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Acetyl-coenzyme A:Polysialic Acid O-Acetyltransferase from K1-positive *Escherichia coli*

THE ENZYME RESPONSIBLE FOR THE O-ACETYL PLUS PHENOTYPE AND FOR O-ACETYL FORM VARIATION*

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The capsular polysaccharide of *Escherichia coli* K1 is a linear polymer of *N*-acetylneuraminic acid in α-2,8 linkage. Certain substrains of *E. coli* K1 (designated OAc+) modify the polysaccharide by *O*-acetylation of the sialic acids. We demonstrate here an acetyl-coenzyme A: polysialosyl *O*-acetyltransferase activity that is found only in *E. coli* K1 OAc+ substrains. When form variation between the *O*-acetyl-positive and -negative states occurred in strain D698:K1, the fluctuations were accompanied by appropriate changes in the expression of enzyme activity. Thus, expression of this enzyme can account for the OAc+ phenotype and for the form variation between OAc+ and OAc−. The enzyme was solubilized in nonionic detergent and freed of endogenous acceptor activity by DEAE-cellulose chromatography, and its general properties were determined. Analysis of the reaction product showed a highly preferential acetylation reaction that was confined to polysialosyl units of >14 residues. Acetyl groups were shown to be transferred to both the 7- and the 9-positions of the sialic acid residues.

The partially purified enzyme was stable even after prolonged incubation at 57 °C. In contrast, any further purification resulted in loss of activity, even at 4 °C. Treatment of the stable enzyme with a polysialic acid-specific endoneuraminidase caused a similar loss of enzyme stability. This effect of the endoneuraminidase could be protected against by the addition of exogenous polysialic acid. This indicates that the partially purified enzyme contains traces of endogenous polysialic acid substrate that are required for the stability of the enzyme. Finally, the enzyme can *O*-acetylate the polysialic acid chains on the eucaryotic protein neural cell adhesion molecule, suggesting that enzymatic recognition of the substrate requires only the polysialic acid sequence.

Sialic acids are a family of 9-carbon carboxylated sugars commonly found as the terminal sugar residues of eucaryotic oligosaccharides (1, 2). More than 25 different types of naturally occurring sialic acids have been identified. This diversity results from the presence of acetyl, methyl, lactyl, and sulfate groups on *N*-acetylneuraminic acid (Neu5Ac[1]) or *N*-glycolylneuraminic acid (Neu5Gc) (2) and a recently described deaminated sialic acid, 3-deoxynonulosonic acid (3). The most common modified sialic acids contain *O*-acetyl substitutions at the 4-, 7-, and 8-, and/or 9-positions of the molecule. These modifications can influence many biological properties of the parent molecule, including activation of alternative complement pathway (4), the activities of enzymes involved in sialic acid metabolism (2, 5), the specificity of recognition of sialyloligosaccharides by influenza viruses (6–8), and the binding of antibodies to gangliosides (9, 10).

Sialic acids are said to have appeared late in evolution and are not generally found in plants, prokaryotes, or most invertebrates (2, 11). In spite of this, certain strains of *Escherichia coli* (K1), *Neisseria meningitidis* (Groups B and C), and streptococcus (group B) contain sialic acids in their capsular polysaccharides (12, 13). These bacterial strains and their capsular sialic acids are of interest for several reasons. All of them are known primary pathogens, causing infections such as meningitis in newborn infants (12). Secondly, these pathogenic bacterial strains are unique among prokaryotes in containing sialic acids. Third, the capsular sialic acids of *E. coli* and *N. meningitidis* bacteria are present as sialic acid homopolymers, which are identical with the outer sugar chains of some eucaryotic proteins such as the neural cell adhesion molecule (N-CAM) (14–16). Finally, certain of these *E. coli* and *N. meningitidis* strains modify their capsular sialic acids by *O*-acetylation at C-7 or C-9 positions (17).

E. coli K1 capsular polysialic acid is a homopolymer of (Neu5Ac α2-8 Neu5Ac)*n*. Substrains (OAc+) which contain *O*-acetylated sialic acids can either be fixed or can undergo a reversible form variation between OAc+ and OAc− at a characteristic frequency (17). The biological roles of the capsular polysialic acid and its *O*-acetylation are not clear. However, it is known that the capsular polysaccharide is poorly immunogenic and fails to activate the alternative pathway of complement (12). There is also evidence that *O*-acetylation can increase the immunogenicity and decrease the pathogenicity of these bacteria (12, 17).

This report describes the partial purification and enzymatic characterization of an *E. coli* K1 polysialosyl *O*-acetyltransferase that can *O*-acetylate polysialic acid sequences and shows that the presence of this enzyme activity in *E. coli* is probably the primary determining factor of the state of *O*-

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[1] The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; Neu5,9Ac$_2$, 9-*O*-acetyl-*N*-acetylneuraminic acid; N-CAM, neural cell adhesion molecule; DP, degree of polymerization of sialic acids; LB medium, Luria-Bertani medium; HPLC, high performance liquid chromatography; BSA, bovine serum albumin.

TABLE I

Comparison of O-acetyltransferase activity in E. coli strains

E. coli strains were grown to stationary phase in 15 ml of LB medium and pelleted by centrifugation at 5000 × *g* for 15 min. The cells were lysed in Buffer C, and the *O*-acetyltransferase activity was determined as described under "Experimental Procedures." The presence of capsular sialic acid and the *O*-acetylation phenotype for each strain was previously determined by others (17).

Strain	Capsular sialic acid	*O*-Acetyl phenotype	*O*-Acetyltransferase activity
			nmol/μg protein/h
C375:K1	+	+	0.762
D698:K1	+	+	1.572
O16:K1	+	−	0.006
C940:K1	+	−	0.066
HB101:K12	−	−	0.03
JM103:K12	−	−	0.012

acetylation. We also provide evidence that the enzyme preferentially recognizes high molecular weight polysialic acid chains and that its stability is dependent upon an association with these chains.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

As described in the Miniprint Section, we have solubilized, partially purified and characterized an *O*-acetyltransferase from an *E. coli* K1 OAc+ strain and shown that it preferentially acetylates high molecular weight polymers of sialic acid.

E. coli K1 OAc+ Phenotype Is Associated with High Levels of O-Acetyltransferase Activity—To study the relationship between the levels of *O*-acetyltransferase activity and the state of *O*-acetylation in capsular polysialic acid, several *E. coli* strains (K1 and K12) were examined for the presence of *O*-acetyltransferase activity (Table I). Only C375 and D698 (both previously shown to be K1, OAc+) contained high levels of *O*-acetyltransferase activity.

The O-Acetyl Form Variation in E. coli K1 Is Related to the Level of Expression of Polysialosyl O-Acetyltransferase Activity—Certain *E. coli* K1 (OAc+) strains undergo a reversible form variation between OAc+ and OAc⁻ (15). To demonstrate that this reversible *O*-acetylation phenotype is correlated with an appropriate change in the level of enzyme activity, a cloned OAc+ isolate (generation I) from the parent D698 K1 (OAc+) was followed through four generations of alternating OAc+/OAc− phenotypic changes during growth in LB media. From each generation, individual colonies were picked, expanded, and screened for the activity of the polysialosyl *O*-acetyltransferase. In each generation, the clone which had undergone a major change in the level of enzyme activity was propagated and expanded for picking the next generation. Fig. 5 shows the *O*-acetyltransferase activities of the individual clones from generations I through IV. The first generation OAc+ isolate (G-I) with high *O*-acetyltransferase activity yielded upon replating many similar colonies, and a single second generation isolate (G-II) with low *O*-acetyltransferase activity. This G-II OAc− isolate was propagated and replated to give a single G-III OAc+ isolate exhibiting high *O*-acetyltransferase activity. This isolate gave rise to five colonies that were OAc− in the fourth generation G-IV. In each generation, the revertants were verified as being *E. coli* K1 rather than contaminants, by reaction with specific polyclonal antibodies against the K1 capsular polysialic acid (kindly performed by Dr. Charles Davis, UCSD Dept. of Microbiology).

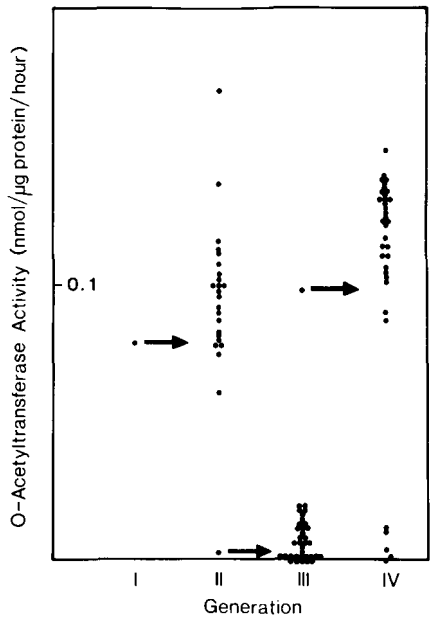


FIG. 5. **Analysis of *O*-acetyltransferase activity through four generations of *O*-acetyl form variation.** *E. coli* strain D698 was cloned by streaking on solid LB medium (in 1.5% agar). One colon (generation I, depicted by an *arrow*) was grown to confluency in 15 ml of LB medium and streaked onto a 9-cm Petri dish containing solid LB medium. Of the resultant second generation colonies, 24 individual colonies (generation II) were isolated, grown to confluency in 15 ml of LB medium, lysed in Buffer C, and assayed for the presence of *O*-acetyltransferase activity as described under "Experimental Procedures." A single colony of generation II with a negligible level of enzyme activity (indicated by an *arrow*) was again propagated and examined for the presence of *O*-acetyltransferase activity as described above. Two more such cycles of cloning and enzyme assays were performed (generations III and IV).

Mixing experiments showed that the loss of expression of the enzymatic activity was not due to the presence of an inhibitor in the OAc− revertants. To confirm that the phenotypic change occurred in parallel with the change in transferase activity, the G-I through G-IV isolates were expanded, and the capsular polysaccharide was purified from each using established procedures (17). The extent of *O*-acetylation of the capsule in each case was measured by degrading the polysaccharide by sequential endo- and exoneuraminidases. The monomers were studied for the presence of *O*-acetylation using the periodate/formaldehyde acetylacetone assay (28). As predicted, the revertants from generations II and IV were deficient in *O*-acetylation while generation I and revertant III were positive (data not shown). The level of *O*-acetylation in the positive strains ranged from 5–12% on various measurements.

These studies demonstrate that the presence of *E. coli* polysialosyl *O*-acetyltransferase activity correlates well with, and is probably the primary basis for, the reversible phenotypic changes observed in *O*-acetyl form variation.

E. coli Polysialosyl O-Acetyltransferase Is Stabilized by Association with Polysialic Acid—The stability of the enzyme in the crude state, or following the DEAE-purification step is in striking contrast to its marked instability to all further attempts at purification. It was also puzzling that the instability became manifest upon using many different purification steps that utilize entirely different principles (*e.g.* cation exchange chromatography, dye-matrix chromatography, affinity chromatography on CoA-Sepharose, and gel filtration.) All attempts at adding back various crude fractions to re-establish

[2] Portions of this paper (including the "Experimental Procedures," part of "Results," and Figs. 1–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

stability also failed (data not shown). Since the partially purified polysialosyl O-acetyltransferase specifically recognizes large polysialic acid fragments, we considered the possibility that such fragments of endogenous origin might be the stabilizing factor. If this were the case, treatment of the stable partially purified O-acetyltransferase by the purified endoneuraminidase might be expected to destroy this stabilizing factor. As shown in Fig. 6, the DEAE-purified *E. coli* polysialosyl O-acetyltransferase shows no loss of activity following preincubation at 37, 49, or 57 °C for 60 min (*open bars*). When endoneuraminidase was added to the preincubation mixture, it did not affect the stability of the enzyme at 37 °C. However, progressive loss of activity occurred upon raising the temperature to 49 and 57 °C (Fig. 6, *closed bars*). (In separate experiments, we noted that the endoneuraminidase itself was stable to incubation at 57 °C for 1 h). Shown in Fig. 7 is the time course of the inactivation of the O-acetyltransferase by endoneuraminidase at 57 °C. After 1 h, 80% of the activity is lost in the presence of endoneuraminidase. To confirm that the effect of endoneuraminidase is due to the enzyme itself, and not due to a trace contaminant such as a protease, excess exogenous colominic acid was added as an alternate substrate for the enzyme. As shown in the same figure, this provided complete protection against inactivation. Taken together, these results indicate that the stability of solubilized *E. coli* polysialosyl O-acetyltransferase is dependent upon a close noncovalent association between the enzyme and polysialic acid of endogenous origin.

E. coli Polysialosyl O-Acetyltransferase Acetylates the Neural Cell Adhesion Molecule—The eucaryotic protein N-CAM (neural cell adhesion molecule) is known to carry polysialic acid sequences in α2-8 linkage similar to the bacterial polysaccharide. We therefore examined the ability of the *E. coli* O-acetyltransferase to acetylate this molecule. As shown in Fig. 8, purified chicken embryonic N-CAM was indeed acetylated by the partially purified *E. coli* enzyme. The ability of chicken N-CAM to serve as a substrate implies that polysialic acid is the sole structural determinant required by this enzyme.

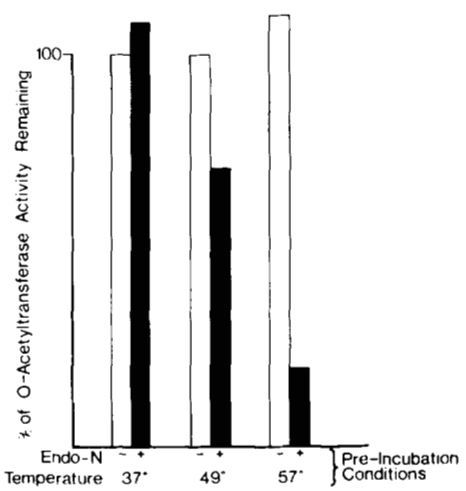


FIG. 6. **Endoneuraminidase inactivation of O-acetyltransferase.** Reaction mixtures (15 μl) containing 2.3 milliunits of DEAE-purified enzyme in 20 mM sodium acetate, pH 6, in the absence (*open bars*) or presence (*closed bars*) of 25 milliunits of endoneuraminidase was preincubated for 30 min at 37, 49, or 57 °C. Following this preincubation, the samples were assayed for O-acetyltransferase activity as described under "Experimental Procedures." The value obtained by incubation at 37 °C in the absence of endoneuraminidase was used to represent 100% activity.

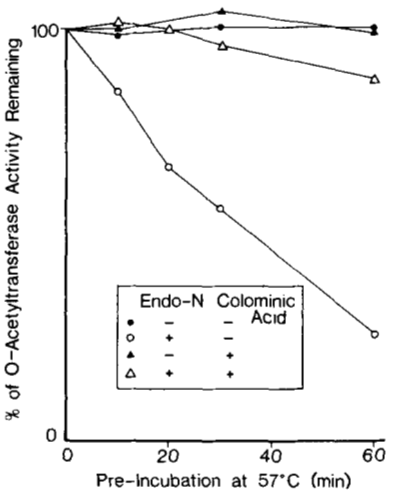
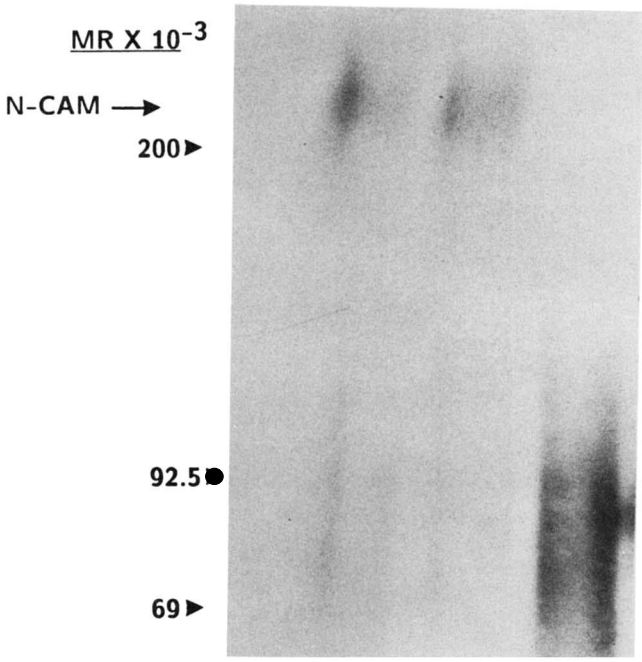


FIG. 7. **Protection of O-acetyltransferase activity from endoneuraminidase inactivation by colominic acid.** Reaction mixtures (27 μl) containing 2.3 milliunits of DEAE-purified O-acetyltransferase in 20 mM sodium acetate, pH 6, in the presence of 25 milliunits of endoneuraminidase and/or 2 μmol of colominic acid were preincubated at 57 °C for 10, 20, 30, or 60 min. Following preincubation, the samples were assayed for O-acetyltransferase activity as described under "Experimental Procedures." The value used for 100% activity was the average of values obtained from 10, 20, 30, and 60 min of preincubation in the absence of endoneuraminidase and in the presence of colominic acid.

DISCUSSION

The biosynthesis of the *E. coli* K1 capsular polysaccharide involving a bacterial sialyltransferase has previously been studied in detail by Troy and others (13, 29, 30). We have described here the solubilization, assay, and partial purification of the enzyme responsible for O-acetylation of the capsular polysialic acid chains. While the enzyme was completely stable in the crude state or after a single DEAE-cellulose step, attempts at further purification of any kind resulted in complete loss of activity. The methods tried have included the cation exchanger CM52, the affinity dye ligands Procion red and Cibacron blue, coenzyme A Sepharose, and gel filtration on Sephacryl S-200. Furthermore, a wide variety of conditions including the presence of different buffers, detergents, glycerol and various ions did not solve the problem. This anomalous behavior is best explained by our finding that the stable form of the enzyme can be specifically inactivated by endoneuraminidase treatment. This treatment presumably destroys polysialic acid molecules of endogenous origin to which the enzyme is bound and which are required for its stability. The stabilizing polysaccharide is either inaccessible for acetylation or must be rather small in amount, since no endogenous acceptor activity was detectable in the DEAE-purified enzyme. Thus, any purification step (other than DEAE-cellulose to which polysialic acids would bind) would presumably cause a separation of the enzyme from this small amount of stabilizing substrate. This also suggests that any further purification of this enzyme would require the presence of polysialic acid in all buffers and fractions at every step. However, we have so far been unsuccessful in using this approach to further purify the enzyme. Attempts at reconstitution of inactivated enzyme by the addition of polysialic acid have also been unsuccessful. We have therefore presented the enzymatic characterization of the partially purified enzyme, that is free of detectable endogenous acceptor in the standard assay.

Analysis of the O-acetylated colominic acid product by DEAE-Sephadex and HPLC showed that the enzyme has preferential substrate specificity toward large molecular



E.COLI ENZYME	+	+	+
N-CAM	+	+	-
TIME (MIN)	120	180	120

FIG. 8. **O-Acetylation of chicken embryonic N-CAM by DEAE-purified *E. coli* O-acetyltransferase.** Reaction mixtures (40 μl) containing [*acetyl*-^{3}H]acetyl-coenzyme A (1×10^6 cpm), 117.5 mM cacodylic acid, pH 7.5, and 2.3 milliunits of DEAE-purified *O*-acetyltransferase were incubated with (*lanes 1* and *2*) or without (*lane 3*) 5 μg of purified chicken embryonic N-CAM at 37 °C for the times indicated. The reaction mixtures were analyzed by 8% nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the procedure of Laemmli (34) and studied by autoradiography with EnHance (Du Pont-New England Nuclear) following the manufacturer's procedure.

weight polysialic acids. No acetylation was apparent in polysialic acids of DP < 14. In view of this result, the apparent K_m obtained with a mixture of polysialic acid of various sizes with an average length of 15 does not reflect the true K_m for large molecular weight polysialic acids. The requirement of the enzyme for such long chain substrates is interesting. Brisson *et al.* (31) have recently used ^{13}C NMR to demonstrate that the two terminal disaccharides of $(Neu5Ac)_{10}$ differ in conformation from the inner residues, and that the immunologically functional part of the molecule resides in the inner six residues. Thus, it is possible that the *O*-acetyltransferase recognizes a conformational state of the polysialic acid chain that is generated only when its length is above a required minimum.

Some *E. coli* K1 OAc+ substrains are characterized by their ability to undergo OAc+/OAc− reversible phenotypic change at a given frequency (17). We examined the relationship between the reversible phenotypic change and the presence of *O*-acetyltransferase activity. A cloned *E. coli* K1 OAc+ isolate was propagated through four generations, and, at each generation, 24–36 colonies were examined for the presence of *O*-acetylation and for the expression of polysialosyl *O*-acetyltransferase activity. Most of the colonies retained their original phenotype and the reversion frequencies observed were on the average 1 in 14. In each case, the *O*-acetylation phenotype correlated with the expression of *O*-acetyltransferase activity. Unlike the first three generations which gave a single revertant in 24–36 colonies, the fourth generation yielded five revertants. This increase in the frequency may reflect the process of selection through the propagation of the revertants. The underlying stimulus which influences the state of *O*-acetylation is not known; however, it is evident that it occurs spontaneously under confluent growth conditions in complete media.

Vimr and co-workers (32, 33) have shown that the endoneuraminidase and the polysialosyl sialyltransferase from *E. coli* can be used as probes for the presence of polysialic acid chains on eucaryotic membranes and cell surfaces. The minimum chain lengths recognized by these enzymes would be DP > 3–5. Since the enzyme that we have described here requires chains of DP > 14, it could also be used as a probe for such structures, particularly those with longer chain length. Indeed, we have demonstrated that this is the case for the chicken embryonic N-CAM.

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SUPPLEMENTARY MATERIAL TO

ACETYL-COENZYME A : POLYSIALIC ACID O-ACETYLTRANSFERASE FROM K1-POSITIVE

ESCHERICHIA COLI : THE ENZYME RESPONSIBLE FOR THE O-ACETYL PLUS PHENOTYPE AND FOR

O-ACETYL FORM VARIATION

Herman Higa and Ajit Varki*

EXPERIMENTAL PROCEDURES

Materials. The following materials were obtained from the sources indicated: [acetyl-^{3}H]acetyl-coenzyme A (24Ci/mmol), ICN; [^{14}C]N-acetylneuraminic acid (56.8mCi/mmol), New England Nuclear; [^{14}C]acetic acid (57mCi/mmol), Amersham; [^{3}H]sodium borohydride (5Ci/mmol), American Radiolabelled Chemicals; *Arthrobacter ureafaciens* neuraminidase, Calbiochem-Behring; *Clostridium perfringens* neuraminidase, colominic acid and phenylmethylsulfonyl fluoride, Sigma Chemical company; Acetyl-coenzyme A, Pharmacia; and N-acetylneuraminic acid, Kantoishi Pharmaceuticals (Tokyo, Japan). Purified endoneuraminidase(18) was a generous gift of Dr. Frederic A. Troy (University of California, Davis). Purified chicken embryonic N-CAM was kindly provided by Dr. Urs Rutishauser (Case Western Reserve). *E.coli* strains C375:K1, D698:K1, O16:K1, and C940:K1 were generous gifts of Dr. Richard Silver (FDA). [acetyl-^{3}H]9-O-acetyl-N-acetyl-neuraminic acid (24Ci/mmol) was prepared as described earlier from labelled rat liver Golgi vesicles(19). All other chemicals were of reagent grade and were purchased from commerical sources.

Buffers. Buffer A: 20mM Tris/HCl pH 7.4, 5% glycerol, 2mM EDTA and 1mM phenylmethylsulfonyl fluoride; Buffer B: Buffer A containing 0.05% Triton X-100; Buffer C: Buffer A containing 2% Triton X-100.

Determination of Colominic Acid Concentration. The heterogeneous mixture of polymer sizes present in the colominic acid preparation complicated the unit expression of the acceptor substrate. Therefore, the concentration of colominic acid was uniformally expressed as total sialic acid present in the preparation. The concentration of total sialic acid was determined by the orcinol/Fe+3 method with Neu5Ac as standard (20).

Determination of Average Size of Colominic Acid. The number of colominic acid chains was determined by a reducing sugar assay (21), and total sialic acid was quantitated by the orcinol/Fe+3 assay (20) with Neu5Ac as standard. The average length of the colominic acid chains was determined by dividing total sialic acid content by the number of reducing termini. The average length calculated by this method was 15 sialic acid residues per polymer.

Extraction of the Enzyme for Quantitation of O-acetyltransferase Activity. For the quantitation of enzyme level in *E.coli* strains, cells were grown to stationary phase in 15ml of LB medium at 37°C, and harvested by centrifugation at 5,000 x g for 15min. Pelleted cells were incubated for a 2-3 days at 4°C in the presence of 100ul of Buffer C. Lysis of cells was apparent by the visual appearance of a clumped precipitate of nucleic acid. The supernatant was assayed for the presence of enzyme by the O-acetyltransferase assay as described below.

O-acetyltransferase Assay. Unless otherwise specified, reactions were incubated for 6min and the amount of enzyme varied such that less than 15% of the donor substrate was consumed during reaction. The reaction mixture (40ul) containing 21mM sodium colominic acid (sialic acid content, see above), 33,000cpm of [^{3}H]acetyl-coenzyme A(1mM final), 117mM cacodylate pH 7.5 and enzyme was incubated at 37°C for 6min, and quenched by freezing on ethanol/dry ice. The samples were thawed and immediately applied onto 4ml Sephadex G-50 *columns (0.3x13cm)* equilibrated in 0.1M NaCl containing 0.02% sodium azide. The sample was washed in with 60ul of column buffer followed by 0.4ml of the same buffer. The labelled product was eluted directly into a scintillation vial with 2ml of the same buffer and counted for the presence of radioactivity. One unit of activity is defined as the transfer of 1µmole of acetate per min.

Preparation of [^{3}H]reducing end-labelled colominic acid. A reaction mixture (100ul) containing 200nmol of [^{3}H]sodium borohydride and 300ug of colominic acid (1 umol of sialic acid residues, and 70nmol of reducing termini) was incubated in 1mM NaOH at 22°C for 16h, and the reduction completed with 50ul of 1M sodium borohydride made in 1mM NaOH. The reaction mixture was incubated at 22°C for an additional 2 hrs, neutralized with 100ul of 2M acetic acid and lyophilized. The sample was reconstituted with 50mM pyridine acetate pH 5, relyophilized and finally reconstituted with 2ml of distilled water. The sample was applied onto a 1ml column of DEAE-Sephadex A-25 equilibrated in 10mM Tris/HCl pH 7.4, washed in with 4ml of the same buffer, and eluted with 5ml of 0.5M NaCl followed by 5ml of 2M NaCl made in the same buffer. Fractions (125ul) were collected and 5ul aliquots were counted for the presence of radioactivity. The labelled peak eluted with 0.5M NaCl was pooled and lyophilized.

Preparation of [^{3}H-acetyl]O-acetylcolominic acid. Preparation of [^{3}H-acetyl]O-acetylcolominic acid was accomplished under identical conditions as described for standard O-acetyltransferase assay except that the reaction mixture (40ul) contained 20 x 10^{6}cpm of [^{3}H-acetyl]acetyl-coenzyme A, and the reaction mixture was incubated at 37°C for 60min. The reaction mixture was applied onto a 4ml column (0.6 x 13cm) containing Sephadex G-50 equilibrated in 0.1M NaCl containing 0.02% sodium azide, and eluted with the same buffer. Fractions of 0.5ml were collected and 1ul aliquots were assayed for radioactivity. Fractions encompassing the first peak were pooled and lyophilized. In a typical preparation, 45% of the [^{3}H-acetyl] label added was recovered as product.

Partial Purification of O-acetyltransferase. All steps were conducted at 4°C.
Step 1-Extraction of the Enzyme. *E.coli* cells were grown to confluency in 1L of LB medium and harvested by centrifugation at 5,000 x g for 20min. Cells (2G) were reconstituted in 10ml of Buffer B, frozen rapidly on ethanol/dry ice and ground with a mortar and pestle. Freeze-thaw and grinding was repeated at least three times, or until the cells were ground to a paste. Disrupted cells were centrifuged at 30,000 x g for 20min and the supernatant was saved. The pellet was reextracted with 10ml of Buffer B as described above except without freeze-thaw. The supernatants from first and second extractions were pooled.

Step 2-DEAE-Cellulose Chromatography. Pooled supernatant (10ml) was applied to a 30ml column (1.6 x 15cm) containing DE-52 equilibrated in Buffer B. The column was washed with 200ml of the same buffer and the enzyme eluted with 200ml of a 200mM to 500mM NaCl gradient made in the same buffer. Fractions (2ml) were collected and 15ul aliquots were assayed at 37°C for 60min by the transferase assay as described above. Two peaks were obtained; the fractions encompassing the second peak (which had a higher specific activity) were pooled and used for further studies of the enzyme.

Determination of Protein. Protein content was determined by Amido-Schwartz dye-binding assay (22) with BSA as standard.

High-pressure Liquid Chromatography (HPLC).
System 1. A column (4 x 30cm, AX-5, Varian Micropack) was eluted in the isocratic mode with acetonitrile:water:0.25M sodium di-hydrogen phosphate (64:20:16) at a flow rate of 1ml per min over a period of 25min(23).
System 2. A column of Biorad HPX-72-S (0.78cmx 30cm) was eluted in the ioscratic mode with 100mM Na_2SO_4 at 1ml per minute.
System 3. A column (5 x 50mM, Mono-Q HR 5/5, Pharmacia) was eluted with a programmed linear gradient of 50mM Tris/HCl pH 7.5 and 50mM NaCl to 50mM Tris/HCl pH 7.5 and 400mM NaCl, over 20min. at a flow rate of 1ml/min. followed by isocratic flow for an 10 additional min This is very similar to the system described in detail by Hallenbeck et. al. (ref 24).

Detection of HPLC eluted Peaks. Unlabelled sialic acids and polymers were detected by absorbance at 200nM. ^{3}H- or ^{14}C-labelled compounds were detected by collecting fractions or with an on-line radioactive detector (Radiomatic, Flo-one Beta) with Monofluor scintillant: Effluent in a ratio of 4:1.

Enzymatic Degradation of Polysialic Acids to Monomeric Form for Study by HPLC. A reaction mixture (100-200ul) containing 50mU of endoneuraminidase, 0.1M sodium acetate pH 5.5 and labelled or unlabelled colominic acid was incubated at 37°C for 6h, afterwhich 30ul of *Clostridium perfringens* neuraminidase(5mUnit/ml) and 30ul of *Arthrobacter ureafaciens* neuraminidase(1mUnit/ml) were added and the incubation continued at 37°C for an additional 6-24h. The reaction mixtures were filtered through Amicon microconcentrator filter (Centricon 10) in preparation for HPLC analysis.

RESULTS

Assay of Polysialosyl O-acetyltransferase. The enzyme catalyzes the transfer of acetyl groups from acetyl-coenzyme A to the acceptor substrate, polysialic acid. An assay was developed to quantitate the [^{3}H]acetyl group transfer from [^{3}H]AcCoA to an exogenous acceptor substrate, colominic acid(a commercially available mixture of polysialic acid fragments). Following incubation of [^{3}H]AcCoA, colominic acid and a detergent extract of *E.coli*, the mixture was chromatographed on a small Sephadex G-50 column. The putative product [^{3}H-acetyl]colominic acid eluted in the void volume, and was well-separated from the residual [^{3}H]AcCoA and [^{3}H]acetate. In the absence of exogenous colominic acid the crude *E.coli* K1 O-Ac+ extract contained significant endogenous acceptor activity. This endogenous acceptor activity was eliminated upon partial purification of the enzyme of DEAE-cellulose (see below). For routine assays, fractions were not collected, but the product in the first peak was collected batchwise into a single scintillation vial.

Partial Purification of the Enzyme. In the absence of non-ionic detergents, no activity could be detected in a cell lysate. However Triton X-100 at 0.05% activated and solubilized most of the enzyme. This suggests that it is a membrane-bound or membrane-associated protein. The enzyme was extracted from the cells, passed over a DEAE-cellulose column and eluted with linear a salt gradient as described under "Experimental Procedures". The activity eluted in two peaks of which the second containing the higher specific acitivity was pooled, resulting in a ~100-fold purification over the total cellular protein. In this partially purified preparation, the endogenous acceptor activity was not detectable (Figure 1, lower panel). Unfortunately, all further attempts at purification of the enzyme with a variety of different methods resulted in complete loss of the activity(this problem and its probable explanation are discussed in greater detail below). Consequently, the DEAE-purified *E.coli* O-acetyltransferase which was free of endogenous acceptor activity was used for the initial characterization.

Characterization of the product of the O-acetyltransferase Reaction. A preparative reaction was carried out, and the product isolated by Sephadex G-50. One aliquot was analyzed by gradient elution from DEAE-Sephadex A-25 as described by others for colominic acid fragments(25). As shown in figure 1 (upper panel), the radioactive product was not eluted from the DEAE column at concentrations of up to 400mM NaCl. Another aliquot of the labelled product was subjected to sequential treatment with bacteriophage endo-neuraminidase (which cleaves colominic acid internally to small oligomers of DP3-4) (16,22) and a mixture of bacterial exoneuraminidases. Endoneuraminidase treatment alone released most of the label as fragments which eluted in the region expected for small oligomers (Figure 1, center panel). Subsequent treatment with exoneuraminidases almost completely degraded the [^{3}H]O-acetyl-colominic acid to a single peak. The radioactivity in this peak co-migrated with 9-O-acetyl-N-acetyl-neuraminic acid ($Neu5,9Ac_2$) by HPLC analysis in System 1 (data not shown).

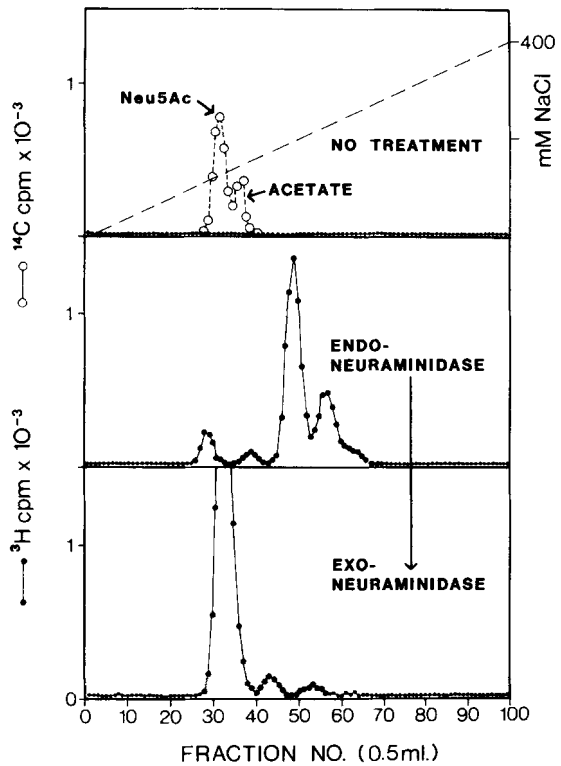

Figure 1. DEAE Sephadex A-25 Chromatography of O-acetyltransferase Enzyme Product. Radioactive samples from an O-acetyltransferase reaction were applied onto 3ml columns (0.6 x 13cm) containing DEAE Sephadex A-25 (acetate form) equilibrated in 10mM Tris/HCl pH 7.2 with or without various enzyme treatments. Samples were eluted from the column with a linear gradient of 0-400mM NaCl made in the same buffer at a rate of 0.5ml per min. Fractions of 0.5ml were collected and monitored for radioactivity. The samples applied were as follows: Top Panel, A mixture of [^{14}C]Neu5Ac (3,000cpm) , [^{14}C]acetate (1,500cpm) and 10,000cpm of the putative [^{3}H-acetyl]O-acetylcolominic acid product; Middle Panel, 10,000cpm of [^{3}H-acetyl]O-acetylcolominic acid product treated with endoneuraminidase; Bottom Panel, 10,000cpm of [^{3}H-acetyl]O-acetylcolominic acid product treated with endoneuraminidase followed by *Clostridium perfringens* and *Arthrobacter ureafaciens* neuraminidase(see Experimental Procedures" for details).

The acetylated polysialic acid product of the reaction could contain 7- or 9-O-acetylated sialic acids, since the α 2-8 linkage in the colominic acid excludes O-acetylation at the 8-position. However, migration of sialic acids from the 7- to the 9-position(2,23) could have occurred following release of the monomer during the prolonged incubation. Furthermore, 7-O-acetylated Neu5Ac migrates only about 0.5min earlier than the 9-O-acetylated molecule in the HPLC System 1 used above(see ref 26). For these reasons, we could not be certain that all of the initial O-acetylation took place exclusively at the 9-position. To address this issue [^{14}C-acetyl]-labelled colominic acid was generated using [^{14}C]Acetyl-CoA and the *E.Coli* enzyme. This [^{14}C]labelled product was subjected to sequential degradation by endo- and exoneuraminidases as described above. The incubation was performed in the presence of an internal standard mixture of [^{3}H-acetyl]7- and 9-O-acetylated Neu5Ac prepared from rat liver Golgi(23). The products were then studied by chromatography in HPLC system 2, which we have found can separate the two isomers. As shown in Figure 2, 75% of the ^{14}C-labelled product was found to be be in 9-O-acetylated Neu5Ac, while the remainder eluted in the position of the 7-O-acetylated compound. The ^{3}H-labelled internal standard (see upper panel of Figure 2) showed < 2% migration of O-acetyl groups from the 7- to the 9-position during the work-up of the sample (data not shown).

These results indicate that the product of the reaction contains O-acetyl groups at both the 7- and the 9-position. This raises the possibility that more than one enzyme is present in the preparation.

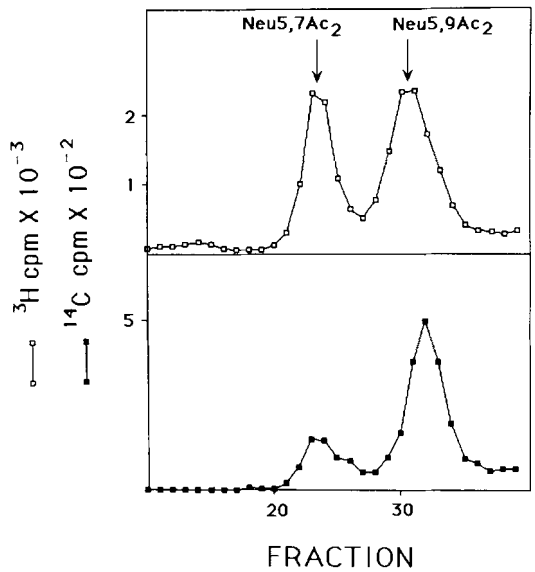

Figure 2. HPLC Identification of 7- and 9-O-acetyl[^{14}C-acetyl]sialic acids released from the O-acetyltransferase Enzyme Product. 5000cpm of [^{14}C-acetyl]O-acetylcolominic acid was prepared and digested with endoneuraminidase followed by *Clostridium perfringens* and *Arthrobacter ureafaciens* neuraminidase(see Experimental Procedures" for details). The digestion mixture included an internal standard of 10,000cpm of a mixture of 7- and 9-O-acetylated [^{3}H O-acetyl]sialic acids prepared from rat liver Golgi as previously described(26). The sample was filtered through an Amicon microconcentrator filter(Centricon 10), and an aliquot was analyzed by HPLC on System 2 as described under "Experimental Procedures". ^{3}H and ^{14}C radioactivity in the effluent were monitored in 0.5ml fractions.

Enzymatic Characterization of the DEAE-purified E.coli O-acetyltransferase. The partially-purified enzyme has a pH optimum of 7-7.5 and does not require divalent cations for activity. The activity is inhibited by coenzyme A-SH, with a half maximal inhibition observed at 100uM. The apparent Km for acetyl-coenzyme A was 0.3mM. The partially-purified enzyme is stable to heating at 60°C for one hour and to storage at 4°C for at least six months.

The O-acetyltransferase Preferentially Acetylates High Molecular Weight Polysialic Acids. The apparent Km of the enzyme for the colominic acid mixture was 3.7mM (expressed as concentration of total sialic acid). However, the commercial colominic acid used as acceptor substrate in O-acetyltransferase assay is a mixture of polysialic acid oligomers and polymers. We determined that the average length (DP=degree of polymerization) of the batch used for these studies is 15 sialic acid residues. The natural acceptor capsular polysialic acids, on the other hand, are probably linear arrays composed of 100-200 sialic acids residues(27).

We had already noted that the product of the reaction did not elute from DEAE-Cellulose at a concentration of 400mM, implying that the target chain length for O-acetylation must be high. When total sialic acid content was monitored across the profile of a typical Sephadex G-50 assay, it was found that the acceptor activity did not follow the broader profile of the total sialic acid concentration, but was localized to the void volume region of the column (Figure 3). Furthermore, when the acceptor was pre-treated with a mixture of bacterial exoneuraminidases, the activity was markedly reduced, even though relatively large oligomers were still left behind after a partial digestion (data not shown). These results further suggest that the apparent Km derived from the commercial colominic acid mixture is erroneous, and that acceptor activity is confined to the very large fragments. This data also shows that the large fragments do not represent a disproportionately large amount of the total sialic acid.

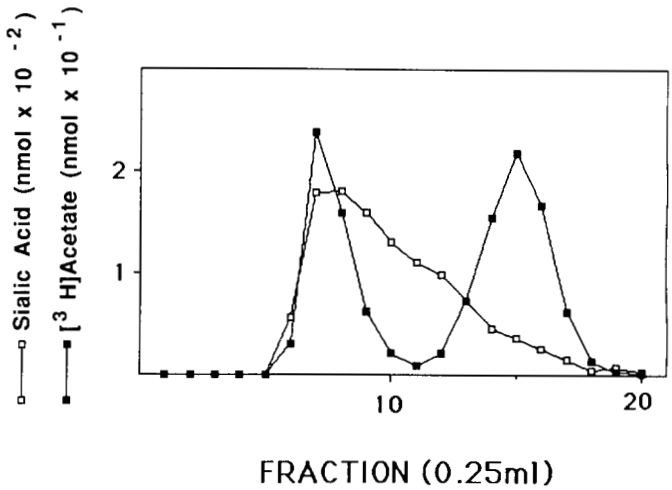

Figure 3. Sephadex G-50 Elution Profile of O-acetyltransferase Reaction Mixtures. An O-acetyltransferase reaction (40ul) was carried out under standard reaction conditions as described under "Experimental Procedures", except that the reaction mixture contained 1.2 umol of colominic acid (expressed as total sialic acid). After incubation at 37°C for 60min, the reaction mixture was applied to a small Sephadex G-50 column (4ml). The eluant was collected as fractions of 250ul; 40ul aliquots were counted for the presence of [^{3}H] label, while the remaining 210ul of eluant in each tube was assayed for total sialic acid by the orcinol/Fe+3 assay (20). (See Experimental Procedures for Details").

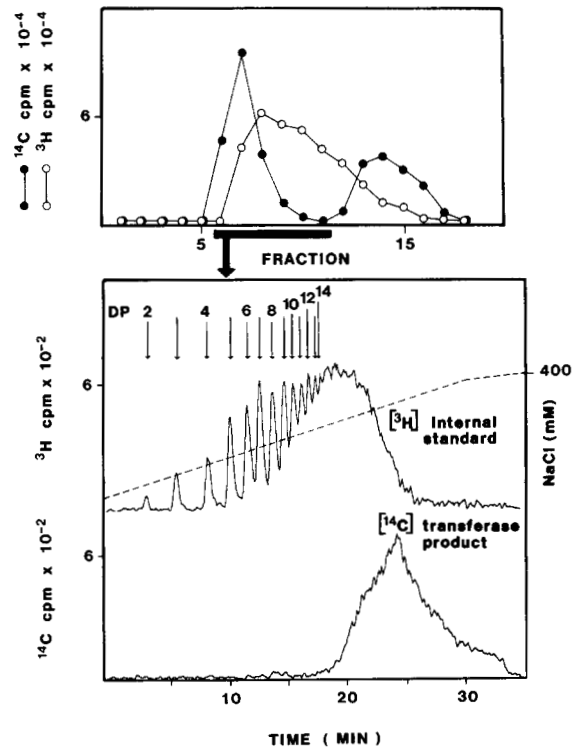

Figure 4. HPLC Analysis of the Size of the O-acetyltransferase Acceptor Substrate. [^{3}H]end-labelled colominic acid (500,000cpm) and [^{14}C-acetyl]O-acetylcolominic acid (500,000cpm) (prepared as described under "Experimental Procedures"), were applied onto a 4ml column (0.3 x 13cm) containing Sephadex G-50 equilibrated in 0.1M ammonium acetate pH 6.4 and eluted with the same buffer. Fractions of 250ul were collected and 5ul aliquots monitored for radioactivity (top panel). All fractions containing the product were pooled as shown, and an aliquot containing 90,000cpm of ^{3}H and 70,000cpm of ^{14}C label analyzed by HPLC in System 3. The [^{3}H]end-labelled colominic acid (middle panel) and [^{14}C-acetyl]O-acetylcolominic acid product (bottom panel) were detected with an on-line radioactive detector as described under "Experimental Procedures". The column was previously calibrated with [^{14}C]Neu5Ac and [^{3}H]end-labelled di-sialic acid (DP 2).

To examine in greater detail the effect of chain length of the acceptor polysialic acid on the transfer activity, an enzyme reaction was carried out with [^{14}C]AcetylCoA. At the end of the reaction, colominic acid end-labelled with NaB^3H_4 was added as an internal marker, and the reaction mixture was applied to a Sephadex G-50 column (Figure 4, upper panel). In keeping with the preceding experiments, the profile of the ^{14}C-acetylated product was substantially skewed towards the elution position of the larger [^{3}H]-labelled oligomers. To obtain a more detailed assessment of this finding, the product was pooled as shown (figure 4, upper panel), and studied on a recently described HPLC system (system 3, ref 24) that resolves colominic acid fragments of various sizes. It can be seen (Figure 4, lower panel) that the major proportion of the ^{14}C radioactivity eluted in the position of polymers of DP >14. Taken together, these results in figures 3 and 4 indicate that the *E.coli* polysialosyl O-acetyltransferase preferentially acetylates large molecular weight polysialic acid.