JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1986, p. 330–332
0095-1137/86/090330-03$02.00/0

Vol. 24, No. 3

Rapid Microprocedure for Isolating Detergent-Insoluble Outer Membrane Proteins from *Haemophilus* Species

GEORGE M. CARLONE,* MYRTLE L. THOMAS, HELLA S. RUMSCHLAG, AND FRANCES O. SOTTNEK

Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333

Received 31 March 1986/Accepted 29 May 1986

A rapid microprocedure for isolating detergent (sodium *N*-lauroyl sarcosinate)-insoluble major outer membrane proteins from *Haemophilus* species produced results qualitatively identical to those obtained with a commonly used preparative isolation procedure. Proteins isolated by both procedures were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after staining with Coomassie brilliant blue R-250. The time for outer membrane protein isolation was substantially reduced with the rapid procedure, allowing a larger number of membrane preparations to be obtained rapidly for routine analysis.

Membrane proteins are important determinants of pathogenicity in many bacteria. *Haemophilus* species can be subtyped (2, 3, 12, 18) and often differentiated (4) on the basis of these proteins. In general, gram-negative bacterial outer membrane proteins are isolated by preparative techniques (9–11, 13, 22, 24), some of which require an initial enrichment step that exploits a selective solubility of cytoplasmic membrane proteins in a particular detergent (1, 5–7, 21, 22). The conditionally insoluble membranes contain integral and likely some peripheral proteins which are released by relatively small changes in pH or ionic strength and are referred to as outer membrane proteins and, more appropriately, detergent-insoluble membrane proteins (6, 15). Phenotypic and epidemiologic information can be obtained by comparing the protein profiles of these sodium dodecyl sulfate (SDS)-solubilizable membranes (6, 8) after SDS-polyacrylamide gel electrophoresis (PAGE) and staining (2, 4, 12, 14, 15, 18, 23).

We compared the membrane protein profiles of three species of *Haemophilus* obtained by a rapid microprocedure (rapid outer membrane protein [ROMP] procedure) with profiles obtained by a commonly used preparative isolation procedure (4) to determine whether the two procedures produced comparable results.

MATERIALS AND METHODS

Bacterial cultures and cell harvest. *H. influenzae* type b KC525 and *H. aegyptius* NCTC 8502 were obtained from the culture collection of the Bacterial Reference Laboratory, Meningitis and Special Pathogens Branch, Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Ga., and *H. parainfluenzae* ATCC 7857 was obtained from the Sexually Transmitted Diseases Laboratory Program, Center for Infectious Diseases. Cells stored at −70°C were inoculated onto chocolate agar plates (BBL Microbiology Systems, Cockeysville, Md.) and incubated at 37°C for 24 h in a candle extinction jar. Isolated colonies (n = 2 to 4) were inoculated into 10 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 5 μg each of hemin and NAD (Sigma Chemical Co., St. Louis, Mo.). The inoculated tubes were slowly rotated (100 rpm) overnight (circa 18 h) at 37°C with the caps loosened; after 18 h of growth, the cultures were in the stationary phase. A chocolate agar plate was streaked from each culture and incubated as described above to check for purity. The optical density of each culture was measured and adjusted to between 0.5 and 0.6 at 600 nm by dilution with sterile supplemented brain heart infusion broth. A 10-ml sample of the adjusted bacterial suspension was centrifuged at 5,000 × g for 10 min at 4°C. The supernatant fluid was decanted, the pellet was suspended in 1.5 ml of cold (4°C) 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4), and the cell suspension was transferred to a 1.5-ml centrifuge tube. The cells were then washed once by centrifugation at 15,600 × g for 2 min at 4°C in a microcentrifuge (Brinkmann Instruments, Inc., Westbury, N.Y.), and the supernatant fluid was decanted. The cell pellets were used immediately or stored at −70°C.

Preparative outer membrane protein and SDS-PAGE. The procedures for detergent-insoluble membrane protein preparation (outer membrane proteins), SDS-PAGE (4), and gel analysis (19) have been previously described.

ROMP procedure. Sodium *N*-lauroyl sarcosinate (Sarkosyl; Pfaltz and Bauer, Stamford, Conn.)-insoluble proteins (referred to as outer membrane proteins) were obtained from the cell pellets by a modification of the procedures of Filip et al. (6) and Portnoy et al. (20). Cell pellets were suspended in 1 ml of 10 mM HEPES buffer (pH 7.4) and, while kept on ice, sonicated (six bursts, 10 s each at 40 W) with a Sonifier cell disruptor (Heat Systems Co., Melville, N.Y.) fitted with a microtip. The unbroken cells and debris were removed by centrifugation at 15,600 × g for 2 min at 4°C. The supernatant fluid was transferred to a 1.5-ml centrifuge tube, and the cell membranes were sedimented from the supernatant fluid by centrifugation at 15,600 × g for 30 min at 4°C in a microcentrifuge (Brinkmann). The supernatant fluid was decanted, and the cell membrane pellets were thoroughly suspended in 0.2 ml of 10 mM HEPES buffer (pH 7.4) by repeated pipetting. The cytoplasmic membranes were solubilized by addition of an equal volume of 2% Sarkosyl in 10 mM HEPES (pH 7.4) and incubated at room temperature for 30 min with intermittent mixing. The outer membranes were then pelleted by centrifugation as described above; the supernatant fluid was decanted, and the membranes were washed once (without resuspending the pellet) with 0.5 ml of 10 mM HEPES buffer. The membrane pellets were then suspended in 50 to 100 μl of 10 mM HEPES buffer.

* Corresponding author.

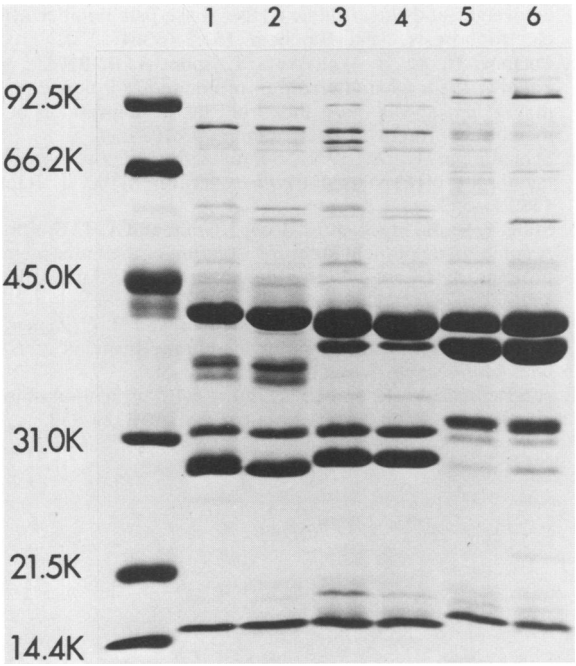

FIG. 1. Outer membrane proteins of *H. aegyptius* NCTC 8502 (lanes 1 and 2), *H. influenzae* type b KC525 (lanes 3 and 4), and *H. parainfluenzae* ATCC 7857 (lanes 5 and 6) were prepared by a preparative procedure (4) (lanes 2, 4, and 6) or a ROMP procedure (lanes 1, 3, and 5). The proteins were separated by SDS-PAGE with 4% stacking and 11% separating gels and stained with Coomassie brillant blue R-250. Protein standards: phosphorylase b (92.5 kilodaltons [kDa]), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

The ROMP procedure routinely yielded preparations containing 2 to 4 mg of protein per ml. The membrane suspensions were diluted in distilled water to contain 50 μg of protein in 25 μl. After an equal volume of solubilization buffer was added (20% glycerol, 10% 2-mercaptoethanol, 4% SDS in 0.125 M Tris [pH 6.8] and 10 μl of 0.05% bromphenol blue per ml of buffer), the suspensions were heated to 100°C for 5 min and loaded onto an SDS-polyacrylamide gel. The remainder of the membrane preparation was stored at −70°C.

Protein determination. The concentration of protein in the membrane preparation was determined by the method of Markwell et al. (16) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

A comparison of a commonly used preparative procedure for outer membrane protein isolation with the ROMP procedure for three species of *Haemophilus* yielded qualitatively identical protein profiles on Coomassie blue-stained SDS-polyacrylamide gels (Fig. 1). Outer membranes were isolated at least five times by each procedure from the three *Haemophilus* species to determine protein band variability; Fig. 1 is a representative gel pattern. There were quantitative differences in the amounts of protein detected in some of the minor bands, although the same bands were observed by either isolation procedure. Similar variability was also observed with strains of *Escherichia coli* when screening for plasmid-encoded outer membrane proteins (data not shown).

The commonly used preparative procedures for outer membrane protein isolation are somewhat cumbersome since they are time consuming and require relatively large amounts of cells and expensive equipment (an ultracentrifuge) (2, 4, 5, 15). The ROMP procedure required approximately 2 h to complete; the other procedure we used (4) required, on average, five times as long to prepare an equivalent number of preparations. For most replicate analyses there is no need to isolate preparative amounts of membrane protein since the anionic dye Coomassie brilliant blue R-250, the most commonly used protein stain, can detect as little as 0.5 μg of protein in a sharp band (8) resolved by SDS-PAGE, and silver staining is approximately 100 times more sensitive (17). The ROMP procedure allows large numbers of membrane preparations to be obtained rapidly for routine outer membrane protein analysis without requiring large amounts of cells or an expensive ultracentrifuge. This allows outer membrane protein analysis to be used, when appropriate, in the diagnostic laboratory as an adjunct to epidemiologic investigations.

ACKNOWLEDGMENTS

We thank William O. Schalla and Leo Pine for many constructive comments and suggestions in preparing the manuscript.

LITERATURE CITED

1. **Achtman, M., S. Schwuchow, R. Helmuth, G. Morelli, and P. A. Manning.** 1978. Cell-cell interaction in conjugating *Escherichia coli*: con⁻ mutants and stabilization of mating aggregates. Mol. Gen. Genet. **164**:171–183.
2. **Barenkamp, S. J., R. S. Munson, Jr., and D. M. Granoff.** 1981. Subtyping isolates of *Haemophilus influenzae* type b by outer membrane protein profiles. J. Infect. Dis. **143**:668–676.
3. **Barenkamp, S. J., R. S. Munson, Jr., and D. M. Granoff.** 1982. Outer membrane protein and biotype analysis of pathogenic nontypable *Haemophilus influenzae*. Infect. Immun. **36**: 535–540.
4. **Carlone, G. M., F. O. Sottnek, and B. D. Plikaytis.** 1985. Comparison of outer membrane protein and biochemical profiles of *Haemophilus aegyptius* and *Haemophilus influenzae* biotype III. J. Clin. Microbiol. **22**:708–713.
5. **Erwin, A. L., and G. E. Kenny.** 1984. *Haemophilus influenzae* type b isolates show antigenic variation in a major outer membrane protein. Infect. Immun. **46**:570–577.
6. **Filip, C., G. Fletcher, J. L. Wulff, and G. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115**:717–722.
7. **Goding, J. W.** 1983. Monoclonal antibodies: principles and practice, p. 134–187. Academic Press, Inc., New York.
8. **Hames, B. D.** 1981. An introduction to polyacrylamide gel electrophoresis, p. 1–91. *In* B. D. Hames and D. Rickwood (ed.), Gel electrophoresis of proteins—a practical approach. IRL Press, Arlington, Va.
9. **Hekcles, J. E.** 1977. The surface properties of *Neisseria gonorrhoeae*: isolation of the major components of the outer membrane. J. Gen. Microbiol. **99**:333–341.
10. **Helenius, A., and K. Simons.** 1975. Solubilization of membranes by detergents. Biochim. Biophys. Acta **415**:29–79.
11. **Hjelmeland, L. M., and A. Chrambach.** 1984. Solubilization of functional membrane proteins. Methods Enzymol. **104**:305–318.
12. **Honberg, P. Z.** 1985. Subtyping of Danish *Haemophilus influenzae* type b by their 4500 and 4600 molecular weight proteins. Acta Pathol. Microbiol. Immunol. Scand. **93**:175–179.
13. **Johnston, K. H., and E. C. Gotschlich.** 1974. Isolation and characterization of the outer membrane of *Neisseria gonorrhoeae*. J. Bacteriol. **119**:250–257.
14. **Loeb, M. R., and D. H. Smith.** 1980. Outer membrane protein composition in disease isolates of *Haemophilus influenzae*: pathogenic and epidemiological implications. Infect. Immun.

30:709–717.
15. **Lutenberg, B., J. Meijers, R. Peters, P. V. D. Hoek, and L. V. Alphen.** 1975. Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* K12 into four bands. FEBS Lett. **58**:254–258.
16. **Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87**:206–210.
17. **Morrissey, J. H.** 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117**:307–310.
18. **Murphy, T. F., K. C. Dudas, J. M. Mylotte, and M. A. Apicella.** 1983. A subtyping system for nontypable *Haemophilus influenzae* based on outer-membrane proteins. J. Infect. Dis. **147**:838–846.
19. **Plikaytis, B. D., G. M. Carlone, P. Edmonds, and L. W. Mayer.** 1986. Robust estimation of standard curves for protein molecular weight and linear-duplex DNA base-pair number after gel electrophoresis. Anal. Biochem. **152**:346–364.
20. **Portnoy, D. A., H. Wolf-Watz, I. Bolin, A. B. Beeder, and S. Falkow.** 1984. Characterization of common virulence plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. Infect. Immun. **43**:108–114.
21. **Schnaitman, C. A.** 1971. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. J. Bacteriol. **108**:545–552.
22. **Stull, T. L., K. Mack, J. E. Haas, J. Smit, and A. L. Smith.** 1985. A comparison of techniques for isolation of the outer membrane proteins of *Haemophilus influenzae* type b. Anal. Biochem. **150**:471–480.
23. **van Alphen, L., T. Riemens, J. Poolman, and H. C. Zanen.** 1983. Characteristics of major outer membrane proteins of *Haemophilus influenzae*. J. Bacteriol. **155**:878–885.
24. **van Renswoude, J., and C. Kempf.** 1984. Purification of integral membrane proteins. Methods Enzymol. **104**:329–339.