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Identification of an Inducible Catabolic System for Sialic Acids (*nan*) in *Escherichia coli*

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***Escherichia coli* K-12 and K-12 hybrid strains constructed to express a polysialic acid capsule, the K1 antigen, were able to efficiently use sialic acid as a sole carbon source. This ability was dependent on induction of at least two activities: a sialic acid-specific transport activity, and an aldolase activity specific for cleaving sialic acids. Induction over basal levels required sialic acid as the apparent inducer, and induction of both activities was repressed by glucose. Induction also required the intracellular accumulation of sialic acid, which could be either added exogenously to the medium or accumulated intracellularly through biosynthesis. Exogenous sialic acid appeared to be transported by an active mechanism that did not involve covalent modification of the sugar. Mutations affecting either the transport or degradation of sialic acid prevented its use as a carbon source and have been designated *nanT* and *nanA*, respectively. These mutations were located by transduction near min 69 on the *E. coli* K-12 genetic map, between *argG* and *glnF*. In addition to being unable to use sialic acid as a carbon source, aldolase-negative mutants were growth-inhibited by this sugar. Therefore, the intracellularly accumulated sialic acid was toxic in aldolase-deficient *E. coli* strains. The dual role of aldolase in dissimilating and detoxifying sialic acids is consistent with the apparent multiple controls on expression of this enzyme.**

The sialic acids (*N*-acylneuraminic acids) are a family of 2-keto-3-deoxy-5-amino-nonulopyranosonic sugar acids that are differentiated by their types of N and O acylations. *N*-Acetylneuraminic acid (NeuNAc; 5-acetamido-3,5-dideoxy-D-*glycero*-D-galactononulosonic acid) is one common sialic acid frequently found as a terminal nonreducing sugar residue in complex glycoproteins and glycolipids of higher eucaryotes. Most lower eucaryotes, plants, and most procaryotes do not appear to synthesize sialic acids (6). Sialic acid has been found in a few genera of bacteria, notably *Neisseria* and *Escherichia*, in which it exists as a capsule of relatively long unbranched homopolymeric chains of α-2,8- or α-2,9-ketosidically linked sialyl residues (reviewed in reference 24). These sialyl polymers have been identified as pathogenic determinants in *Neisseria meningitidis* serogroups B and C and *Escherichia coli* K1 strains, and considerable information is available about their chemical structure and biosynthesis (13, 20, 24). Much less is known about the mechanisms of assembly of sialyl polymers in vivo and the organization and regulation of genes involved in polymer and polymer precursor biosynthesis.

We have been investigating the biosynthesis of sialyl polymer in *E. coli* K1 serotypes (reviewed in reference 24). The K1 antigen in these bacteria is composed of as many as 200 sialyl residues in α-2,8-ketosidic linkage (20). To understand the mechanisms of polymer initiation and elongation, we attempted to identify potential intermediates in the biosynthetic pathway by labeling K1-producing hybrid strains of *E. coli* with radiolabeled sialic acid. We observed that <10% of the incorporated label appeared in polysialic acid. Uptake followed by rapid catabolism of sialic acid presumably reduced its efficient incorporation into sialyl polymer. This supposition was supported by isolating mutants defective in uptake and degradation of sialic acids. In a preliminary report, these mutants were shown to be defective in sialic acid permease and aldolase activities (E. R. Vimr and F. A. Troy, Abstr. Annu. Meet. Am. Soc. Microbiol., 1984, K14, p. 149).

In this communication, we present a more detailed description of the sialic acid catabolic system in *E. coli*, including results of genetic and physiological studies which suggest that sialic acid catabolism is under multiple genetic control. In an accompanying paper (28), we present results suggesting that sialic acid aldolase may, in part, regulate the accumulation of biosynthetically derived sialic acid. The potential overlap in catabolic and anabolic systems is suggested by our finding that sialic acid is toxic if allowed to accumulate in *E. coli*. We propose the designation *nan* (*N*-acylneuraminate) for mutations that affect sialic acid catabolism.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and media. All bacterial strains were *E. coli* K-12 or K-12 hybrid derivatives (Table 1). Hybrid strains expressing the K1, or polysialic acid capsular, antigen (polysialic acid is synonymous with sialyl polymer) are *kps*$^+$, and their construction is described in the accompanying paper (28). *E. coli* K-12 does not express K1 antigen under any known conditions and may entirely lack *kps* DNA sequences (10). Thus, K-12 strains are null for *kps* functions and are designated *kps*^{n} in the text.

All P1 transductions were performed with P1 *vir* as described by Miller (16) except that an agar overlay was not used. Transposon Tn*10* transpositions were performed with the vector λ840 (*nin-5* cI857 P80 *hisG*::High Hopper 104), which gives a 50- to 100-fold-higher transposition frequency than wild-type Tn*10* (11, 22).

Luria broth, minimal E medium, minimal M9 medium, and F top agar have been described (9). Solid media were prepared by including 1.5% agar (Difco Laboratories, De-

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TABLE 1. Bacterial strains used and their origin

E. coli strain	Genotype (phenotype)[a]	Source or reference
AT12-55	Hfr AB312 *thr-1 leuB6 argG75 thi-1 lacY1 rpsL8 supE44*	CGSC 5707[b]
JL3664	*argA22 galP23 rha-200*	J. L. Ingraham
K19	*fadE62 mel-1 supF58*	CGSC 4811
MC4100	*araD139* Δ*(argF-lac)U169 flbB5301 deoC1 rpsL150 relA1 ptsF25 rbsR*	J. Beckwith
NCM195	*ntrA6 (glnF6)*[c]	S. Kustu
NCM196	*ntrA7 (glnF7)*[c]	S. Kustu
EV36	*rpsL9* [*argA*$^+$ *kps*$^+$ *rha*$^+$][d] *galP23*	From JL3664 (28)
EV46	EV36 *nan-1* (Nan$^-$ Nan^s)	MNNG induced
EV47	EV36 *nan-2* (Nan$^-$ Nan^s)	MNNG induced
EV48	EV36 *nan-3* (Nan$^-$ Nan^s)	MNNG induced
EV49	EV36 *nan-5* (Nan$^-$ Nan^s)	MNNG induced
EV50[e]	EV36 *nanA4 uxa* (Nan$^-$ Nan^s)	MNNG induced
EV51	EV50 *nanA*$^+$ *zgj-791*::Tn*10* (Nan$^+$ Nan^r Tet^r)	P1/JL3664 RTP[f] × EV50, select Tet^r, screen Nan$^+$ Nan^r
EV52	EV50 *nanA4 zgj-791*::Tn*10*	As above, screen Nan$^-$ Nan^s
EV55	EV36 *nanT1* (Nan$^-$ Nan^r)	MNNG induced
EV56	EV55 *zgj-791*::Tn*10* (Nan$^-$ Nan^r Tet^r)	P1/EV51 × EV55, select Tet^r, screen Nan$^-$ Nan^r
EV70	EV36 *nanA4 zgj-791*::Tn*10*	P1/EV51 × EV36, select Tet^r, screen Nan$^-$ Nan^s
EV72	EV36 *nanA*$^+$ *zgj-791*::Tn*10*	As above, screen Nan$^+$ Nan^r
EV76	JL3664 *nanA4 zgj-791*::Tn*10*	P1/EV51 × JL3664 (see EV70)
EV77	JL3664 *nanA*$^+$ *zgj-791*::Tn*10*	As above, screen Nan$^+$ Nan^r
EV78	MC4100 *nanA4 zgj-791*::Tn*10*	P1/EV51 × MC4100 (see EV70)
EV79	MC4100 *nanA*$^+$ *zgj-791*::Tn*10*	As above, screen Nan$^+$ Nan^r
EV80	K19 *nanA4 zgj-791*::Tn*10*	P1/EV51 × K19 (see EV70)
EV81	K19 *nanA*$^+$ *zgj-791*::Tn*10*	As above, screen Nan$^+$ Nan^r

[a] Nan, Ability to use sialic acid as carbon source; Nan^s, Nan^r, sensitivity and resistance, respectively, to sialic acid for cells grown on Gro as the carbon source; Tet^r, resistance to tetracycline.

[b] *E. coli* Genetic Stock Center strain number (Yale University School of Medicine, New Haven, Conn.).

[c] In *E. coli*, *ntrA* mutations are called *glnF* (1).

[d] Markers in brackets are known to be derived from an *E. coli* strain other than K-12.

[e] EV50 was unable to use galacturonic acid as a carbon source (Uxa$^-$). The Uxa$^-$ phenotype of EV50 was apparently the result of MNNG mutagenesis, because strains bearing other *nan* mutations did not have this phenotype. The *uxa* mutation in EV50 was not cotransducible with *nanA4* or *zgj-791*::Tn*10* (unpublished results). EV50 could be transduced to growth on galacturonic acid with P1 grown on a *uxa*$^+$ strain; such transductants remained Nan$^-$, demonstrating that the *uxa* and *nanA4* mutations were unlinked.

[f] RTP, Random Tet^r population; refers to a population of strain JL3664 with Tn*10* elements randomly inserted within the genome.

troit, Mich.). M9 medium, with the ammonium salts replaced by KCl and containing 0.2% glucose (Glc) and 0.1% L-arginine, was used to score strains with *glnF* mutations. Minimal E medium was supplemented with thiamine hydrochloride and required amino acids in the concentrations described previously (8). Sugars were added as carbon sources at 0.3% unless otherwise indicated. Plate growth tests for assessing various sugars as potential carbon sources were done by plating 0.1 ml of an overnight culture of bacteria in 2.5 ml of F top agar onto a minimal agar plate and placing ca. 1 mg of sugar on the overlay. Growth was scored after overnight incubation. All incubations were carried out at 37°C, and aeration of liquid media was supplied by vigorous shaking.

Mutant isolation. Strain EV36 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as described previously (27). After mutagenesis, survivors were grown overnight in E medium plus Glc and diluted 1:50 into fresh minimal medium containing 0.05 to 0.1% sialic acid. Growth was monitored by the increase in turbidity (A_{600}); once cells were growing exponentially, samples were diluted into fresh medium containing sialic acid plus 200 U of penicillin G per ml to give 2×10^6 to 10×10^6 bacteria per ml. Cultures were grown for 2 to 4 h and washed by low-speed centrifugation at room temperature, and survivors were grown overnight in minimal Glc medium. Dilutions were plated on minimal Glc agar to give 200 to 300 colonies per plate. Colonies were replica plated on the same medium and on minimal medium containing 0.1% sialic acid. Colonies unable to grow on the sialic acid plate were saved as potential mutants with defects in sialic acid catabolism (*nan*).

Enzymes and chemicals. Sialic acid aldolase (*N*-acylneuraminate pyruvate-lyase, EC 4.1.3.3.) isolated from *Clostridium perfringens* was purchased from Sigma Chemical Co., St. Louis, Mo. Sialic acid was purchased from Sigma, Pfhansteihl Laboratories, Inc., Chicago, Ill., and Kantoishi Pharmaceutical Co., Tokyo, Japan. Equivalent results were obtained with each batch of this sugar. The C-1 methyl ester derivative of sialic acid (MeNeuNAc; *C*-1-methyl-*N*-acetylneuraminate) was from Kantoishi. *N*-Glycolylneuraminic acid (NeuNGc) and other sugars were from Sigma. Radiolabeled sialic acid was prepared by hydrolysis of CMP-[9-^{3}H]NeuNAc (18.9 mCi/mmol), CMP-[4-^{14}C]NeuNAc (1.6 mCi/mmol), or CMP-[4,5,6,7,8,9-^{14}C]NeuNAc (319 mCi/mmol) in 0.1 N HCl for 30 to 60 min at 37°C. CMP-[^{3}H]NeuNAc and CMP-[^{14}C]NeuNAC were purchased from New England Nuclear Corp., Boston, Mass. Labeled C-2 alcohol derivatives of sialic acid, i.e., sialitol (*N*-acetylneuraminitol [NeuNAc-OH]), were prepared by reduction with sodium borohydride. To prepare [4-^{14}C]NeuNAc-OH, 30 mg of sialic acid was mixed with 1 μCi of [4-^{14}C]NeuNAc in 0.1 N NaOH; 30 mg of $NaBH_4$ in 0.1 N NaOH was added, followed by an additional 15 mg after 15 min and again after 30 min at room temperature. After a total of 45 min, 0.15 ml of acetone was added to consume excess borohydride, and the sample was desalted by gel permeation chromatography and lyophilized. The sialitol failed to give a color reaction with thiobarbituric acid (29), indicating quan-

titative reduction of the C-2 keto group to an alcohol. Radiochemical purity was determined by descending paper chromatography and high-voltage borate paper electrophoresis, as previously described (25). [2-^{3}H]NeuNAc-OH was similarly prepared by reducing sialic acid, obtained after mild acid hydrolysis of colominic acid, with 10 mCi of KB^3H_4 (100 mCi/mmol; Amersham Corp., Arlington Heights, Ill.). Preparation of the partial acid hydrolysate and purification of sialitol by DEAE chromatography have been described (26). Tritium-labeled sialitol was desalted and analyzed as described above.

Measurement of sialic acid permease and aldolase activity. Permease activity was measured by a membrane filtration assay (15). Unless otherwise indicated, the following protocol was used. Exponentially growing cells with an A_{600} between 0.2 and 0.4 were sedimented at room temperature by low-speed centrifugation, washed in E medium lacking a carbon source, and suspended in 1/50 volume of E medium plus 0.2% glycerol (Gro) and any required amino acids. A sample was then added to a tube containing the same medium preincubated at 37°C plus [9-^{3}H]NeuNAc (diluted with unlabeled sialic acid to a specific radioactivity of 1,600 dpm/nmol), so that a final volume of 1 ml would contain 0.2 mM sialic acid. Sufficient cells were added to give a 1-ml final volume with an A_{600} between 0.2 and 0.4. After 1 min, 3 ml of prewarmed E medium was added, and the cells were filtered. The tube was rinsed with an additional 3 ml of E medium, followed by washing the filter with 3 ml of the same medium. Radioactivity on the filters was counted in 5 ml of PCS aqueous scintillant (Amersham). Tritium counting efficiency by this technique was 23.4%. One unit of permease activity represented 1 nmol of sialic acid taken up in 1 min at 37°C. Specific activity was expressed as units per milligram of total cell protein. Proteins were determined independently by a modified Folin procedure suitable for accurate estimation of protein content of whole cells (14). Uptake was proportional to cell protein in the assay. Results shown are the average of duplicate determinations for which variation between samples was <15%.

Aldolase activity was measured with a coupled lactic acid dehydrogenase-NADH assay for pyruvic acid as previously described (5). The source of enzyme was soluble cell extracts prepared by sonication as described in the accompanying paper (28). One unit of aldolase activity represented the formation of 1 nmol of pyruvic acid per min at 37°C. Activities were corrected for background by performing duplicate incubations wherein one lacked exogenous sialic acid as the substrate.

Analytical procedures. Descending paper chromatography on Whatman 3MM paper was developed in ethanol–1 M ammonium acetate (7:3), pH 7.5, as described previously (20). Radioactivity was detected in chromatograms by radiometric scanning with a Packard 7201 Radiochromatogram scanner. The intracellular concentration of sialic acid accumulated by an aldolase-negative strain was calculated with the assumptions that the water space per cell was 6.2×10^{-13} ml (calculated from Table 2 in reference 23) and that 3.4×10^9 cells were equal to 1 mg (dry weight) (12).

Genetic procedures. A Tn*10* element was isolated near *nanA* by published procedures (9). Briefly, a population of 30,000 independent Tn*10* insertions in strain JL3664 was prepared by transposition from λ840 with selection for Tet^r. A P1 transducing lysate was prepared on this population and used to transduce strain EV50 (*nanA4*) to Tet^r. Approximately 6,000 Tet^r transductants were replica plated to minimal medium containing tetracycline plus 0.1% sialic acid as the carbon source. Several Tet^r Nan$^+$ colonies were isolated. One isolate contained a Tn*10* element linked 50% (35 to 70% range in cotransduction frequencies) to *nanA4*. This Tn*10* insertion was called *zgj-791*::Tn*10* according to the *z* notation convention (4).

TABLE 2. Effects of *nan* mutations on sensitivity of *E. coli* to sialic acid toxicity

Strain	Relevant genotype	Aldolase sp act (U/mg of soluble protein)[a]		Zone of inhibition[b] diam (mm)
		Gro	NeuNAc	
EV36	*nan*$^+$ *kps*$^+$	2.6	1,782	ND
EV77	*nan*$^+$ *kps*^{n}	6.6	2,421	ND
EV79	*nan*$^+$ *kps*^{n}	4.3	2,066	ND
EV81	*nan*$^+$ *kps*^{n}	4.5	2,103	ND
EV46	*nan-1 kps*$^+$	5.7	—	15 (fuzzy)
EV47	*nan-2 kps*$^+$	13.5	—	20 (fuzzy)
EV48	*nan-3 kps*$^+$	<0.3	—	24 (fuzzy)
EV49	*nan-5 kps*$^+$	<0.3	—	26 (sharp)
EV50	*nanA4 kps*$^+$	<0.3	—	30 (sharp)
EV76	*nanA4 kps*^{n}	<0.3	—	31 (sharp)
EV78	*nanA4 kps*^{n}	<0.3	—	31 (sharp)
EV80	*nanA4 kps*^{n}	<0.3	—	29 (sharp)

[a] Stationary-phase glycerol-grown cells were diluted 100-fold into fresh Gro medium or into minimal medium containing 1 mM sialic acid (NeuNAc) and grown to an A_{600} between 0.15 and 0.3. —, Assay not done.

[b] Gro-grown cells (50μl) plated in 1 ml of F top agar on 6 ml of minimal-Gro bottom agar in 55-mm petri plates were grown overnight with a 1-cm glass fiber disk soaked with 116.5 μg of sialic acid placed in the center of the plate. ND, (no zone of inhibition detected); fuzzy, edge demarcating zone of inhibition and growth was indistinct; sharp, edge demarcating zone of inhibition and growth was distinct.

RESULTS

Sialic acid is a carbon source for *E. coli*. A variety of *E. coli* strains were tested for growth on sialic acid obtained from several commercial suppliers. Plate growth tests showed that sialic acid served as an efficient carbon and energy source. In fact, judged by colony size, growth at sialic acid concentrations as low as 5×10^{-5} M was equivalent to growth on *N*-acetylglucosamine (GlcNAc). This conclusion was verified by quantitative growth curves which showed that *E. coli* grew with the same doubling time (88 min) and to the same extent on equimolar concentrations of NeuNAc and of GlcNAc (Fig. 1). Growth on either substrate was superior to growth on glucosamine (GlcN) or N-acetylmannosamine (ManNAc) (Fig. 1). We concluded that sialic acid is efficiently transported and catabolized by *E. coli*, implying the presence of a permease and aldolase for sialic acid.

Isolation of mutants defective in sialic acid catabolism. Mutants unable to use sialic acid as a sole carbon or energy source were isolated with a frequency of 10^{-2} after mutagenesis and one cycle of penicillin enrichment. Extracts prepared from mutants of independent origin were screened for loss of aldolase activity (Fig. 2). Wild-type (strain EV36) extract degraded [4-^{14}C]NeuNAc to a faster-migrating radiolabeled species (Fig. 2). The R_f of this component was identical to that of ManNAc produced by treating NeuNAc with a sialic acid-specific aldolase from *C. perfringens* (Fig. 2). We conclude that sialic acid-cleaving activity detected in the wild-type extract was due to an aldolase, i.e., *N*-acylneuraminate pyruvate-lyase. Because the label in the substrate was in the hexosamine moiety of sialic acid, the other product of the aldol cleavage, pyruvic acid, was not detected by radiometric scanning. Identical results were obtained with [4,5,6,7,8,9-^{14}C]NeuNAc as the substrate. An extract from the aldolase-deficient mutant EV50 (*nanA4*)

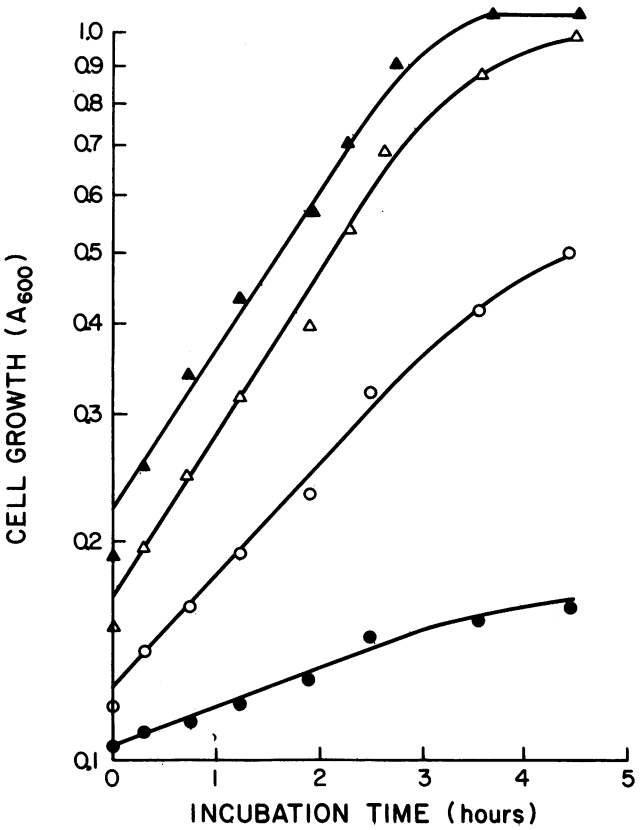

FIG. 1. Utilization of sialic acid as a carbon source. Strain EV36 was grown overnight in minimal E medium containing Glc. Cells were collected by low-speed centrifugation at room temperature and suspended to an A_{600} of ca. 0.1 in Glc-free medium containing 2 mM NeuNAc (△), GlcNAc (▲), GlcN (○), or ManNAc (●).

was unable to degrade radiolabeled sialic acid (Fig. 2). An extract of strain EV55 (*nanT1*) had aldolase activity (Fig. 2) yet did not grow on sialic acid as a carbon source, suggesting that it might be defective in sialic acid uptake.

Sialic acid transport in *E. coli*. Evidence that strain EV55 was transport defective was obtained by measuring the time course of [9-^{3}H]NeuNAc uptake by wild-type and mutant *E. coli* strains (Fig. 3). Results of uptake measurements with strain EV36 (*nan*$^+$) grown on Gro, ManNAc, Glc, or NeuNAc (Fig. 3) suggested that sialic acid or a derivative induced sialic acid permease activity, because the initial rate of uptake by cells grown on NeuNAc was >20 times faster than that by cells grown on the other carbon sources. Net uptake in the NeuNAc-induced cells was linear over the course of the experiment (Fig. 3A), suggesting that internalized NeuNAc was rapidly degraded, a situation that could prevent the establishment of equilibrium conditions. As suggested earlier, strain EV55 was defective in NeuNAc uptake (Fig. 3B). The residual uptake in this strain may represent diffusion of sialic acid, entry by a secondary transport system, or simply remaining uptake activity of mutant permease in the *nanT1* background.

Sialic acid uptake was measured in the Gro-grown aldolase mutant strain EV50 and found to be comparable to induced levels of sialic acid uptake in the wild type, although for EV50 net uptake nearly ceased after 40 min (Fig. 3A). Cessation of net uptake would be expected for a transported substance that was not rapidly metabolized, consistent with the aldolase-negative phenotype of strain EV50. We conclude from these results that in the absence of an active aldolase, sialic acid generated biosynthetically is sufficient to constitutively induce sialic acid permease. In addition, induction of permease appeared to be sensitive to glucose because strain EV50 grown on this sugar had reduced NeuNAc uptake (Fig. 3A).

The relatively high rate of uptake by NeuNAc-induced or by constitutive cells (Fig. 3) suggested that transport was mediated by an active mechanism. If energy were required

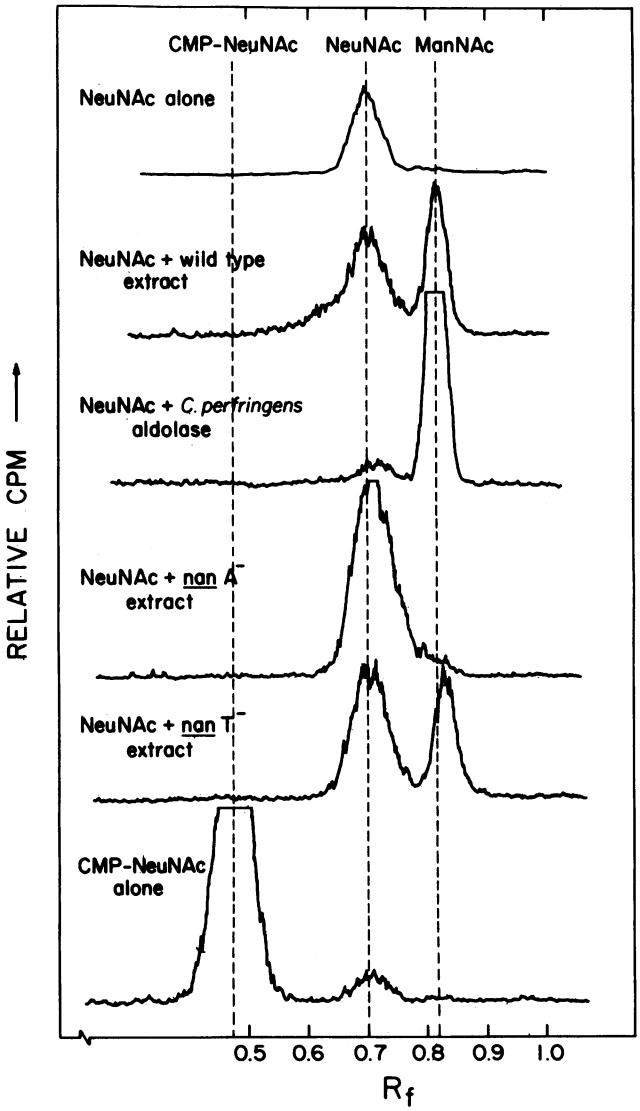

FIG. 2. Analysis of sialic acid degradation products derived from treatment with wild-type and mutant soluble cell extracts. [4-^{14}C]NeuNAc (0.18 mM, 1.6 mCi/mmol) was treated with 0.6 to 1.2 mg of soluble protein from extracts of strains EV36 (wild type), EV50 (*nanA4*), and EV55 (*nanT1*) in a total volume of 60 μl containing 50 mM potassium phosphate, pH 7.2. Extracts were from stationary-phase cells grown overnight in Luria broth. After 1 h at 37°C, the entire reaction mixtures were fractionated by descending paper chromatography. In another 60-μl reaction, 100 U of aldolase from *C. perfringens* was used to generate ManNAc from sialic acid. The R_fs of untreated [^{14}C]NeuNAc and CMP-[^{14}C]NeuNAc are shown for comparison.

for sialic acid uptake, cells should accumulate NeuNAc at concentrations above the extracellular concentration. Assuming that the sample of Gro-grown strain EV50 (Fig. 3A) with an A_{600} of 1.0 contained 1.2×10^9 cells per ml, we calculated that the intracellular concentration of NeuNAc in strain EV50 at 40 min was approximately 50 mM, ca. 500 times higher than the extracellular concentration of sialic acid used in the experiment. Although the assumptions involved in this estimate may have caused our calculation to overestimate the true intracellular concentration, we conclude that sialic acid uptake is likely to require active transport.

Sialic acid transported without modification. The constitutive-uptake, aldolase-negative strain EV50 was used to determine whether NeuNAc transport involved group translocation or posttransport modification. [9-^{3}H]NeuNAc that accumulated in strain EV50 grown in glycerol and labeled under the conditions described for Fig. 3 was extracted and chromatographed on Whatman 3MM as described in Materials and Methods. The radioactivity profile showed two peaks; one was chromatographically immobile and remained at the origin, and a second migrated coincident with authentic NeuNAc (data not shown). The origin peak represented [^{3}H]NeuNAc incorporated into sialyl polymer, as determined by its sensitivity to *endo*-α-2,8-neuraminidase (26). This conclusion was further substantiated by showing that an aldolase-negative, K1-antigen-negative strain (EV76) accumulated only NeuNAc under similar conditions. Additional confirmation that the second peak was free [^{3}H]NeuNAc was obtained by demonstrating that it was sensitive to *C. perfringens* aldolase and that the radioactivity migrated coincident with ManNAc. *C. perfringens* aldolase will not cleave NeuNAc in ketosidic linkage or NeuNAc that has been phosphorylated at C-9 (2, 21). We therefore conclude that sialic acid is transported without covalent modification.

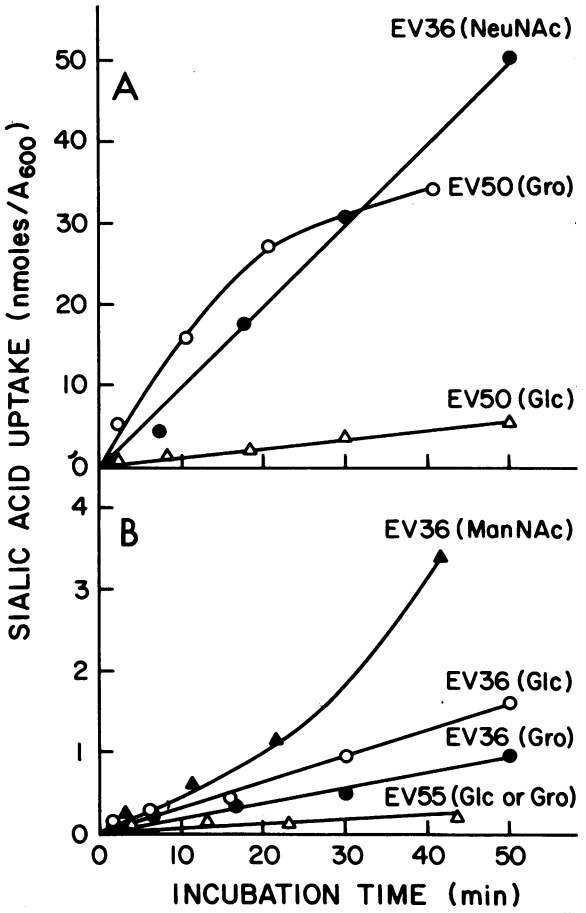

FIG. 3. Uptake of sialic acid by mutant and wild-type *E. coli* strains grown on various carbon sources. Cells were grown overnight in minimal E medium with one of the following carbon sources, each at 0.2%: NeuNAc, Gro, Glc, or ManNAc. Stationary-phase cells were collected by low-speed centrifugation at room temperature, washed once in E medium lacking a carbon source, and suspended to an A_{600} of ca. 0.25. Unlabeled sialic acid was added to cultures preincubated at 37°C to give a final concentration of 0.1 mM. After 5 min, [9-^{3}H]NeuNAc was added so that the specific radioactivity was 3,300 dpm/nmol.

Specificity of sialic acid permease and aldolase activities. Uptake assays with NeuNAc-induced strain EV36 or constitutive strain EV50 were carried out under conditions similar to those described for Fig. 3. At a 10-fold molar excess, various unlabeled sugars were tested as potential competitors of sialic acid uptake. Compounds tested as potential inhibitors of [9-^{3}H]NeuNAc uptake and found to be inactive were Glc, GlcN, GlcNAc, Gro, ManNAc, and pyruvic acid. In contrast, a 10-fold molar excess of unlabeled NeuNAc inhibited [9-^{3}H]NeuNAc uptake 95%. Further evidence for the high specificity of the sialic acid permease and aldolase came from plate growth tests. Strains EV50 (aldolase deficient) and EV55 (sialic acid uptake deficient) were tested for growth on a variety of potential carbon sources, including the compounds listed above plus hexoses (fucose, mannose, rhamnose), hexitols (galactitol, mannitol, sorbitol), pentoses (arabinose, ribose), disaccharides (lactose, melibiose), trisaccharides (raffinose, trehalose), and hexuronic acids (galacturonic acid, glucuronic acid). All the sugars tested were used as carbon sources by the mutants as judged by their growth compared with that of the parent strain EV36, except that strain EV55 did not use ManNAc. The significance of this observation is not clear, because ManNAc did not compete for uptake of sialic acid. Strain EV50 could not use galacturonic acid, but this result was due to an unlinked mutation (see Table 1, footnote *e*). Transduction of the *nanA4* allele from strain EV52 into an unmutagenized background resulted in a strain (EV70) that was able to use galacturonic acid for growth. Together these results suggest that both permease and aldolase possess a high degree of specificity for sialic acid.

The nature of the C-5 amino substituent in sialic acid did not appear to affect transport or degradation. Thus, strain EV36 used both NeuNGc and NeuNAc as a carbon source, as judged by plate growth tests. Surprisingly, MeNeuNAc was also a good carbon source. Purified *C. perfringens* aldolase will not cleave MeNeuNAc (21); therefore, either the *E. coli* aldolase has a different specificity or a demethylase removes the C-1 methyl group from MeNeuNAc before aldolase cleavage.

Two preparations of sialitol were tested as potential permease and aldolase substrates. Neither [2-^{3}H]NeuNAc-OH nor [4-^{14}C]NeuNAc-OH was transported by strain EV50, nor was either labeled alcohol cleaved by extracts of NeuNAc-induced strain EV36 (data not shown). In addition, neither preparation of sialitol supported the growth of EV36 when tested as a carbon source. We conclude from these results and from the results described above that sialic acid permease recognizes sialic acids in their pyranose form, but

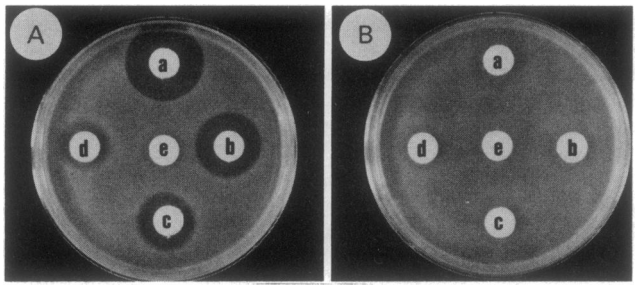

FIG. 4. Sialic acid toxicity and Glc protection. Gro- and Glc-grown cultures of strain EV70 (*nanA4*) were plated in F top agar onto minimal Gro (A) or minimal Glc (B) agar plates, respectively. Filter disks (1 cm) were soaked with 20 μl of water containing (a) 340, (b) 170, (c) 85, (d) 42.5, or (e) 21.25 μg of NeuNAc and placed on the overlay. Photographs were taken after 15 h of incubation.

that the permease may not have stringent recognition specificity for some ring substituents.

Accumulation of sialic acid is toxic. Adding NeuNAc to exponentially growing cultures of aldolase-negative cells resulted in cessation of growth within 30 min. NeuNAc concentrations as low as 0.1 mM produced growth stasis (data not shown). Growth inhibition was dependent on the carbon source and was not observed for cells growing on Glc. This observation suggested that toxicity required transport of sialic acid and that transport was inhibited by growth on Glc. Evidence supporting this supposition is shown in Fig. 4. Gro-grown EV50 cells were sensitive to NeuNAc, with a detectable zone of inhibition at 21.25 μg of sialic acid (Fig. 4A). In contrast, EV50 grown on Glc was only weakly inhibited by 340 μg of sialic acid and was resistant to 21.25 μg (Fig. 4B). Since previous results showed that Glc did not compete for NeuNAc uptake, we conclude that Glc protection occurs at the level of induction of permease activity.

Sialic acid toxicity was solely a function of the particular *nan* mutation. Thus, introduction of the *nanA4* allele by cotransduction with *zgj-791*::Tn*10* yielded strains with identical sensitivity to sialic acid and with undetectable levels of uninduced aldolase activity (Table 2). Strains bearing either the *nan-3* or *nan-5* mutation gave results similar to those for strains bearing the *nanA4* mutation (Table 2). Two other mutants, EV82 (*nan-1*) and EV83 (*nan-2*), had less-pronounced zones of inhibition and essentially wild-type levels of uninduced aldolase activity. Whether aldolase activity in these strains failed to increase when inducer was added was not determined. Only the *nanA4* and *nanT1* mutations have been mapped, so the possibility exists that other *nan* mutations may define additional loci involved in sialic acid catabolism. Therefore, since each *nan* allele was independently isolated, we have refrained from designating these additional mutations *nanA* until they are mapped to this locus. The results (Table 2) prove that sialic acid toxicity is not a phenomenon unique to a single *nan* mutation and strongly suggest that high levels of aldolase are required to escape sialic acid toxicity. In addition, it is pertinent to note that the size and shape of the zone of inhibition correlated with the severity of the particular aldolase defect in a given mutant (Table 2).

Mixing an extract of fully induced NeuNAc-grown strain EV79 with an extract from uninduced EV79 or from an aldolase-negative strain (EV78) did not appreciably augment or reduce aldolase activity in the extract of induced EV79 (data not shown). These results indicate that aldolase is not present in uninduced cells in an inactive form, nor is there likely to be an inhibitor of aldolase in aldolase-negative strains, although these mixing experiments have not been performed with *kps*$^+$ hybrid derivatives.

Induction of permease and aldolase. The previous results showed that permease and aldolase were induced by sialic acid or a sialic acid derivative. Furthermore, permease could be induced by sialic acid accumulated by transport or through biosynthesis (Table 3). The basal level of permease and aldolase in strain EV72 (*nan*$^+$ *kps*$^+$) grown in Gro was unaffected by growth on Glc. In contrast, the permease activity in strain EV77 (*nan*$^+$ *kps*^{n}) was repressed by growth on Glc. We conclude from these results that the basal level of permease in *E. coli* is between 4 and 6 nmol of sialic acid per min per mg of cell protein, which is 5- to 10-fold higher than the residual permease activity of Glc-grown cells or of a mutant with an uptake defect (EV56). Strain EV72 presumably avoided complete Glc repression because of sufficient sialic acid (inducer) produced through biosynthesis, since this strain was *kps*$^+$. Consistent with this interpretation was the level of permease activity in the aldolase-negative strain EV70 (*nanA4 kps*$^+$) grown on Gro. Permease in EV70 was fully induced when the cells were grown on Gro compared with that in NeuNAc-induced EV72 (wild-type aldolase) and EV72 grown on NeuNAc and was even partially induced by growth on Glc (Table 3). These results would be expected if the intracellular concentration of sialic acid (inducer) produced through biosynthesis were higher in the absence of an active aldolase. Data fully in accord with this expectation are presented in the accompanying paper (28). Confirmation that biosynthetically derived sialic acid acted as an inducer of permease was found by comparing the activities of strains EV77 (*nan*$^+$ *kps*^{n}) and EV76 (*nanA4*

TABLE 3. Sialic acid permease and aldolase levels in mutant and wild-type strains of *E. coli* grown on different carbon sources

Strain	Relevant genotype	Permease sp act (U/mg of cell protein)					Aldolase sp act (U/mg of soluble protein)				
		Gro	Glc	Gro (NeuNAc)[a]	Glc (NeuNAc)[a]	NeuNAc[b]	Gro	Glc	Gro (NeuNAc)[a]	Glc (NeuNAc)[a]	NeuNAc[b]
EV72	*nanA*$^+$ *nanT*$^+$ *kps*$^+$	4.1	4.7	54.6	12.6	59.4	4.2	3.2	46.1	9.4	2,341
EV70	*nanA4 nanT*$^+$ *kps*$^+$	69.6	14.3	59.1	ND[c]	ND	<0.3	<0.3	<0.3	ND	ND
EV56	*nanA*$^+$ *nanT1 kps*$^+$	1.2	ND	1.6	ND	ND	4.2	ND	7.0	ND	ND
EV77	*nanA*$^+$ *nanT*$^+$ *kps*^{n}	6.0	0.7	95.8	7.0	70.7	0.7	0.6	1,437	202	3,142
EV76	*nanA4 nanT*$^+$ *kps*^{n}	4.3	0.6	5.7	ND	ND	<0.3	<0.3	<0.3	ND	ND

[a] 0.1 mM NeuNAc was used to induce the cultures for 90 min before the cells were harvested. All cultures were harvested by centrifugation after they had reached an A_{600} of 0.2 to 0.4.

[b] NeuNAc concentration was 1 mM.

[c] ND, Not determined.

TABLE 4. Transductional mapping of *nan* genes in *E. coli*

Transduction no.	Donor strain (relevant genotype)	Recipient strain (relevant genotype)	Selected marker	Unselected marker[a]	% Linkage (no. analyzed)
1	EV70 (*nanA4 zgj-791*::Tn*10*)	AT12-55 (*argG75*)	Tet^r	Arg$^+$	30 (81)
				Nan^s Arg$^+$	0 (23)
2	EV70	AT12-55	Arg$^+$	Tet^r	31 (173)
3	EV50 (*nanA4*)	AT12-55	Arg$^+$	Nan^s	6 (82)
4	EV70	NCM195 (*glnF6*)	Tet^r	Aut$^+$	54 (109)
				Nan$^-$ Aut$^+$	72 (32)
				Nan$^-$ Aut$^-$	61 (23)
5	EV70	NCM196 (*glnF7*)	Tet^r	Aut$^+$	40 (96)
				Nan$^-$ Aut$^+$	74 (19)
				Nan$^-$ Aut$^-$	44 (25)
6	NCM196	AT12-55	Arg$^+$	Aut$^-$	0 (150)
7	NCM196	AT12-55	Arg$^+$	Aut$^-$	0 (84)
8	EV72 (*zgj-791*::Tn*10*)	EV55 (*nanT1*)	Tet^r	Nan$^+$	52 (92)
9	EV70	EV55	Tet^r	Nan$^-$ Nan^s	53 (85)
				Nan$^+$ Nan^r	10 (85)
				Nan$^-$ Nan^r	37 (85)

[a] Arg, Requires arginine for growth; Aut, ability to use arginine as nitrogen source; Nan, ability to use sialic acid as carbon source; Nan^s, unable to grow on Gro containing 0.05% sialic acid, i.e., sialic acid sensitivity; Nan^r, able to grow on Gro-sialic acid medium, i.e., sialic acid resistant; Tet^r, tetracycline resistance.

kps^n). These strains produced no sialic acid biosynthetically, and consequently the aldolase mutation had no effect on permease activity in strain EV76, i.e., there was no induction. Permease and aldolase did respond to exogenous sialic acid in *kps*^{n} strains, demonstrating that functional *kps* genes are not obligatory for regulation of catabolic enzymes. In contrast to the induction of permease, the degree of aldolase induction appeared to be strain dependent. Thus, aldolase in NeuNAc-induced strain EV72 was ca. 20-fold lower than in similarly induced cultures of EV77.

The results (Table 3) demonstrate that permease activity was induced 100-fold by sialic acid regardless of whether sialic acid was accumulated by transport or by biosynthesis. In contrast, the extent of aldolase induction varied over nearly a 1,000-fold range (Table 3), but these data do not permit any conclusions about the induction of aldolase by endogenous sialic acid. Results in the accompanying paper (28) demonstrate that aldolase was induced by biosynthetically derived sialic acid. Attempts to induce permease or aldolase by growing cells on pyruvic acid, ManNAc, or pyruvic acid plus ManNAc were negative. Therefore, the primary degradation products of sialic acid were not inducers, and these results suggested that sialic acid itself induced its own catabolic system. Aldolase activity in the transport-defective strain EV56 (*nanT1 kps*$^+$) was not induced by sialic acid (Table 3), demonstrating that induction required an intracellular accumulation of sialic acid. Finally, induction of both permease and aldolase was sensitive to Glc (Table 3).

Genetic mapping of *nanA* and *nanT*. We initially located *zgj-791*::Tn*10*, which is 50% linked to *nanA* by transduction, near *rpsL* (min 72) by a series of two-factor Hfr crosses. These crosses further suggested that the Tn*10* insertion mapped counterclockwise relative to *rpsL*. P1-mediated cotransduction indicated that *zgj-791*::Tn*10* was linked to *argG* (transductions 1 and 2, Table 4). Since the insertion was linked to *nanA*, and because we observed low linkage of *nanA* and *argG* (transduction 3, Table 4), we conclude that the marker orientation was *argG zgj-791*::Tn*10 nanA*. This orientation was consistent with lack of cotransduction between *nanA4* or *zgj-791*::Tn*10* and *uxaA* (data not shown). Confirmation of this assignment is given in Table 4 (transductions 4 and 5). Thus, *zgj-791*::Tn*10* was linked to *glnF* (*ntrA*) and *nanA* with similar cotransduction frequencies. Scoring *glnF*$^+$ transductants that had coinherited *nanA4* suggested that *nanA* was located within <0.5 map units (ca. 75% cotransduction) of *glnF*. Since the linkage between *nanA4* and *glnF* as unselected markers was approximately equal to the linkage of *nanA4* to *zgj-791*::Tn*10*, i.e., 50% cotransduction frequency, we conclude that the relative positions of *nanA* and *glnF* are as shown in Fig. 5. This assignment is consistent with our inability to detect linkage of *glnF* to *argG* (transductions 6 and 7, Table 4). Evidence that *nanT* was linked to *nanA* is based on cotransduction with *zgj-791*::Tn*10* (transduction 8, Table 4). The ability to independently score *nanT1* (Nan$^-$ Nan^r) and *nanA4* (Nan$^-$ Nan^s) permitted an estimation of linkage between these two markers by a three-factor cross (transduction 9, Table 4). This transduction indicated that *nanA* and *nanT* were tightly linked because only 10% of the recombinants inherited both the *nanA*$^+$ and *nanT*$^+$ alleles and were consequently Nan$^+$ Nan^r. We favor the orientation of *nanT* relative to *nanA* shown in Fig. 5 because the opposite orientation would have required a double recombinational event to generate the *nanA*$^+$ *nanT*$^+$ class of transductants. The data (Table 4) are consistent with the relative placements of *nanA*, *nanT*, and *glnF* shown in Fig. 5, but do not rigorously exclude the possibility that *nanA* and *nanT* map to the opposite side of *glnF*.

DISCUSSION

Identification of an inducible catabolic system for sialic acids in *E. coli* was a direct result of our interest in the biosynthesis of sialic acid-containing capsular polysaccharides. As a consequence, all of the original *nan* mutations were isolated in hybrid strains of *E. coli* K-12 that contained functional *kps* genes. Results in this study have clearly established that the catabolism genes are genetically distinct from the biosynthesis genes. This conclusion is based on transductional analysis involving "pure" K-12 strains that lack functional *kps* genes (Table 4) and may even lack *kps* DNA sequences (10). In addition, the Tn*10* insertion, *zgj-791*::Tn*10*, and its linked *nanA*$^+$ allele were isolated from a pure K-12 strain. Thus, all the K-12 strains tested, and perhaps all other *E. coli* strains, were able to grow on sialic acid as a carbon source regardless of whether they contained the functional genes required to synthesize sialic acid and sialyl polymer. We also tested *Salmonella typhimurium*

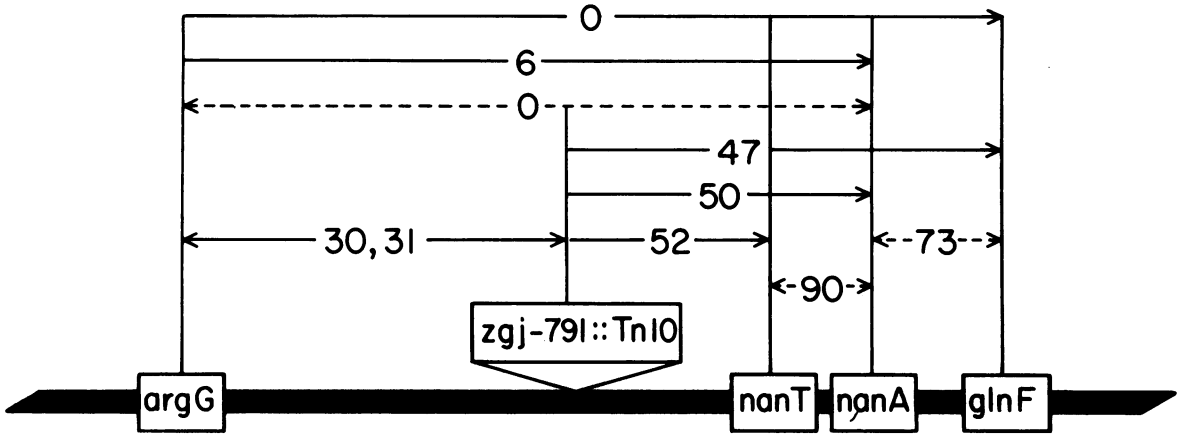

FIG. 5. Map location of *nan* loci relative to other markers. Linkage data from Table 4 were used to construct the genetic organization of *nan* loci relative to *argG* and *glnF*. Solid-line arrows point to the unselected marker, with numbers indicating percent contransduction. A line with arrowheads at each end indicates that transduction was done in both directions. Dashed-line arrows indicate that both markers were unselected. The data from Table 4 for the crosses involving *glnF* mutations have been averaged. The linkage shown between *nanT* and *nanA* is an approximation based on the fact that recombination between these markers was detected in 10% of the transductants (transduction 9, Table 4). A transduction to measure the actual linkage between *nanA* and *nanT* has not been done. The figure is not drawn to scale.

LT-2 and found that it could utilize sialic acid for growth (data not shown).

The relative map positions of markers near *argG* (Fig. 5) were based on cotransduction data (Table 4). We were unable to detect linkage between *argG* and *glnF*, which indicates that *nanA* and *nanT* map to the *argG* side of *glnF*. This result is in apparent conflict with the results of Pahel et al. (19), who reported 22 to 35% cotransduction between *argG* and *glnF*. More recently, Castaño and Bastarrachea reported 45% cotransduction between these two markers (3). These workers were unable, however, to detect linkage between *gltB* and *glnF* (3). Our results (Table 4) are not consistent with such high linkage of *argG* to *glnF*. Since *gltB* is more closely linked to *glnF* than *argG* is (19), crosses with this marker may help confirm the map positions shown in Fig. 5.

The ability to grow on sialic acid appears to be widespread among gram-positive bacteria (17, and references therein). These bacteria also possess an inducible catabolic system. For example, Nees and Schauer (17) presented evidence that free sialic acid induced neuraminidase, aldolase, and permease activities in *C. perfringens*. As in *E. coli*, the aldolase produced by *C. perfringens* was cytosolic and the neuraminidase was extracellular. *E. coli* does not produce detectable neuraminidase activity. Furthermore, neuraminidase and aldolase in *C. perfringens* appear to be coordinately regulated (17), suggesting an operon organization. Neuraminidase excretion presumably results in the release of free sialic acid from glycoconjugates in the gut; the free sialic acid is then transported and degraded by aldolase to supply *C. perfringens* and related organisms with an additional carbon and energy source. The K_m of sialic acid uptake by *C. perfringens* was estimated at 0.3 mM (17), and we estimated the K_m of sialic acid uptake in *E. coli* at between 0.2 and 0.6 mM (unpublished results). This range is similar to the K_m of lactose entry into *E. coli* (18). *E. coli* lacks neuraminidase but presumably competes for free extracellular sialic acids produced by neuraminidase-excreting organisms. Nees and Schauer (17) showed that induction of permease activity in *C. perfringens* lagged 20 to 40 min after sialic acid was added to the culture medium. In contrast, induction of *E. coli* permease activity was detected less than 5 min after the addition of 0.1 mM sialic acid to cells growing on Gro as the carbon source (unpublished results). Thus, *E. coli* may effectively compete with other bacteria having sialic acid-inducible permeases with similar K_ms by the rapidity with which it induces its own permease. Whether the *E. coli* and *C. perfringens* inducible systems have common regulatory features remains an intriguing possibility for future investigation.

We presented evidence that sialic acid was transported without modification, apparently ruling out involvement of the phosphoenolpyruvate-phosphotransferase system. However, sialic acid might be transported by this system as the 9-phosphate derivative, which is then dephosphorylated with unusual rapidity. Definitive proof that sialic acid transport does not involve phosphorylation will require experiments with appropriate pleiotropic mutants defective in group translocation.

Elucidation of the exact mechanism of sialic acid induction will require the isolation of mutants with regulatory defects. Indeed, we have not proved that the *nanT* or *nanA* mutation is in the structural gene coding for permease and aldolase, respectively. However, it seems unlikely that both types of mutants could be the result of mutations in regulatory genes. The fairly complete map information for *nanA* and *nanT* suggests obvious approaches for further study on regulation of the *nan* system with recombinant DNA techniques.

The unexpected finding that sialic acid is toxic in aldolase-negative strains suggests that another function of this enzyme is to prevent the accumulation of intracellular sialic acid. The dual role of aldolase in dissimilating and detoxifying sialic acids may explain the apparent degree of genetic controls for aldolase production. Most accumulated sugars are toxic as their phosphorylated derivatives (7). Apparently, sialic acid toxicity does not require such modification, since most of the accumulated free sugar was unmodified.

Finally, the observation that aldolase-negative strains are sensitive to sialic acid suggests a simple bioassy for this sugar. Plotting the sizes of the zones of inhibition in Fig. 4A versus the amount of sialic acid applied yielded a straight line. Standard chemical methods for measuring sialic acid permit the detection of ca. 2 μg (29). Reducing the volume of

agar and of detector strain in the bioassay may allow sialic acid to be detected at nearly this level. Such an assay might also be less sensitive to interference by contaminating substances than are the chemical assays and may provide an alternative way to quantitate free sialic acids in certain clinical and experimental situations.

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