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Selective Synthesis and Labeling of the Polysialic Acid Capsule in *Escherichia coli* K1 Strains with Mutations in *nanA* and *neuB*

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The enzymes required for polysialic acid capsule synthesis in *Escherichia coli* K1 are encoded by region 2 *neu* genes of the multigenic *kps* cluster. To facilitate analysis of capsule synthesis and translocation, an *E. coli* K1 strain with mutations in *nanA* and *neuB*, affecting sialic acid degradation and synthesis, respectively, was constructed by transduction. The acapsular phenotype of the mutant was corrected in vivo by exogenous addition of sialic acid. By blocking sialic acid degradation, the *nanA* mutation allows intracellular metabolite accumulation, while the *neuB* mutation prevents dilution by the endogenous sialic acid pool and allows capsule synthesis to be controlled experimentally by the exogenous addition of sialic acid to the growth medium. Complementation was detected by bacteriophage K1F adsorption or infectivity assays. Polysialic acid translocation was observed within 2 min after addition of sialic acid to the growth medium, demonstrating the rapidity in vivo of sialic acid transport, activation, and polymerization and translocation of polysaccharide to the cell surface. Phage adsorption was not inhibited by chloramphenicol, demonstrating that de novo protein synthesis was not required for polysialic acid synthesis or translocation at 37°C. Exogenous radiolabeled sialic acid was incorporated exclusively into capsular polysaccharide. The polymeric nature of the labeled capsular material was confirmed by gel permeation chromatography and susceptibility of sialyl polymers to K1F endo-*N*-acylneuraminidase. The ability to experimentally manipulate capsule expression provides new approaches for investigating polysialic acid synthesis and membrane translocation mechanisms.

The *kps* cluster for polysialic acid (PSA) synthesis in *Escherichia coli* K1 is composed of a central group of *neu* genes flanked on either side by region 1 or 3 *kps* genes that encode polypeptides for general functions in PSA synthesis or translocation (4, 22, 25). Homologous region 1 and 3 genes have been detected in a variety of other *E. coli kps* clusters coding for synthesis of polysaccharides that are structurally unrelated to PSA (3). One phenotype of mutants with defects in many of the flanking genes is accumulation of intracellular polysaccharides (3, 4, 25), indicating that region 1 and 3 gene products may function directly in translocating structurally unrelated polysaccharides to the cell surface. This possibility is supported by primary sequence similarities of the region 3 gene products KpsM and KpsT with those of the components of other translocation systems that hydrolyze ATP (3). However, mutations in *kpsT* and several region 1 genes are pleiotropic, as shown by reduced activity of the *neuS*-encoded polysialyltransferase and an overall decrease in PSA synthesis (25), suggesting that at least some flanking gene products function in polymer assembly reactions, such as initiation, termination, or covalent modifications with lipid or acetyl groups (25). Furthermore, epistasis observed in a double mutant with defects in *kpsT* (region 3) and *kpsS* (region 1), resulting in loss of polysialyltransferase activity and absence of intracellular PSA (25), indicates that the pleiotropy described above is not caused by feedback inhibition of polysialyltransferase by intracellular PSA. The problem of distinguishing between a direct involvement of *kps*-flanking gene products in translocation or as structural and catalytic components of a glycosyltransferase complex limits the ability to understand both synthesis and translocation mechanisms. Together, the observations indicate that in vivo translocation assays are needed to better define the molecular mechanisms of PSA synthesis and export.

PSA is a cell-associated homopolymer of 100 to 200 α2,8-linked sialyl residues (12, 13) that may terminate at the reducing end in an acid-labile phosphodiester linkage to 1,2-diacylglycerol (6, 14). Other potential chemical modifications include a variable degree of O-acetylation in some K1 strains (10) and the possibility of intrachain esters or lactones (8, 9). The monotonic structure of PSA and the absence of other sialylated compounds in *E. coli* indicated that exogenous sialic acid could be used for in vivo labeling and selective synthesis of the capsular K1 antigen (20).

Initial attempts to label the *E. coli* K1 capsule in vivo with radioactive sialic acid resulted in only about 10% incorporation of the radioactivity into polysaccharide (27). However, these experiments revealed a highly regulated sialic acid catabolic system that mapped near *argG* at min 72 (27, 28). The two genes in this system, *nanT* and *nanA*, code for a sialic acid membrane transporter and an intracellular aldolase, respectively (27). Aldolase-deficient mutants still transport exogenously added radiolabeled sialic acid, but unlike in the wild type, radioactivity is incorporated exclusively into PSA (28). Thus, sialic acid's primary fate in a *nanA* mutant background is polymerization catalyzed by the sialyltransferase complex (27, 28).

In this article, the construction and properties of a double mutant with defects in sialic acid synthesis and degradation are described. PSA is selectively synthesized after exogenous addition of sialic acid to exponentially growing cells. Capsule expression is detected by phage K1F adsorption or infectivity assays. By addition of labeled sialic acid, de novo PSA synthesis is detected radiometrically. The ability to experimentally manipulate K1 capsule expression is facili-

tating analysis of PSA synthesis and translocation mechanisms (20).

MATERIALS AND METHODS

Bacterial and phage strains. All bacterial strains were *E. coli* K-12-derived K1 hybrids, as described previously (27, 28). EV138 (*nanA4 neuB25*) was constructed by P1 *vir* transduction with an EV52 (*nanA4 zgj-791*::Tn*10*) donor and EV24 (*neuB25*) recipient. Tetracycline-resistant (10 μg/ml) transductants were screened for the aldolase-negative phenotype as described previously (27). The PSA-specific phage K1F has been described previously (26). Phage-produced, soluble endo-*N*-acylneuraminidase (endo-*N*) was prepared as described previously (25).

Complementation assays. (i) Infectivity. Bacteria from a single colony were inoculated into 3 ml of LB medium (GIBCO, Paisely, Scotland) and grown overnight at 37°C with vigorous aeration by rotation in a New Brunswick model G76 water bath. Cultures were diluted 1:50 in minimal M63 medium (16) containing all required supplements (27, 28) plus 0.4% glycerol and, unless indicated otherwise, 1% (wt/vol) N-Z-Amine AS (Sigma) to stimulate growth. After overnight incubation, cultures were diluted in minimal medium to give A_{600} readings of 0.01 to 0.1 and grown to between 0.1 and 0.4 A_{600} units. Sialic acid was stored as a 10-mg/ml frozen stock in M63 medium. Aliquots of this stock were added directly to cultures to give the indicated final sialic acid concentrations. For infectivity assays, K1F was added at a multiplicity of infection of ~10. Lysis was monitored as the decrease in A_{600} per unit of time with a Beckman DU-30 spectrophotometer.

(ii) Binding. Cell cultures, grown as above, were diluted 1:1 at different times after sialic acid addition into 0.5 ml of minimal medium to contain a final titer of 2×10^3 to 3×10^3 PFU of K1F. After 3 min of adsorption at room temperature without shaking, cells and bound phage were pelleted for 1 min at 12,500 rpm in a Sorvall Microspin 24S centrifuge. Unbound K1F were measured by immediately pipetting 100 μl of the supernatant into 200 μl of EV36 indicator cells, previously grown to stationary phase in LB medium, and then plated with 2 ml of nutrient broth top agar (0.7%, wt/vol) onto nutrient broth agar (1.5%, wt/vol). Alternatively, 0.4-ml samples were pipetted into sterile microcentrifuge tubes and stored on ice for up to 1 h before plating. Plaques were counted after incubation for a few hours at 37°C or after overnight incubation at 32°C. Data were expressed as the bound fraction per unit of time, obtained by subtraction.

(iii) Eclipse. Cultures at the indicated cell densities were exposed at time zero to an initial phage concentration (P_0) of 3×10^7 K1F per ml. Samples were diluted 10^4-fold into chloroform-saturated 0.85% NaCl, and the titer of unadsorbed phage was determined on EV36 indicator cells, as described above. The general aspects of this assay are described in detail in reference 21.

Labeling with exogenous sialic acid. Cultures were grown as described above and exposed to a continuous pulse of ^{3}H-labeled sialic acid (22.2 mCi/mmol) at the concentrations indicated. Labeled sialic acid was prepared as described previously (27).

Gel permeation chromatography. Columns of Sephadex G-25 (fine) were equilibrated with 10 mM Tris, pH 7.6, containing 50 mM NaCl and 1% sodium dodecyl sulfate. Cultures of EV138 (1 to 5 ml) were exposed for 1 h to 0.16 mM sialic acid containing 1.1×10^6 dpm of ^{3}H-labeled sialic acid per ml. Cells were collected by centrifugation, washed once with ice-cold 10 mM Tris, pH 7.6, resuspended to 0.25 ml with the same buffer, and disrupted by sonication, as described previously (27). Membrane and soluble fractions were prepared by ultracentrifugation, and where indicated, the fractions were treated with excess endo-*N* to hydrolyze PSA (25). Samples were solubilized with an equal volume of 2% sodium dodecyl sulfate, loaded directly onto the column, and chromatographed by gravity flow. Fractions were collected at a flow rate of 0.5 ml/min; the radioactivity in 250-μl samples was counted by liquid scintillation spectrometry in 5 ml of Aquasol (Amersham). Void volumes (V_0) were determined with blue dextran. Columns were also calibrated by elution of radiolabeled sialic acid or CMP-sialic acid markers, as described previously (28).

To avoid potential shearing of PSA chains during cell disruption, EV138 harboring pLysS (Novagen, Madison, Wis.) was lysed with 1/10 volume of chloroform. pLysS expresses a low level of intracellular T7 lysozyme from the structural gene cloned into pACYC184 (5). Treating cells harboring this plasmid with agents that do not normally disrupt bacteria, such as chloroform and nonionic detergents, causes rapid cell lysis and release of intact PSA chains. After cell lysis with chloroform, 1/10 volume of DNase (type I; Sigma) was added to a final concentration of 100 μg/ml. Samples were solubilized and fractionated as described above.

RESULTS

Construction of EV138 (*nanA4 neuB25*). PSA-synthetic functions are encoded by *neu* genes located near min 64 in a central biosynthetic "cassette" (region 2) of the multigenic *kps* cluster (23). This cassette includes genes for sialic acid synthesis, activation, and polymerization (25). The gene products of two of these loci, *neuB* and *neuC*, were previously implicated in monosaccharide synthesis, since acapsular mutants were complemented for PSA synthesis after exogenous addition of sialic acid to the medium (25, 34). Presumably, sialic acid was transported by NanT, activated by conversion to CMP-sialic acid, and polymerized to yield PSA, which was then translocated to the outer membrane in combination with other *kps* gene products of the sialyltransferase complex (Fig. 1). These observations suggested that a strain with mutations in *nanA* and *neuB* would be labeled more efficiently with exogenous sialic acid than a strain with *nanA* alone, since the *neuB* mutation would prevent dilution by the endogenous sialic acid pool (Fig. 1). More importantly, *neuB* would block PSA synthesis unless sialic acid was added to the culture. The strain designated EV138 (*nanA4 neuB25*) was constructed by transducing *nanA4* into EV24 (*neuB25*) and selecting for the linked tetracycline resistance element *zgj-791*::Tn*10* (27). EV138 was used as the test strain in all subsequent experiments. Other *nanA neuB* mutant strains are described in reference 20.

Complementation of EV138. The susceptibility of EV138 to the PSA-specific phage K1F was used to assess complementation. Since previous studies showed that NanT activity was repressed by glucose (27), cultures were grown in minimal medium containing glycerol. Infection of the capsulated EV36 control strain caused a rapid decrease in cell density (Fig. 2), consistent with the phage's 15- to 20-min latent period. As expected, EV138 was K1F^r (Fig. 2), since this strain lacked sialic acid-synthetic ability and thus did not produce the PSA capsule. However, exogenous addition of 13 or 320 μM sialic acid resulted in cell lysis (Fig. 2),

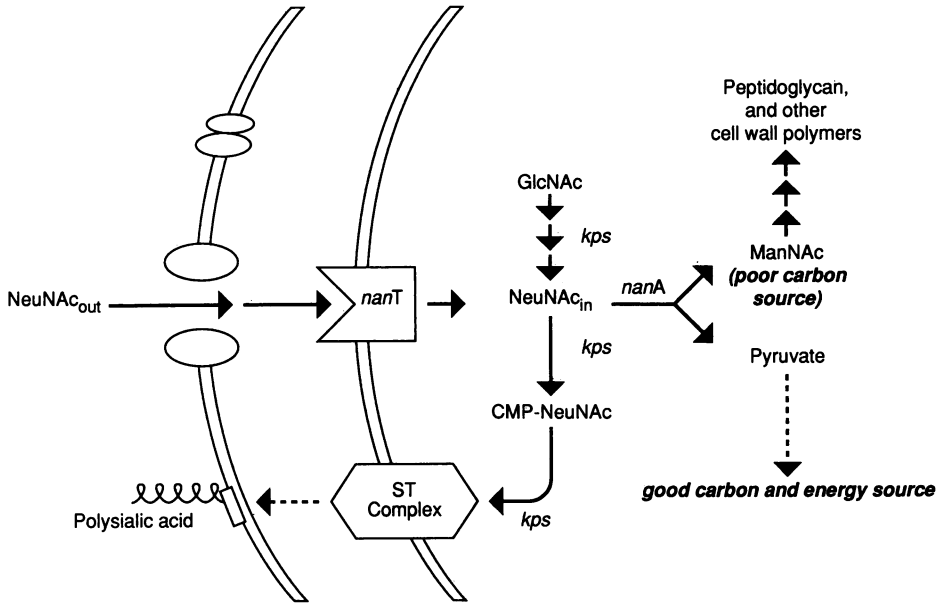

FIG. 1. Sialic acid metabolism in *E. coli* K1. External *N*-acetylneuraminic acid (NeuNAc$_{out}$) diffuses into the periplasm through outer membrane pores and is transported by the sialic acid permease encoded by *nanT*. Internal sialic acid (NeuNAc$_{in}$), transported or produced endogenously by *kps* gene products, is either degraded by the aldolase encoded by *nanA* or activated to the sugar nucleotide precursor CMP-NeuNAc. Sialic acid is polymerized and translocated to the outer membrane as PSA by the sialyltransferase (ST) complex. The aldolase cleavage products either enter intermediary metabolic pathways or are disseminated into surface polysaccharides.

indicating that PSA was synthesized and transported to the cell surface, where it acted as a phage receptor. Cell lysis was dependent on intracellular sialic acid accumulation, since a culture exposed to K1F at time zero was not lysed until sialic acid was added 1.5 h later (Fig. 2, arrow). Control experiments demonstrated a requirement for the *neuB* mutation, since the acapsular mutants EV5 and EV15, with defects in sialic acid activation and PSA assembly, respectively (25), were not complemented by exogenous sialic acid. These results also showed that sialic acid did not convert K1F to an infective state. Furthermore, exogenous addition of colominic acid (1 mg/ml) to EV5, EV15, or EV24 did not produce K1F sensitivity, demonstrating that PSA receptors must be cell associated to be functional. Attempts to complement the *neuB* defect in EV24 or EV138 with exogenous *N*-acetylmannosamine (ManNAc), a sialic acid precursor (2), were unsuccessful, although EV138 but not EV24 became K1F^s during growth in LB, suggesting that this medium contained a low level of sialic acid or a sialic acid precursor. These results indicated that the sialic acid metabolic defect caused by *neuB* might occur after ManNAc synthesis, as suggested previously for *neuC* (34).

K1F adsorption on EV138. Phage adsorption can be defined by the integrated form of the first-order rate equation $\log P/P_0 = -(1/2.3)kNt$, where the linear decrease with time of the logarithm of the fraction of unadsorbed phage, P/P_0, is proportional to the adsorption rate constant, k, and the number of bacteria in the mixture, N (21). An average rate constant for K1F adsorption to EV36 at cell densities of 0.5×10^8 and 1.0×10^8 per ml (Fig. 3, inset) was calculated as 8.2×10^{-9} cm^3/min. At 6.4×10^8 cells per ml, adsorption was so rapid (Fig. 3, inset) that an accurate rate constant could not be calculated. The K1F rate constant was 3.3 times higher than the k for phage T4, consistent with the smaller size of K1F and the PSA capsule on EV36, factors which are expected to increase the K1F diffusion coefficient and the

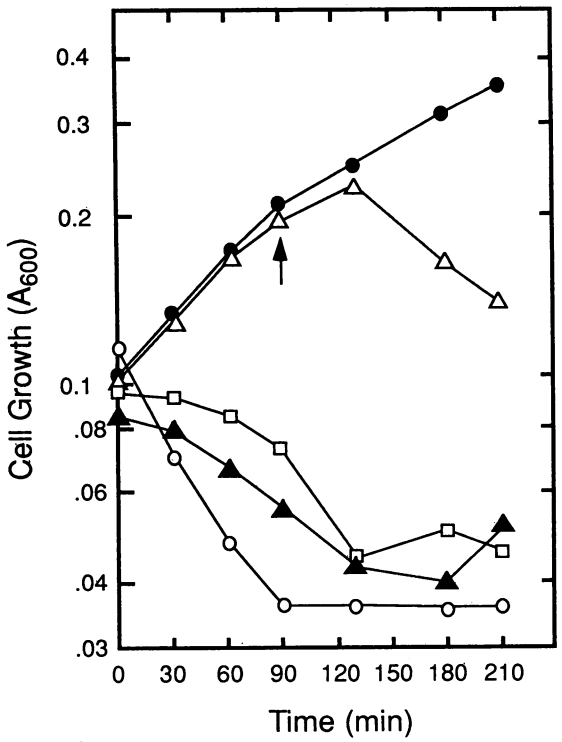

FIG. 2. Effects of exogenous sialic acid on sensitivity to K1F. Growth of an EV138 culture (●) is shown relative to that of similar cultures of EV138 exposed at −20 min to 0 (△), 13 (□), or 322 (▲) μM sialic acid. K1F was added at time zero to these cultures and to a culture of the capsulated control strain EV36 (○). After 90 min, sialic acid was added (13 μM final concentration) to the culture of EV138 that had been exposed to K1F at time zero (△), as indicated by the arrow. N-Z-Amine AS was not included in the medium.

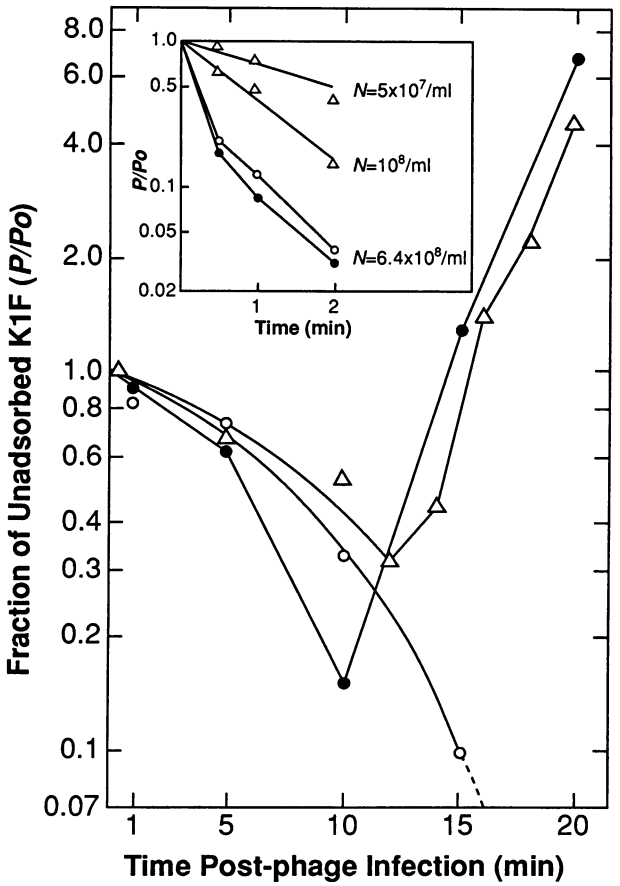

FIG. 3. K1F adsorption kinetics. Cultures of EV138 were exposed to 0.16 mM sialic acid 1 min prior to phage addition at time zero. The titer of unadsorbed phage was determined after dilution into chloroform-saturated saline, as described in Materials and Methods. Chloramphenicol was added to a final concentration of 100 μg/ml 20 min before sialic acid exposure (○). For each experiment, N was 1.5×10^8 (○, △) or 5.2×10^8 (●). Dashed line indicates extrapolation of the curve to 20 min, at which time the fraction of unadsorbed phage was 0.003. The inset shows K1F adsorption to EV36 (○, △) at the indicated cell densities. The cultures were not exposed to exogenous sialic acid. Solid symbols indicate exposure to chloramphenicol, as described above, for 10 min prior to phage addition.

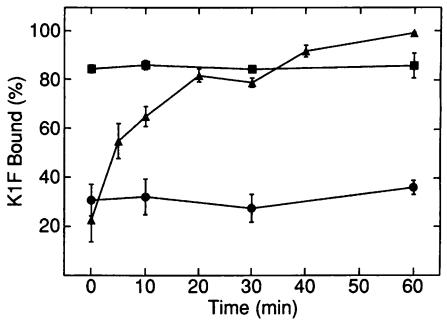

FIG. 4. Time course of PSA synthesis by EV138 after exposure to exogenous sialic acid. EV138 (▲) was exposed to 0.06 mM sialic acid at time zero and assayed for K1F binding. Cultures of capsulated EV1 (●) and EV138 (■), neither exposed to exogenous sialic acid, served as positive and negative controls, respectively. Data are the means ± standard deviation for one representative experiment (n = 3).

effective radius of the target cell, respectively, relative to those for T4 and thus increase k (21).

In contrast to the linear decrease in P/P_0 observed with EV36 (Fig. 3, inset), the effective concentration of EV138 before sialic acid addition is zero; consequently, K1F does not enter eclipse under these conditions, and P/P_0 remains 1. However, after sialic acid addition, capsule expression becomes dependent on the separate rates for sialic acid transport, activation, and polymerization and transit of PSA to the outer membrane (Fig. 1). The results (Fig. 3) show that numbers of PSA chains sufficient to support phage eclipse are present within 2 min after sialic acid addition. The convex adsorption curves in Fig. 3 indicate that K1F binding is a stochastic process, in which N and k increase continuously until phage exit the eclipse period or are exhausted from the adsorption mixture. Therefore, while all bacteria in the population are expected to be synthesizing PSA, they are not all equally susceptible to K1F infection, suggesting that the number of chains being translocated per cell varies or that other factors, such as the spatial organization of polysaccharide on the cell surface, may influence K1F binding or DNA ejection. K1F virions also contain the PSA depolymerase endo-N (26). Whether depolymerization without infection could affect the observed adsorption kinetics is unknown. Although the molecular details of phage binding and DNA ejection are obscure, the results in Fig. 3 clearly demonstrate the utility of K1F adsorption as a sensitive assay for PSA translocation.

When chloramphenicol was added to the culture before sialic acid or phage, virus maturation was prevented, which permitted the nonlinear adsorption kinetics to be observed until virtually all phage in the mixture entered eclipse (Fig. 3). A control experiment showed that K1F eclipse was not affected by chloramphenicol (Fig. 3, inset). These results demonstrate that de novo protein synthesis is not required for sialic acid uptake, indicating that basal expression of *nanT* is sufficient to support capsule synthesis in vivo. The results (Fig. 3) further show that neither sialic acid polymerization nor PSA translocation is dependent on de novo protein synthesis, indicating that, unlike its role as an inducer of the *nan* system (27), sialic acid is not an obligate inducer of the *kps* cluster. This conclusion and the results described above indicate that the only effect of the *neuB25* mutation on PSA production is its abrogation of sialic acid synthesis, a phenotype which can be bypassed (complemented) with exogenous monosaccharide. EV138 and related strains thus provide systems for investigating polysialytransferase function (20) and PSA translocation under in vivo conditions.

K1F adsorption on EV138 was indirectly measured by adding samples to mixtures of phage particles at various times after exposure to exogenous sialic acid. After a few minutes for adsorption, the samples were centrifuged briefly, and the titer of unadsorbed phage was determined. Although this method was complicated by PSA synthesis continuing during the adsorption period and a high background of nonspecifically bound phage, the results of the time course experiments shown in Fig. 4 were consistent with those of the above eclipse experiments and further indicated that K1F binding is dependent on the number of susceptible cells in the population. This number increases with time of exposure to exogenous sialic acid, as predicted from the results in Fig. 3.

Quasi proportionality between virus adsorption and receptor density has been observed when the number of receptors was varied experimentally from 0 to about 10^3 per cell, above which virtually every virus-cell collision resulted in a productive infection (15, 33, and references cited therein). On the basis of membrane sialic acid concentration measurements (28), the number of PSA chains per cell was estimated to be 5×10^4 (30), where each chain was assumed to contain 200 sialic acid residues. In contrast, lipopolysaccharide has been estimated at $>1 \times 10^6$ molecules per cell (18). The results (Fig. 3 and 4) show that by 20 to 30 min after sialic acid addition, the EV138 surface has been extensively repopulated with capsular polysaccharide. To demonstrate this directly, EV138 was grown on agar containing anti-PSA antibodies (25) and 0.16 mM sialic acid. The colonies that arose on this medium had precipitin halos, which are indicative of extracellular PSA (25), that were one-half to two-thirds the diameter of those of EV36 grown on the same medium without sialic acid. EV138 produced no detectable halos on the unsupplemented medium. These observations are consistent with repopulation of the EV138 surface with PSA in the presence of exogenous sialic acid. As predicted from the above results, K1F adsorption on EV138 grown overnight with 0.16 mM sialic acid was essentially identical ($k = 8.0 \times 10^{-9}$ cm^3/min) to the adsorption kinetics for EV36 shown in Fig. 3 (data not shown).

In vivo labeling of PSA. Since *nanA* prevents metabolic scrambling of the sialic acid carbon backbone (Fig. 1), cultures of EV138 exposed to radioactive sialic acid should direct label exclusively into the PSA-biosynthetic pathway. Figure 5 shows the results of a chromatographic analysis of detergent-treated membrane and soluble fractions that were prepared by sonication from a 5-ml culture of EV138. Virtually all of the labeled sialic acid from the membranes eluted in the column void volume (Fig. 5A) but was retained when the sample was pretreated with endo-*N*, which converted larger PSA chains to a heterogeneous mixture of sialyl oligomers (Fig. 5B). In contrast, all of the polymeric sialic acid in the soluble fraction migrated as relatively short chains that were nevertheless larger than about 10 sialyl residues, as shown by their susceptibility to endo-*N* (Fig. 5C and D). While these shorter PSA chains could, in principle, represent intermediates in PSA assembly, this was unlikely, because short chains were not detected when mutant or wild-type K1 was extracted by a mild chemical procedure (11). Therefore, it was probable that these shorter chains were produced from intact PSA during cell disruption by sonication. Alternatively, short chains might be produced if sialic acid were limiting for PSA synthesis.

To better characterize the PSA synthesized from exogenous sialic acid, a 1-ml culture of EV138 harboring pLysS was labeled as above and gently lysed with chloroform. This method avoids the high shear forces of sonication and should therefore allow more accurate assessment of PSA chain length. After treatment of the sample with DNase to reduce viscosity, the entire mixture was solubilized in 1% SDS and fractionated as described above. The results (Fig. 6) show that the internalized sialic acid was converted to CMP-sialic acid and that most or all of the polymeric product was relatively long PSA chains eluting in the column void volume. The absence of detectable short chains strongly implies efficient reconstitution of PSA synthesis with exogenous sialic acid, as indicated by the phage adsorption experiments. Investigating whether there is any chain length heterogeneity or the possibility of intermediates in the PSA assembly and translocation pathways will obviously require higher-resolution analytical techniques. However, by altering sialic acid-specific activity and using different pulse and fractionation procedures, it should be possible to better define these pathways by using the general approaches described in this article.

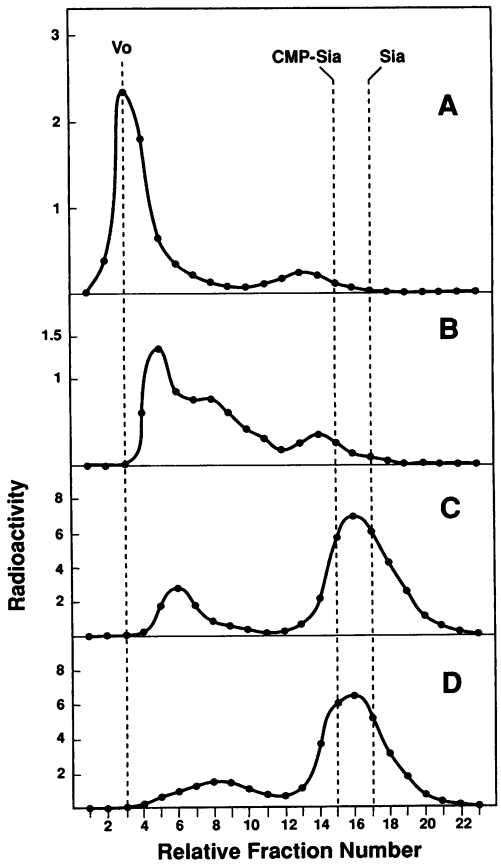

FIG. 5. Polymeric composition of PSA synthesized by EV138 from exogenous sialic acid. A 5-ml culture of EV138 was labeled as described in Materials and Methods and fractionated into membrane and soluble subcellular fractions, which were then analyzed by gel permeation chromatography (fraction size, 0.5 ml; column dimensions, 1 by 28 cm). (A) Membrane-associated PSA; (B) membranes pretreated with endo-*N*; (C) soluble PSA; (D) soluble PSA pretreated with endo-N. Radioactivity is presented as kcpm of ^{3}H per fraction. Sia, sialic acid; V_0, void volume.

DISCUSSION

The major findings of these studies are the following. (i) *E. coli* K1 strains with mutations in *nanA* and *neuB* (see also reference 20) are acapsular until sialic acid is added to the culture medium, after which capsular polysaccharide production at levels sufficient to support K1F infection is detected within 2 min. (ii) Capsule is synthesized continuously after the addition of exogenous sialic acid, as shown by increasing phage binding and eclipse over time. (iii) Sialic acid uptake, activation, and polymerization and PSA translocation are independent of de novo protein synthesis. (iv) Radioactive sialic acid can be used to specifically label PSA chains in vivo. These findings demonstrate selective synthesis and labeling of the PSA capsule in an essentially unperturbed system and thus allow capsule expression to be manipulated under defined culture conditions. The rapidity

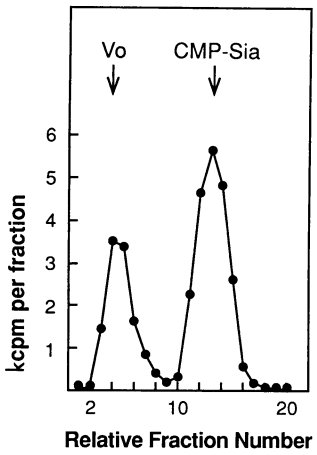

FIG. 6. Absence of short PSA chains in extracts of EV138 prepared by gentle cell lysis. A labeled culture (1 ml) of EV138 harboring pLysS was lysed with chloroform and fractionated by gel permeation chromatography, as described in the legend to Fig. 5 (fraction size, 0.65 ml; column dimensions, 1 by 25 cm). Radioactivity is presented as kcpm of ^3H per fraction.

of PSA synthesis and translocation detected by phage-binding experiments, while not necessarily excluding discrete assembly or translocation intermediates, appears to be compatible with a concerted pathway in which PSA synthesis may be coupled with translocation. Phage adsorption also appears to be a simpler and more sensitive indicator of PSA synthesis than previously reported radiometric assays (1, 20).

Polysaccharides encoded by type II *kps* clusters are not synthesized at temperatures below about 25°C (7, 31). If chloramphenicol is added to cultures of K1 or K5 strains prior to a shift from the nonpermissible temperatures to 37°C or during a 15- to 30-min period after temperature upshift, polymer synthesis and translocation, respectively, are blocked (7, 31). In contrast, the results (Fig. 3) show that neither PSA synthesis nor translocation is affected by chloramphenicol, indicating that certain *kps* genes are not expressed at low temperature and that these gene products are required for PSA assembly and translocation. This conclusion is consistent with the previous correlation between K1 capsule expression and de novo membrane protein synthesis after temperature upshift (32). The genetic mechanism(s) of temperature regulation is currently being investigated in this laboratory. However, it is already certain from the results in this communication that *kps* temperature regulation involves transcriptional or translational controls in addition to any potential posttranslational modifications that might exist.

Since little is known about the energetics or compartmentalization of PSA synthesis and translocation, the approaches described in this communication are a powerful addition to established genetic and biochemical procedures (20). Furthermore, there are few limitations on the combination of mutations that could be tested in a *nanA neuB* background to facilitate future studies (20). It should therefore be possible to reassemble the sialyltransferase complex in vivo by testing its functional properties at each stage of genetic reconstitution, starting with *neuS* (20). Such experiments will be useful for future in vitro attempts to reconstitute part or all of the synthesis and translocation pathways.

The present results, together with those published previously (25, 28), clearly indicate that the *neuB25* mutation blocks sialic acid synthesis, although it is uncertain whether the wild-type allele encodes the sialic acid synthase that presumably condenses ManNAc and phosphoenolpyruvate (2). Sialic acid synthase assays with *E. coli* K1 extracts as the enzyme source gave ambiguous results (24), unlike the results with *Neisseria meningitidis*, which has a readily detected synthase activity (2). Although ManNAc is a sialic acid precursor for meningococci (2) and mammalian cells (29), exogenous addition of this sugar did not complement the defect in EV24 or EV138. Presumably, exogenous ManNAc is transported by the sugar phosphotransferase system and would exist as the 6-phosphate sugar derivative, which is not a substrate for the meningococcal sialic acid synthase (2). In contrast, sialic acid is transported without apparent covalent modification (27). Thus, while it is tempting to conclude that the failure to complement *neuB* or *neuC* (34) mutations with ManNAc means that the metabolic defect occurs after ManNAc synthesis, it is possible that the ManNAc-6-phosphate expected to accumulate in vivo is not a substrate for the K1 enzyme(s). Together, the observations imply that there may be important differences between microbial sialic acid synthases. Work is in progress to characterize the defect in *neuB* strains as a way to better understand sialic acid synthesis, since an ability to specifically inhibit this process could eventually have clinical relevance (17, 25).

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