JOURNAL OF BACTERIOLOGY, June 1988, p. 2646–2653
0021-9193/88/062646-08$02.00/0

Vol. 170, No. 6

Polyacrylamide Gel Electrophoresis of the Capsular Polysaccharides of *Escherichia coli* K1 and Other Bacteria

SINIKKA PELKONEN,[1]†* JUKKA HÄYRINEN,[2] AND JUKKA FINNE[1,2]

Department of Biochemistry, Biocenter, University of Basel, CH-4056 Basel, Switzerland,[1] and Department of Biochemistry, University of Kuopio, SF-70211 Kuopio, Finland[2]

Received 2 December 1987/Accepted 8 March 1988

Methods were developed for the polyacrylamide gel electrophoretic analysis of capsular polysaccharides of bacteria with *Escherichia coli* K1 as a model. Conditions were determined for the rapid and gentle extraction of the K1 polysaccharide by incubation of the bacteria in a volatile buffer and for the subsequent removal of the putative phospholipid moiety attached to the reducing end of the polysaccharide. Detection of the polysaccharides after gel electrophoresis was carried out by fluorography of samples labeled by sodium borotritiide reduction or by combined alcian blue and silver staining. The smallest components could be detected only by fluorography, owing to diffusion during staining. Components of the *E. coli* K1 polysialic acid capsule ranging from monomers to 80 sialic-acid-unit-containing polymers could be separated as distinct bands in a ladderlike pattern. A maximum chain length of 160 to 230 sialyl residues was estimated for the bulk of the K1 polysaccharide from the nearly linear reciprocal relationship between the logarithm of the molecular size and the distance of migration. Gel electrophoresis of capsular polysaccharides of other bacterial species revealed different electrophoretic mobilities for each polysaccharide, with a ladderlike pattern displayed by the fastest-moving components. There are many potential applications of this facile method for the determination of the sizes of molecules present in a polydisperse polysaccharide sample. When combined with the simple method for the isolation of the capsule, as in the case of the K1 capsule, it provides an efficient tool for the characterization and comparison of the capsular polysaccharides of bacteria.

Capsular polysaccharides are important bacterial surface determinants that consist of polymers with repeating units of one or more monosaccharides (15, 16, 30). Characterization of bacterial proteins and lipopolysaccharides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis is a commonly applied method in comparative and structural studies. Similar methods have not been available for capsular polysaccharides, and their characterization has therefore been hampered by the need for cumbersome analytical procedures. The chain length of the polysaccharides has been determined with methods like gel filtration, ion-exchange chromatography, determination of the ratio of the terminal and internal residues in the polysaccharide chain, and high-performance liquid chromatography (13, 19, 20, 26, 30).

One of the most extensively studied capsules is the *Escherichia coli* K1 capsule, a homopolymer with up to 200 sialic acid residues and α2-8 linkages (4, 21, 26). Capsular antibodies and capsule-specific bacteriophages seem to recognize conformational determinants in the polysaccharide chain. In the case of the K1 capsule, mono- and polyclonal anti-K1 antibodies (8, 27) require about eight sialyl residues for binding (6, 7), whereas different K1-specific bacteriophages containing capsule-degrading endosialidases differ in their minimum substrate size and capsule-splitting patterns (7, 12, 18). Although a high-performance liquid chromatographic method to determine the chain length of sialic acid oligomers was recently described (13), a simple method applicable for both capsular oligosaccharides and capsular polysaccharides is needed for the characterization of strains differing in the amount of capsular material exposed on their surfaces (17, 25, 33). Such a method would also facilitate studies of the molecular interactions in which sialic acid is involved.

Recently, several reports of high-resolution electrophoretic methods for the separation of glycosaminoglycan oligosaccharides have been published (5, 14, 23). Polyacrylamide gel electrophoresis techniques adapted from procedures used in nucleic acid research separate polysaccharides on the basis of size and charge/mass ratio. Since capsular acidic polysaccharides of different chain lengths should have identical charge/mass ratios, we investigated whether they could be characterized by gel electrophoresis. Using the *E. coli* K1 polysaccharide as a model, we describe the application of polyacrylamide gel electrophoresis for the characterization of capsular polysaccharides of bacteria. This method represents a convenient and efficient approach for analysis of chain length and general characterization of capsular polysaccharides.

MATERIALS AND METHODS

Materials. Bacteriophage PK1B (11) was kindly provided by B. Rowe, Central Public Health Laboratory, Colindale, London, England. *E. coli* IH3088 (rough:K1), IH3044 (O2:K1), IH3056 (O7:K1), and IH3080 (O18:K1) were kindly supplied by P. H. Mäkelä, National Public Health Institute, Helsinki, Finland. *E. coli* K235 (O1:K1) and IH3036 (O18:K1) have been described elsewhere (1). *Neisseria meningitidis* group A and group C polysaccharides were obtained from Merck Sharp & Dohme Research Laboratories, West Point, Pa., and group B polysaccharide was obtained from Connaught Laboratories, Swiftwater, Pa. *E. coli* K5 polysaccharide was kindly provided by K. Jann, Max-Planck-Institut für Immunbiologie, Freiburg, Federal

* Corresponding author.
† Present address: National Veterinary Institute, P.O. Box 368, SF-00101 Helsinki, Finland.

Republic of Germany, and group B streptococcal polysaccharides of types Ia, Ib "core", Ic, II, and III were provided by D. L. Kasper, Channing Laboratory, Boston, Mass. Colominic acid (sodium salt) and *N*-acetylneuraminic acid were purchased from Sigma Chemical Co., St. Louis, Mo. Electran acrylamide and *NN'*-methylenebisacrylamide were from BDH Chemicals Ltd., Poole, England, and *NNN'N'*-tetramethylethylenediamine was from Bio-Rad Laboratories, Richmond, Calif. NaB^3H_4 (5 to 10 Ci/mmol) was obtained from Amersham International, the Superose column was from Pharmacia, Uppsala, Sweden, and Bio-Gel gel filtration media were from Bio-Rad Laboratories. 2,5-Diphenyloxazole was from E. Merck AG, Darmstadt, Federal Republic of Germany, *Vibrio cholerae* neuraminidase was from Behringwerke AG, Marburg, Federal Republic of Germany, and X-Omat AR film was from Eastman Kodak Co., Rochester, N.Y. Alcian blue (catalog no. 12021) was from Serva, Heidelberg, Federal Republic of Germany, and the silver stain kit was from Bio-Rad Laboratories.

Polyacrylamide gel electrophoresis. A modification of the method described recently for the analysis of glycosaminoglycans (14) was used. A stock solution of 50% acrylamide containing 495.9 g of acrylamide and 4.1 g of bisacrylamide per liter was prepared. A 10-fold concentrated electrophoresis buffer contained 0.89 M Tris base–0.89 M boric acid–0.02 M EDTA (pH 8.3). To prepare 20 ml of gel solution we mixed 10 ml of the acrylamide stock solution, 2 ml of the concentrated buffer, and 7.9 ml of H_2O and initiated polymerization by adding 7.5 μl of tetramethylethylenediamine and 75 μl of 10% ammonium persulfate. Mixtures were poured into glass plates (15-cm wide and 0.8-mm thick) differing in length (14, 25, and 37 cm) at room temperature. The longer plates were swept with dimethyldichlorosilane solution (BDH Chemicals Ltd.) to facilitate the detachment of the gel from the plate. After polymerization at room temperature the sample wells were carefully rinsed. Samples (2.5 to 30 μg in a volume of 5 to 15 μl) were mixed with 0.1 volume of 2 M sucrose in 0.089 M Tris–borate–EDTA buffer. Trypan blue (final concentration, 0.05%), xylene cyanol, bromphenol blue, bromcresol purple, and phenol red (final concentration of each, 0.02%) were used as tracking dyes and applied to the wells with no sample. Electrophoresis was performed at 4°C under cooling by a fan. The gel was preelectrophoresed at 12 V/cm for about 1 h, and the samples were layered onto the bottoms of the sample slots containing the running buffer. Electrophoresis was done at 25 to 40 V/cm. Running times varied from 3.5 to 18 h.

Fluorography and staining. After electrophoresis the gel was immediately immersed in prewarmed 20% (wt/vol) 2,5-diphenyloxazole in glacial acetic acid at 35°C and gently shaken for 6 to 8 min. The 2,5-diphenyloxazole in the gel matrix was precipitated in distilled water for 8 min. The gel was immediately dried in a conventional vacuum gel dryer and kept at −70°C with X-Omat AR film. The 2,5-diphenyloxazole was recycled in the manner described before (29). A new solution was prepared for each gel; the 2,5-diphenyloxazole was recrystallized after use with distilled water, recycled, and reused. In some experiments the wet gels were wrapped in a plastic film (Saran Wrap) and subjected without drying to fluorography. Reexposure of such gels was usually not possible because of distortion of the bands owing to diffusion during thawing. Staining of the gels was performed by the combined alcian blue-silver staining method described recently (23).

Extraction of the K1 capsule. The *E. coli* strains were grown in a strongly buffered defined medium (10 g of Casamino Acids [Difco Laboratories, Detroit, Mich.], 5 g of glucose, 12.5 g of $Na_2HPO_4 \cdot 2H_2O$, 0.9 g of KCl, and 0.6 g of $MgSO_4 \cdot 7H_2O$ per liter [pH 7.8]). The high buffering capacity of the medium keeps the pH at 7.4 during incubation, preventing acid-catalyzed hydrolysis of the polysaccharide (31). An overnight culture was diluted 1:250 in the defined medium and incubated at 37°C on a rotary shaker for 16 h. The culture was cooled to 4°C and pelleted at 1,500 × *g* for 15 min at 4°C. After two washes with cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.4]) and one wash with cold 0.1 M pyridine acetate (pH 5.0) at 1,500 × *g* for 15 min at 4°C, the bacteria were suspended in 0.1 volume of prewarmed pyridine acetate (0.1 M, pH 5.0) and incubated with intermittent gentle shaking in a 37°C water bath for 1 h. The supernatant obtained after centrifugation (10,000 × *g*, 10 min, 4°C) was lyophilized, and the sialic acid content was measured by a resorcinol reaction (22) with *N*-acetylneuraminic acid as a standard. The isolated K1 capsules were treated with 5 μl of 1% acetic acid-hydrochloride (pH 2) for 1 h on ice to disrupt the putative phospholipid-polysaccharide bond, kept on ice for 1 h at pH 10 (by adding 500 μl of cold 0.1 M NaOH) to saponify lactone rings, and finally neutralized with 6 μl of 0.1 M acetic acid. After lyophilization the material was desalted (see below) and analyzed by gel electrophoresis.

High-performance liquid chromatography. Lyophilized capsular polysialic acid (550 nmol) was dissolved in 400 μl of 20 mM sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl and fractionated on a Superose 6 column (10 by 300 mm) eluted with the same buffer (20 ml/h, pump P-500; Pharmacia Fine Chemicals, Piscataway, N.J.). Fractions of 400 μl were collected and analyzed for sialic acid content by a resorcinol reaction (22).

Radiolabeling of polysaccharides. The polysaccharides were ^3H labeled at their reducing ends by borotritiide reduction (7). To 100 μg of polysaccharide in 50 μl of H_2O 2 mCi of NaB^3H_4 in 100 μl of 10 mM NaOH was added on ice, and the reaction mixture was kept at room temperature for 4 to 5 h. The reaction was stopped by adding on ice 100 μl of cold 10 mM acetic acid, and the mixture was lyophilized. For the removal of salt the samples were dissolved in 100 μl of 50 mM ammonium bicarbonate–100 mM ammonium acetate (pH 7.2) and desalted by centrifugation (500 × *g*, 4 min) through a dry microcolumn (bed volume, 1 ml) prepared from a suspension of Bio-Gel P-2 in the same buffer (24).

Digestion of the K1 polysaccharide with phage-bound endosialidase. Phage PK1B was propagated on *E. coli* IH3088 and purified by polyethylene glycol precipitation by standard methods (19). The purified stock contained 7.4×10^{11} PFU/ml. Radiolabeled K1 polysaccharide (10 μg) and 10^9 PFU were incubated in 50 μl of 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 1% chloroform at 37°C for 24 h and lyophilized. Controls without phage were included.

Determination of the membrane-bound sialic acid content. For determination of the membrane-bound sialic acid content the bacteria were first washed by centrifugation (1,500 × *g*, 15 min) in phosphate-buffered saline. The amount normally corresponded to 500 μl of the original culture. The bacteria were then digested with *V. cholerae* neuraminidase (15 mU/500 μl of culture) in 0.1 M sodium acetate buffer (pH 5.5) containing 25 μg of bovine serum albumin per ml, 5 mM $CaCl_2$, and 5 mM NaN_3 at 37°C for 24 h. The supernatant obtained after pelleting the bacteria (10,000 × *g*, 5 min) was assayed for sialic acid content (22).

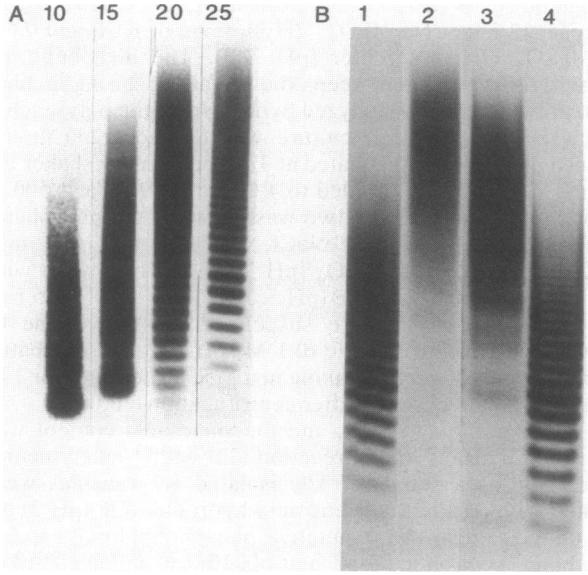

FIG. 1. (A) Effect of polyacrylamide concentration on the separation of ^{3}H-labeled colominic acid. Samples containing 5 μg of colominic acid were electrophoresed in gels with the indicated percentages of polyacrylamide at 10 mA and 4°C and subjected to fluorography for 1 to 2 days. (B) Effect of internal esters on the separation of colominic acid. ^{3}H-labeled colominic acid was treated with 0.1 M HCl at room temperature for 2 h and neutralized with 0.1 M $NaHCO_3$ or kept at an alkaline pH for 15 min before neutralization. Ten micrograms of each sample was electrophoresed at 35 V/cm for 3.5 h in a 25% gel at 4°C. The film was exposed for 1.5 days. Lanes: 1, not treated; 2, treated with acid; 3, treated with acid and neutralized; 4, treated with acid and kept at an alkaline pH before neutralization.

RESULTS

Gel electrophoresis of colominic acid. For initial studies colominic acid, a commercially available preparation of the capsular polysaccharide of *E. coli* K1, was used. Different acrylamide and bisacrylamide ratios and total polyacrylamide concentrations were first tested to determine the gel composition which gave the best resolution. The resolution of the individual oligosaccharide components increased with the percentage of acrylamide (Fig. 1A). Gels containing 25% polyacrylamide were used in further experiments, since a further increase in percentage did not improve the resolution significantly but extended the electrophoresis time. The percentage of the cross-linker bisacrylamide relative to total polyacrylamide had to be kept as low as 0.82% to prevent cracking during drying. The gel system required efficient cooling; otherwise, uneven running of the samples because of heat differences between the middle and lateral parts of the gel was very prominent.

Exposure of colominic acid to a low pH before electrophoresis was found to abolish its separation into discrete bands during electrophoresis (Fig. 1B). This result was apparently due to the presence of internal esters in polysialic acid (21), since smearing was abolished by alkali treatment. Therefore, all the samples exposed to a low pH were subsequently treated with alkali prior to electrophoresis.

To identify the bands observed for colominic acid, we used reference oligosaccharides purified by ion-exchange chromatography (7) (Fig. 2). The electrophoretic mobility exhibited by each oligosaccharide correlated with its relative molecular size. It can be concluded that the adjacent bands represented molecules differing in chain length by one sialic acid unit, i.e., the smallest molecules having the greatest mobility (Fig. 2). Band mobility was approximately a linear function of the logarithm of the molecular size (Fig. 3).

Since the tracking dyes interfered with fluorography, they were applied only into wells with no sample. In the 25% gel system used their mobilities repeatedly matched those of oligomers with the following numbers of sialyl residues: phenol red, 4; bromcresol purple, 11.5; bromphenol blue, 19; and xylene cyanol, 52. Thus, although the positions of the dyes may vary according to gel percentage, these dyes could be used as markers for the determination of the molecular sizes in each type of gel.

Extraction of the K1 polysaccharide. For analysis of capsular chain length we needed a gentle and rapid extraction method that would yield representative samples and that could be used for large numbers of samples in the screening of capsular mutants. When bacteria are grown some of the capsular antigen is released into the culture medium (30). This shedding phenomenon encouraged us to try to extract the capsule simply by incubation of the bacteria in buffer.

Preliminary experiments with neutral and alkaline buffers (0.05 M Tris hydrochloride [pH 8.6], 0.05 M glycine-NaOH [pH 9.8], and phosphate-buffered saline [pH 7.5]) indicated that the time and temperature of incubation were far more important in capsule release than the nature of the buffer. To avoid the adverse effect of salts on electrophoresis, we next investigated volatile buffers.

Pyridine acetate (0.1 M; pH 5.0) had a slightly better detaching capacity than 1 M ammonium bicarbonate (1 M; pH 8.8) (results not shown). Using pyridine acetate buffer, we assessed the amount of capsule released and the lengths of the released polysaccharide chains at different temperatures (Fig. 4). The simultaneous analysis of chain length by

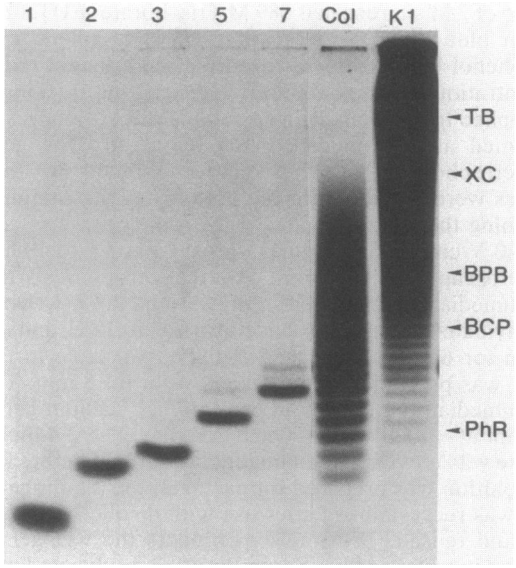

FIG. 2. Electrophoretic mobility of sialic acid oligomers. Lanes 1, 2, 3, 5, and 7, 1 to 3 μg of ^{3}H-labeled sialic acid oligomers containing 1, 2, 3, 5, and 7 sialyl residues, respectively; lane Col, 4 μg of ^{3}H-labeled colominic acid; lane K1, 30 μg of ^{3}H-labeled K1 polysaccharide. The gel (25%) was run at 35 V/cm for 3 h at 4°C and subjected to fluorography for 3 days. The tracking dyes were trypan blue (TB), xylene cyanol (XC), bromphenol blue (BPB), bromcresol purple (BCP), and phenol red (PhR).

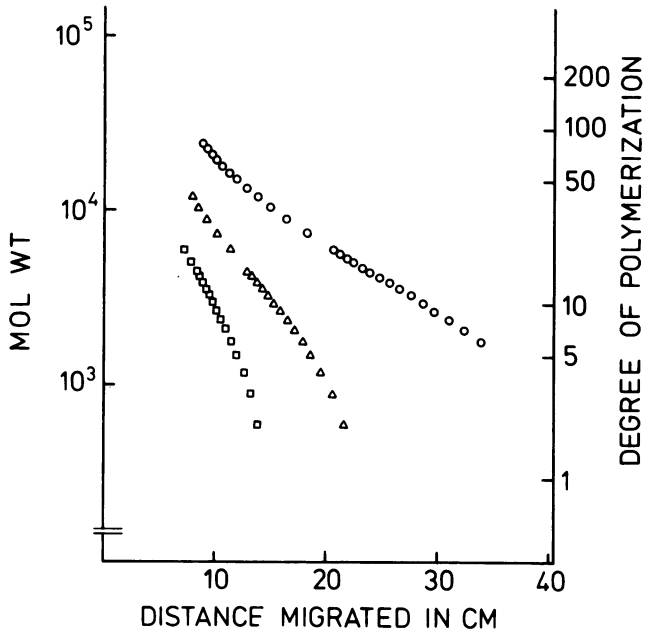

FIG. 3. Electrophoretic mobilities of polysialic acid as a function of molecular weight and degree of polymerization. The degree of polymerization was determined with the tracking dye phenol red, which has the same electrophoretic mobility as a sialyl tetramer in this gel system. Electrophoretic mobilities were determined with 25% polyacrylamide in a 14-cm gel (□), a 25-cm gel (△), and a 37-cm gel (○) and plotted against the molecular weight (MOL WT) on a logarithmic scale.

high-performance liquid chromatography and gel electrophoresis showed that incubation at 37°C, even for 4 h, caused only a slight shortening of the chains, whereas at 56°C many of the glycosidic bonds were cleaved during the first hour of incubation (Fig. 4). It was concluded that extraction at 37°C for 1 h in pyridine acetate buffer released a representative amount of polysaccharide (58%) with relatively little degradation of the glycosidic bonds.

Pretreatment of the K1 polysaccharide with mild acid. The pyridine acetate-extracted capsular antigen could be radiolabeled by borotritiide reduction and separated by gel electrophoresis without any further treatments (Fig. 5, lane 1). However, when the labeled polysaccharide was digested with endosialidase, a major band moving a little faster than the oligomer of eight sialyl residues was obtained (Fig. 5, lane 2). This result was unexpected for polymers having sialic acid in their reducing ends, since such material is degraded to smaller units by endosialidase (7). After digestion of the products with *V. cholerae* neuraminidase, the major band became even more prominent, and the bands of the expected oligosaccharides faded (results not shown). These results suggested that most of the labeled substance represented a component to which the polysaccharide had been bound at its reducing end and which therefore prevented the introduction of label into the cleavable sialyl polymer. In many gram-negative bacteria a phospholipid moiety had been suggested to anchor the capsule to the outer membrane (10, 28). The phospholipid-polysaccharide linkage can be disrupted by dilute acid (10). To remove the putative lipid, we treated the extract with acid (pH 2) and subsequently with alkali to saponify the lactone rings formed during acid treatment. Conditions as mild as possible were sought to prevent hydrolysis of the polysaccharide at an acidic pH. Removal of the lipid moiety after the mild acid treatment was assessed by labeling of the free reducing end of the polysaccharide by borotritiide reduction, digestion with phage endosialidase, and analysis of the undigested and digested polysaccharides by gel electrophoresis. Treatments at 0, 20, and 37°C for 1 to 60 min were compared. A slight increase in the amount of polysaccharide released was observed during incubation at 0°C, whereas treatment at higher temperatures seemed to induce some degradation of the glycosidic bonds with no apparent increase in the amount of the non-sialic acid component removed (results not shown). Treatment at pH 2 on ice for 1 h, followed by alkali treatment and neutralization, removed the non-sialic acid moiety quantitatively, as seen from the appearance of the expected (7) labeled cleavage products containing five to seven sialyl residues (Fig. 5, lanes 3 and 4). Control experiments with alkali treatment alone did not produce the same effect.

Gel electrophoresis of the K1 capsular polysaccharide. In the capsule extractions the yields of polysialic acid varied from 3.3 to 7.8 μg ml of bacterial culture for smooth *E. coli* K1 and 13 to 15 μg/ml of bacterial culture for rough *E. coli* IH3088 under the standard extraction conditions. These values correspond to 26 to 57% and 50 to 76% of the total membrane-bound sialic acid present, respectively. In gel

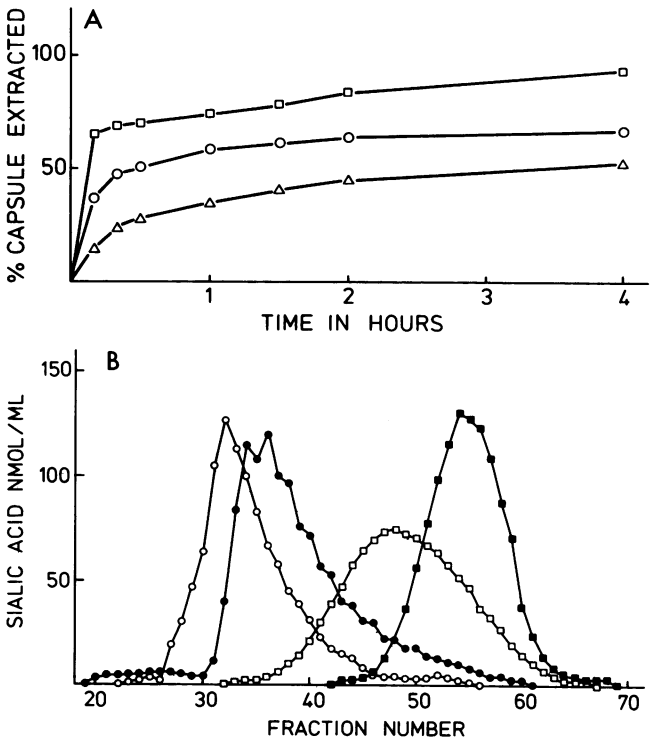

FIG. 4. Effect of time and temperature on the K1 polysaccharides extracted. (A) The K1 capsule of IH3088 (rough:K1) was extracted at 20°C (△), 37°C (○), and 56°C (□) in 0.1 M pyridine acetate buffer (pH 5.0). At the times indicated, samples were withdrawn and centrifuged, and the supernatants were tested for sialic acid. Results are expressed as the percentage of material released as compared with the total membrane-bound sialic acid content determined by *V. cholerae* neuraminidase digestion before extraction. Each point represents the geometric mean of two assays. (B) The capsular material (about 450 nmol of sialic acid) extracted at 37°C for 1 h (○) and 4 h (●) and at 56°C for 1 h (□) and 4 h (■) was analyzed by high-performance liquid chromatography.

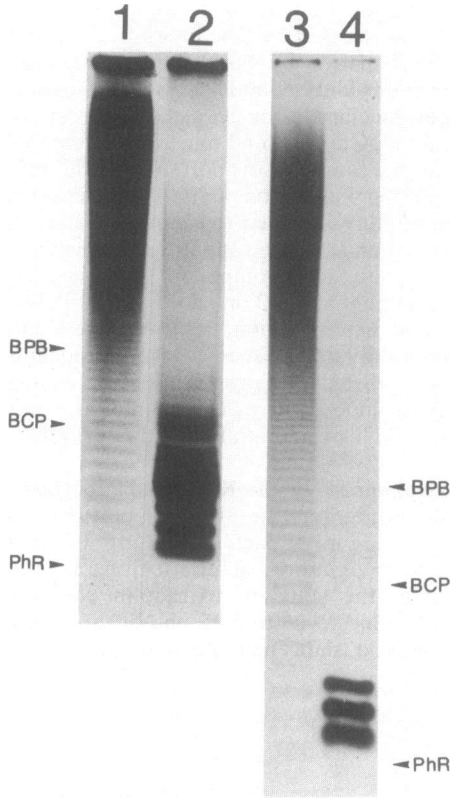

FIG. 5. Effect of mild acid treatment on the extracted K1 capsule. Lanes: 1, ^{3}H-labeled K1 extract, not treated; 2, as in lane 1 but digested with phage endosialidase; 3, K1 extract treated with mild acid before ^{3}H labeling; 4, as in lane 3 but digested with phage endosialidase. Ten micrograms of each sample was electrophoresed in a 25% gel at 35 V/cm and 4°C. Samples in lanes 1 and 2 were run in a 14-cm gel for 3.5 h, and samples in lanes 3 and 4 were run in a 25-cm gel for 7.5 h. The films were exposed for 3 days. For tracking dyes, see the legend to Fig. 2.

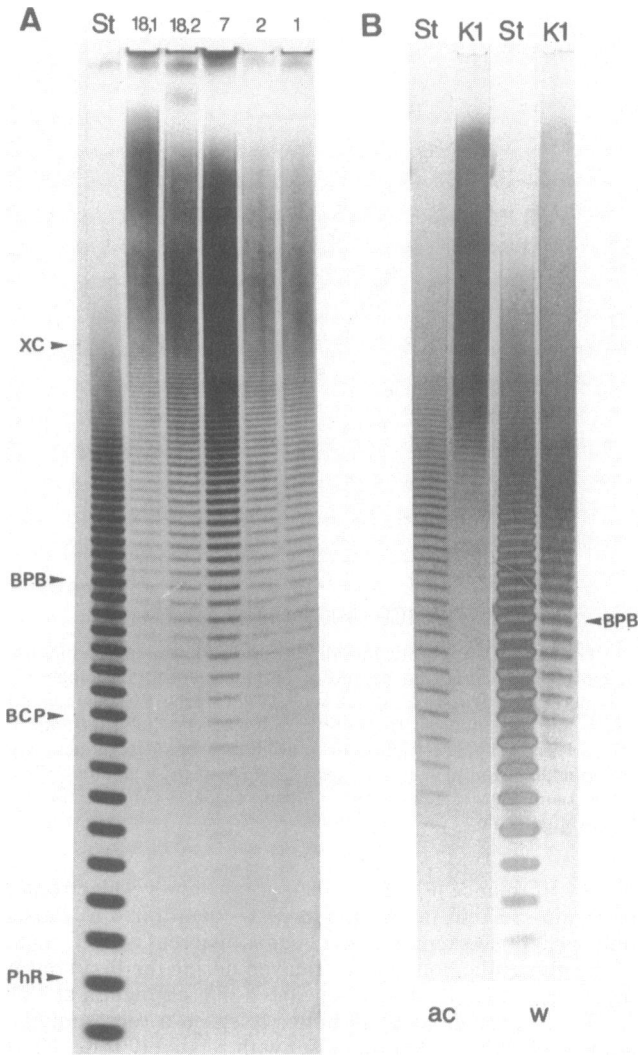

FIG. 6. Banding patterns of the K1 capsules from different *E. coli* strains. (A) The K1 capsule was extracted from strains IH3080 (O18) (lane 18, 1), IH3036 (O18) (lane 18, 2), IH3056 (O7) (lane 7), IH3044 (O2) (lane 2), and K235 (O1) (lane 1). Ten micrograms of each ^{3}H-labeled extracts was loaded. Lane St was loaded with 5 μg of partially hydrolyzed (pH 2, 80°C, 10 min) K1 polysaccharide labeled with NaB^3H_4. The samples were run in a 37-cm gel (25%) at 24 to 32 V/cm for 14 h at 4°C and subjected to fluorography for 14 days. (B) Lanes: St, 10 μg of partially hydrolyzed K1 polysaccharide; K1, 10 μg of the K1 capsule extracted from strain IH3088 (rough). The 37-cm, 25% gel was run at 25 V/cm for 18 h at 4°C. Before being stained with silver, one-half of the gel was fixed with alcian blue in 2% acetic acid (ac), and the other half was fixed with alcian blue in water (w). For tracking dyes, see the legend to Fig. 2.

electrophoresis samples of 2.5 to 30 μg were used. Thus, the minimum culture volume needed for analysis of chain length was about 5 ml, depending on the amount of capsule produced by the strain.

Fluorography of the results of gel electrophoresis of the K1 extract revealed a ladderlike pattern (Fig. 6A). Bands corresponding to up to 80 sialyl residues could be seen under illumination of the X-ray film of a 37-cm gel. Slower-moving, larger molecules did not separate into visible bands. Performing the electrophoresis for longer times (1 to 2 days) did not separate these compounds into distinct bands and only decreased the resolution, probably because of diffusion. When the polyacrylamide concentration was decreased to 15% the largest compounds moved more quickly, but the resolution of the smaller ones was poorer than in the 25% polyacrylamide gel. From the mobilities of the largest compounds it can be extrapolated (Fig. 3) that the polysaccharides that had migrated 3 cm in a 37-cm gel had molecular weights in the range of 5×10^4 to 7×10^4, which means that they were composed of approximately 160 to 230 sialic acid residues. The susceptibility of all the compounds to digestion with phage endosialidase confirmed that they consisted solely of polysialic acid (Fig. 5, lanes 3 and 4).

Analysis of the K1 capsule extracted from *E. coli* strains with the O serotypes O1, O2, O7, and O18 (Fig. 6A) revealed that the capsular chain length among these strains was about the same. A similar experiment with unlabeled samples and alcian blue-silver staining yielded the same result. Thus, the method of capsule extraction with subsequent gel electrophoresis proved to be generally applicable to *E. coli* K1 strains irrespective of the O antigen.

Comparison of the banding patterns obtained by alcian blue-silver staining and fluorography revealed that the intensity of staining and radioactivity were distributed differently in the samples (Fig. 6), owing to two factors. First, while the radioactivity is supposed to be similar for each fragment on a molar basis, the staining intensity is expected to increase with the growing chain length of the polysaccharides, owing

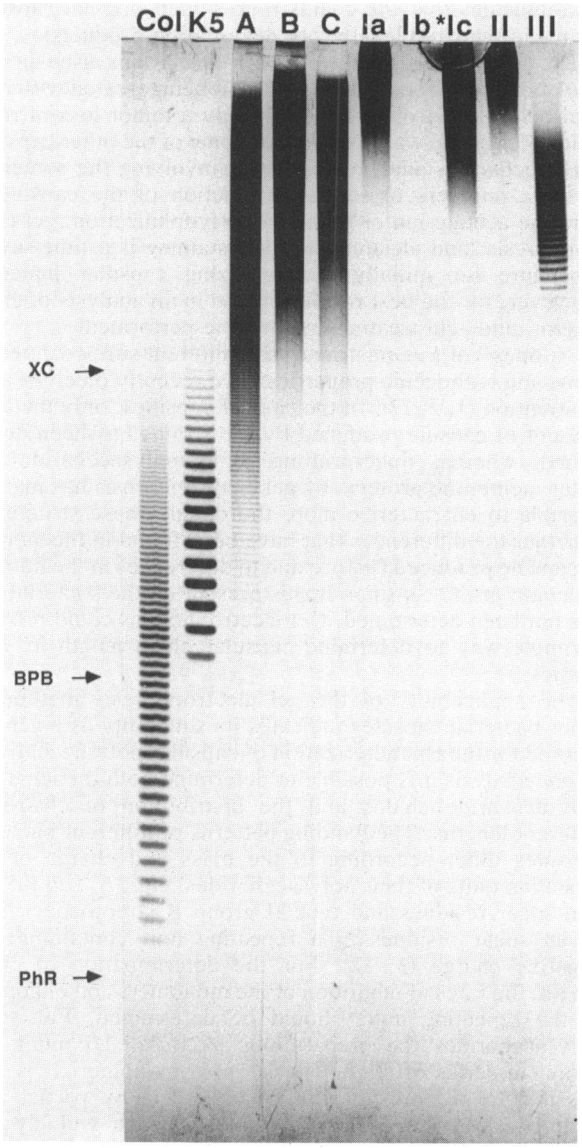

FIG. 7. Gel electrophoresis of bacterial polysaccharides. Polysaccharides (3.2 μg) pretreated with mild acid were subjected to gel electrophoresis in 15% polyacrylamide gels (1-mm thick, 37-cm long) at 600 V (22 mA) for 7 h at 4°C, and the gels were stained with alcian blue in water followed by silver. Lanes: Col, colominic acid; K5, *E. coli* K5 polysaccharide; A, B, and C, meningococcal group A, B, and C polysaccharides, respectively; Ia, Ib*, Ic, II, and III, group B streptococcal polysaccharides of types Ia, Ib, Ic, II, and III, respectively (Ib is represented by the desialylated core polysaccharide). For tracking dyes, see the legend to Fig. 2.

to an increased binding capacity for the dye. Second, since alcian blue is unable to fix the smallest fragments, they diffuse out from the gel during the staining procedure.

Alcian blue, dissolved in either water or 2% acetic acid, has been used to fix polysaccharides before staining with silver (23). Alcian blue in acetic acid resulted in uniform brownish staining of the K1 polysaccharide, whereas alcian blue in water not only fixed the capsular material but also stained it faintly. The latter method resulted in a two-colored appearance after silver staining; the center of the band was bluish, and it was surrounded by a brownish area (Fig. 6B). This broad appearance of the bands decreased resolution. The efficiency of the two alcian blue solutions in fixing oligosaccharides varied somewhat from time to time, but alcian blue in water was regularly more efficient in fixing the smaller fragments (Fig. 6B). It was possible to detect bands down to the size of trimer with alcian blue in water, whereas the heptamer was the smallest oligomer detected when alcian blue in acetic acid was used as a fixative. This staining difference is in agreement with the results reported for hyaluronate oligomers by Min and Cowman (23).

Gel electrophoresis of other bacterial polysaccharides. To evaluate whether the gel electrophoresis method could be applied to other bacterial species, we subjected capsular polysaccharides isolated by classical methods from different sources to gel electrophoresis and stained them with alcian blue-silver. Gels containing 15% polyacrylamide instead of 25% polyacrylamide had to be used; otherwise, the most slowly migrating polysaccharides could not be separated. All samples analyzed produced a distinct pattern in gel electrophoresis (Fig. 7). By careful examination of the gel a series of discrete bands could be seen in the region containing the fastest-moving components, whereas the bulk of the material in most samples appeared as progressively poorly separated bands merging into a smear of slowly migrating material. That the material revealed by staining in fact represented capsular polysaccharides was supported by the findings that a similar pattern was also obtained by fluorography of the radioactively labeled polysaccharides and that the capsule-specific antibodies for the polysaccharides tested (*E. coli* K1 and K5, meningococcal group B, and group B streptococcal types Ia, II, and III) immunoprecipitated 40 to 90% of the total radioactivity applied to the gel (J. Häyrinen and J. Finne, unpublished results). The desialylated Ib core polysaccharide of group B streptococci (3) was only revealed by fluorography and not by staining with alcian blue-silver (Fig. 7). This result was apparently due to the absence of negative charges that could serve as binding sites for the cationic dye. Since capsular polysaccharides are as a rule negatively charged (15, 16, 30), the alcian blue-silver staining method should be applicable to the staining of most polysaccharides.

As expected from their similar chemical structures, colominic acid and the meningococcal group B polysaccharide had a similar distance between adjacent bands. Interestingly, the meningococcal group C polymers were separated from each other by a slightly wider distance (not visible in Fig. 7). Since the latter differ from the former only by the position of the glycosidic bond between the *N*-acetylneuraminic acid units (α2-9 in meningococcal group C and α2-8 in meningococcal group B and colominic acid), it appears that the rate of migration is influenced not only by charge and mass but also by the structure of the polysaccharides. In the meningococcal group A polysaccharide, composed of phosphodiester-linked *N*-acetylmannosamine residues, the components were slightly less densely spaced than in the meningococcal group B polysaccharide and colominic acid, and up to 110 bands could be detected. However, the exact length of the group A polymer could not be determined because the position of the monomer was not known. As estimated from the relative mobility (Fig. 3), most of the material in the meningococcal group B polysaccharide sample migrated at a position corresponding to 130 to 200 sialyl residue long chains, with the longest components represented by approximately 300 sialyl residues.

DISCUSSION

Gel electrophoresis provides a facile and rapid method for the study of bacterial polysaccharides. The amount of ma-

terial needed for analysis depends on the distribution of chains of different lengths in the polydisperse polysaccharide samples and on the detection method used, but less than 1 μg of borotritiide-reduced oligosaccharide and 5 to 10 μg of unlabeled polysaccharide can be detected by fluorography or by alcian blue-silver staining, respectively. Tracking dyes can be used as markers for the determination of molecular sizes if their electrophoretic mobilities relative to the polysaccharides under study have first been determined in the gel system used.

Special attention must be paid to two factors when gel electrophoresis is applied. First, a high percentage (25%) of polyacrylamide in the gel is needed for good resolution. Consequently, a relatively high voltage is required to perform electrophoresis in a reasonable time, and this in turn requires that efficient cooling be provided. Second, since the smallest oligosaccharides cannot be fixed, they diffuse out of the gel quickly after electrophoresis. Therefore, the gel must be processed in a minimum time for fluorography, which is the only reliable detection method for small oligosaccharides. If only large polysaccharides are analyzed, staining with alcian blue in acetic acid combined with silver is superior to fluorography; no radioactivity is needed, the result is obtained in a few hours, and the sensitivity is comparable to that of fluorography with sodium borotritiide-labeled polysaccharides. The use of gels with lower polyacrylamide concentrations may be advantageous for some bacterial capsules that exhibit low mobility in the 25% gels.

In the methods commonly used for the isolation of acidic capsular polysaccharides, the capsular material is first precipitated and then purified further by methods involving several steps (9, 30). Such procedures are not suitable for studies in which many bacterial strains have to be characterized simultaneously. The method developed for the extraction of the K1 capsule proved to be rapid and easy to perform. Further purification of the capsule preparation was not required for gel electrophoresis. Gel electrophoresis of the K1 capsule extracted revealed that the bulk of the material consisted of long polysialic acid chains, with an estimated maximum length of 160 to 230 sialyl residues. This length is consistent with the reported length of the K1 capsular polysaccharide, which consisted of 165 sialyl residues and could be elongated in vitro to long chains of 200 residues (26). The capsule had been isolated from *E. coli* K235, which was also included in our study. This result indicated that the fragile glycosidic linkages had not been cleaved to a major extent by the conditions of extraction and mild acid treatment prior to electrophoresis.

Borotritiide reduction of the reducing ends of the capsular chains in the K1 extract was almost completely inhibited by a non-sialic acid moiety that became labeled instead. The K1 polysaccharide has been shown to contain fatty acids (28), but the existence of a phospholipid anchor between the capsule and the bacterial cell wall has not been confirmed. *E. coli* K92 and *N. meningitidis* groups B and C, which also bear polysialic acid capsules, all have phospholipid moieties in acid-labile bonds attached to the reducing ends of their capsular polysaccharides (10). The K1 polysaccharide could be freed of the non-sialic acid structure by treatment with mild acid, suggesting that the structure could be similar to that of the other capsules. The fact that there were almost no reducing ends available for reduction in the untreated K1 polysaccharide indicates that most of the polysialic acid chains were detached from the bacterium with the putative phospholipid moiety attached. The absence of reducing ends also indicates that the chains represented original capsular chains in their full length, not degradation products.

The K1 capsule of different *E. coli* strains used in this work could even be analyzed without being treated with mild acid before electrophoresis with only a minor loss of resolution. The same was also true for some of the other capsular polysaccharides analyzed. In work involving the screening of large numbers of strains, extraction of the capsule in pyridine acetate buffer followed by lyophilization, gel electrophoresis, and alcian blue-silver staining is a time-saving procedure for initially characterizing capsular material. However, for the best resolution and in an analysis of chain length, mild acid treatment should be performed.

"Clones" of *E. coli* strains with different surface determinants and pathogenic properties have recently received a lot of attention (1, 2, 17). In the case of capsules, only the total amount of capsule produced by the strains has been determined, whereas fingerprinting of lipopolysaccharide and outer membrane proteins by gel electrophoresis has made it possible to characterize more thoroughly these structures. Whether the differences that have been found in the amount of capsule produced (17) are due to differences in the number or length of the capsular chains present on the bacterial cell has not been determined. Gel electrophoresis could provide a simple way to determine capsular chain length in such studies.

The applicability of the gel electrophoresis method to other bacterial capsules indicates its suitability as a general method for the characterization of capsular polysaccharides. In one analysis it is possible to determine both the length of polysaccharide chains and the distribution of chains of different lengths. The banding patterns of different bacterial capsules differ according to the mass and charge of the repeating units of their polysaccharides; e.g., *E. coli* K5 has two sugar residues and type II group B streptococci have seven sugar residues in a repeating unit containing one negative charge (3, 32). For the determination of chain length, the rates of migration of the monomers and oligomers of the repeating units should be determined. For some polysaccharides the preparations as isolated contain the complete series of oligomers down to monomer. It is also possible to prepare a series of oligomers by partial acid hydrolysis or degradation by bacteriophage endoglycosidases (7, 19, 20). Within each type of polysaccharide, the charge/mass ratio of the chains differing in length is constant, allowing electrophoretical separation to take place according to molecular size.

ACKNOWLEDGMENTS

This study was supported by grants from the Swiss National Foundation (3.020-0.84), the Sigrid Jusélius Foundation, and the Academy of Finland.

We thank S. Weber for help in high-performance liquid chromatography and L. Schwoerer for typing the manuscript.

LITERATURE CITED

1. **Achtman, M., A. Mercer, B. Kusecek, A. Pohl, M. Heuzenroeder, W. Aaronson, A. Sutton, and R. P. Silver.** 1983. Six widespread bacterial clones among *Escherichia coli* K1 isolates. Infect. Immun. **39**:315–335.
2. **Achtman, M., and G. Pluschke.** 1986. Clonal analysis of descent and virulence among selected *Escherichia coli*. Annu. Rev. Microbiol. **40**:185–210.
3. **Baker, C. J., and D. L. Kasper.** 1985. Group B streptococcal vaccines. Rev. Infect. Dis. **7**:458–467.
4. **Barry, G. T., and W. F. Goebel.** 1957. Colominic acid, a substance of bacterial origin related to sialic acid. Nature

(London) **179**:206–208.

5. **Cowman, M. K., M. F. Slahetka, D. M. Hittner, J. Kim, M. Forino, and G. Gadelrab.** 1984. Polyacrylamide-gel electrophoresis and alcian blue staining of sulphated glycosaminoglycan oligosaccharides. Biochem. J. **221**:707–716.
6. **Finne, J., D. Bitter-Suermann, C. Goridis, and U. Finne.** 1987. An IgG monoclonal antibody to group B meningococci cross-reacts with developmentally regulated polysialic acid units of glycoproteins in neural and extraneural tissues. J. Immunol. **138**:4402–4407.
7. **Finne, J., and P. H. Mäkelä.** 1985. Cleavage of the polysialosyl units of brain glycoproteins by a bacteriophage endosialidase. J. Biol. Chem. **260**:1265–1270.
8. **Frosch, M., I. Görgen, G. J. Boulnois, K. N. Timmis, and D. Bitter-Suermann.** 1985. NZB mouse system for production of monoclonal antibodies to weak bacterial antigens: isolation of an IgG antibody to the polysaccharide capsules of *Escherichia coli* K1 and group B meningococci. Proc. Natl. Acad. Sci. USA **82**:1194–1198.
9. **Gotschlich, E., T. Y. Liu, and M. S. Artenstein.** 1969. Human immunity to meningococcus. III. Preparation and immunochemical properties of the group A, group B, and group C meningococcal polysaccharides. J. Exp. Med. **129**:1349–1365.
10. **Gotschlich, E. C., B. A. Fraser, O. Nishimura, J. B. Robbins, and T.-Y. Liu.** 1981. Lipid on capsular polysaccharides of gram-negative bacteria. J. Biol. Chem. **256**:8915–8921.
11. **Gross, R. J., T. Cheasty, and B. Rowe.** 1977. Isolation of bacteriophages specific for the K1 polysaccharide antigen of *Escherichia coli*. J. Clin. Microbiol. **6**:548–550.
12. **Hallenbeck, P. C., E. Vimr, F. Yu, B. Bassler, and F. A. Troy.** 1987. Purification and properties of a bacteriophage-induced endo N-acetylneuraminidase specific for poly-α-2.8-sialosyl carbohydrate units. J. Biol. Chem. **262**:3553–3561.
13. **Hallenbeck, P. C., F. Yu, and F. A. Troy.** 1987. Rapid separation of oligomers of polysialic acid by high-performance liquid chromatography. Anal. Biochem. **161**:181–186.
14. **Hampson, I. N., and J. T. Gallagher.** 1984. Separation of radiolabelled glycosaminoglycan oligosaccharides by polyacrylamide-gel electrophoresis. Biochem. J. **221**:697–705.
15. **Jann, K., and B. Jann.** 1983. The K antigens of *Escherichia coli*. Prog. Allergy **33**:53–79.
16. **Kasper, D. L.** 1986. Bacterial capsule—old dogmas and new tricks. J. Infect. Dis. **153**:409–415.
17. **Kusecek, B., H. Wloch, A. Mercer, V. Vaisänen, G. Pluschke, T. Korhonen, and M. Achtman.** 1984. Lipopolysaccharide, capsule, and fimbriae as virulence factors among O1, O7, O16, O18, or O75 and K1, K5, or K100 *Escherichia coli*. Infect. Immun. **43**:368–379.
18. **Kwiatkowski, B., B. Boschek, H. Thiele, and S. Stirm.** 1983. Substrate specificity of two bacteriophage-associated endo-*N*-acetylneuraminidases. J. Virol. **45**:367–374.
19. **Kwiatkowski, B., and S. Stirm.** 1987. Polysialic acid depolymerase. Methods Enzymol. **138**:786–792.
20. **Lifely, R. M., U. Nowicka, and C. Moreno.** 1986. Analysis of the chain length of oligomers and polymers of sialic acid isolated from *Neisseria meningitidis* group B and C and *Escherichia coli* K1 and K92. Carbohydr. Res. **156**:123–135.
21. **McGuire, E. J., and S. B. Binkley.** 1964. The structure and chemistry of colominic acid. Biochemistry **3**:247–251.
22. **Miettinen, T. A., and I.-T. Takki-Luukkainen.** 1959. Use of butyl acetate in determination of sialic acid. Acta Chem. Scand. **13**:856–858.
23. **Min, H., and M. K. Cowman.** 1986. Combined alcian blue and silver staining of glycosaminoglycans in polyacrylamide gels: application to electrophoretic analysis of molecular weight distribution. Anal. Biochem. **155**:275–285.
24. **Neal, H. W., and J. R. Florini.** 1973. A rapid method for desalting small volumes of solution. Anal. Biochem. **55**:328–330.
25. **Pelkonen, S., and J. Finne.** 1987. A rapid turbidimetric assay for the study of serum sensitivity of *Escherichia coli*. FEMS Microbiol. Lett. **42**:53–57.
26. **Rohr, T. A., and F. A. Troy.** 1980. Structure and biosynthesis of surface polymers containing polysialic acid in *Escherichia coli*. J. Biol. Chem. **255**:2332–2342.
27. **Schiffer, M. S., E. Oliveira, M. P. Glode, G. H. McCracken, Jr., L. M. Sarff, and J. B. Robbins.** 1976. Relation between invasiveness and the K1 capsular polysaccharide of *Escherichia coli*. Pediatr. Res. **10**:82–87.
28. **Schmidt, A. M., and K. Jann.** 1982. Phospholipid substitution of capsular (K) polysaccharide antigens from *Escherichia coli* causing extraintestinal infections. FEBS Microbiol. Lett. **14**:69–74.
29. **Skinner, M. K., and M. D. Griswold.** 1983. Fluorographic detection of radioactivity in polyacrylamide gels with 2,5-diphenyloxazole in acetic acid and its comparison with existing procedures. Biochem. J. **209**:281–284.
30. **Troy, F. A.** 1979. The chemistry and biosynthesis of selected bacterial capsular polymers. Annu. Rev. Microbiol. **33**:519–560.
31. **Troy, F. A., and M. A. McCloskey.** 1979. Role of a membranous sialytransferase complex in the synthesis of surface polymers containing polysialic acid in *Escherichia coli*. Temperature-induced alteration in the assembly process. J. Biol. Chem. **254**:7377–7387.
32. **Vann, W. F., M. A. Schmidt, B. Jann, and K. Jann.** 1981. The structure of the capsular polysaccharide (K5 antigen) of urinary tract-infective *Escherichia coli* O10:K5:H4. A polymer similar to disulfoheparin. Eur. J. Biochem. **116**:359–364.
33. **Vimr, E. R., and F. A. Troy.** 1985. Regulation of sialic acid metabolism in *Escherichia coli*: role of *N*-acylneuraminate pyruvate-lyase. J. Bacteriol. **164**:854–860.