *Five structural classes of major OMPs in N. meningitidis. The numbers on the top, 1 through 15, are protein serotypes as described in the legend for Fig. 1. Five structural classes based on the peptide maps of the proteins are represented by five different symbols on the right of protein bands: class 1, ○; class 2, *; class 3, △; class 4, ◇; and class 5, □. Protein bands without symbols were not mapped.*

proteins. Although some unique peptides in class 3 proteins are hydrophobic, most hydrophobic peptides were common peptides. The lower halves of maps could be very different, such as those of class 2 proteins, or similar, as for those of class 3 proteins. Occasionally, identical lower halves as well as upper halves were observed between two proteins, such as in the 41K maps of M986 and M1011 in Fig. 2 and 5, respectively, indicating that these same MW proteins may be identical in their primary structure.

From the above results it is concluded that the major OMPs of the same classes from different serotype strains are structurally related and that some of them may be identical proteins.

Although three out of six major proteins in strain M1080, i.e., the 30K, 28K, and 27K proteins, had very similar peptide maps (Fig. 3), all of the major proteins within each of 20 other strains examined were structurally distinct.

DISCUSSION

Peptide mapping after radioiodination and proteolytic digestion of proteins within polyacrylamide gel slices is a convenient method for comparative structural analysis of membrane proteins. In the present studies more than 60 proteins from meningococcal outer membranes were analyzed, and five structural classes were revealed among these proteins. It was not feasible to carry out peptide analysis on this many proteins by conventional methods because of the difficulty in purifying these membrane proteins. A possible limitation of ^{125}I-peptide mapping of proteins is that radioiodination of proteins by chloramine T labels primarily tyrosine residues as well as some histidine and phenylalanine residues (9); therefore, peptides without these amino acids will not be detected.

Comparative structural analysis of all major proteins from over 20 strains of group B *N.*

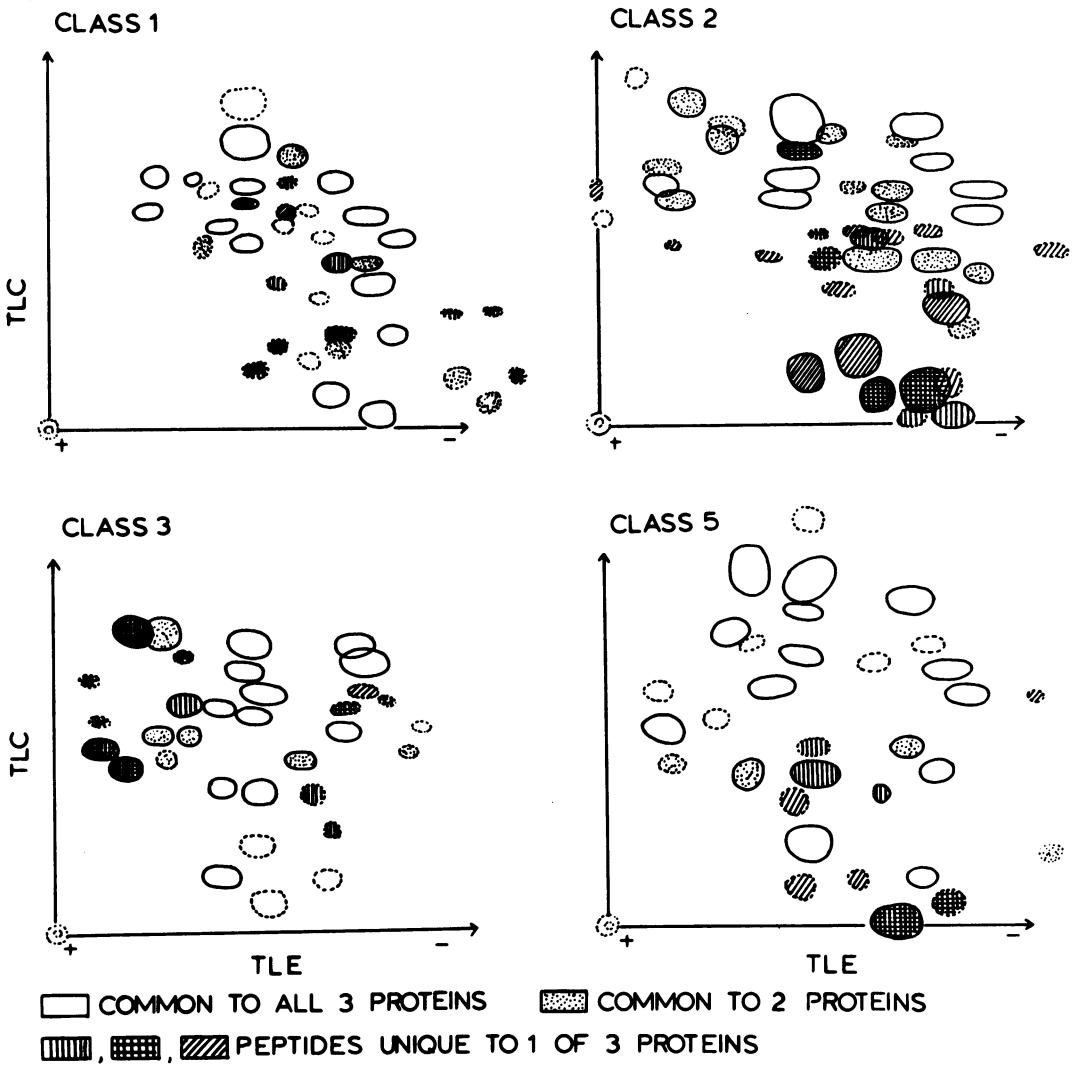

FIG. 9. *Unique and common peptides among OMPs of the same classes, as depicted by a composite peptide map for three representative proteins from each class. The four classes are class 1, 2, 3, and 5 as shown on the top left corners of the maps. TLE, Thin-layer electrophoresis; TLC, thin-layer chromatography.*

meningitidis revealed that within a strain the three to four major OMPs differed in their primary structure. These observations suggest that the different MW major OMPs within a strain may be coded by different genes. This notion was further supported by the chymotryptic peptide mapping results on 65K minor OMPs of S6317 (nontypable) and BB-114 (nontypable), which had another class of peptide map distinct from those presented in Fig. 8 (unpublished data). OMPs may therefore be used as markers for genetic and epidemiological studies of *N. meningitidis*.

The peptide mapping results showed that the same or very similar MW proteins from different strains were closely related but not identical in their primary structure. These differences in structure are most likely due to genetic variations in a common ancestral gene resulting in a group of very similar, but not identical proteins.

Although there was considerable variation between strains in their OMP patterns on SDS-PAGE, proteins of the same or very similar MW could be grouped into a common class. To facilitate our ongoing structural studies, we have designated the following OMP classes: (i) class 1, 45K to 47K proteins; (ii) class 2, 40K to 42K proteins; (iii) class 3, 37K to 39K proteins, (iv) class 4, 32K to 34K proteins; and (v) class 5, 27K to 29K proteins. The class 2 proteins and class

5 proteins are heat-modifiable proteins (8). The class 2 and 3 proteins may well be the porins because they form trimers and are largely embedded within the membrane, as shown by insolubility in deoxycholate and in situ resistance to enzymatic cleavage (unpublished data). No strain has thus far been found to contain both class 2 and class 3 major proteins.

Cell surface labeling experiments of intact meningococci of M986 (type 2) with lactoperoxidase in the presence of $^{125}I^-$ and hydrogen peroxide suggest that OMPs of classes 1, 2, 4, and 5 are cell surface proteins (unpublished data). Whether class 3 proteins are also surface proteins is under investigation.

The different outer membrane antigens used for serotyping usually contain proteins of classes 1, 2 (or 3), and often 5 (7, 14). The structural similarities between proteins of the same class are probably responsible for much of the cross-reactivity observed in the enzyme-linked immunosorbent assay (7, 14).

Comparative structural analysis of the major OMPs revealed not only that the proteins of the same classes from different serotype strains had many common hydrophobic peptides, but also that many of the variable unique peptides were hydrophilic (Fig. 9). A cell surface OMP is probably oriented in such a way that hydrophilic regions protrude from the cell wall into an aqueous environment, whereas hydrophobic regions are embedded in the lipid bilayer. Exposed hydrophilic segments of the OMPs probably carry the serotype determinants, which may reside in the different peptides of their variable hydrophilic peptides (see Fig. 9).

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