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Redirection of sialic acid metabolism in genetically engineered *Escherichia coli*

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Most microorganisms do not produce sialic acid (sialate), and those that do appear to use a biosynthetic mechanism distinct from mammals. Genetic hybrids of nonpathogenic, sialate-negative laboratory *Escherichia coli* K-12 strains designed for the *de novo* synthesis of the polysialic acid capsule from *E. coli* K1 proved useful in elucidating the genetics and biochemistry of capsule biosynthesis. In this article we propose a dynamic model of sialometabolism to investigate the effects of biosynthetic *neu* (*N*-acetylneuraminic acid) and catabolic *nan* (*N*-acylneuraminate) mutations on the flux of intermediates through the sialate synthetic pathway. Intracellular sialate concentrations were determined by high pH anion exchange chromatography with pulsed amperometric detection. The results indicated that a strain carrying a null defect in the gene encoding polysialyltransferase (*neuS*) accumulated > 50 times more CMP–sialic acid than the wild type when strains were grown in a minimal medium supplemented with glucose and casamino acids. Metabolic accumulation of CMP–sialic acid depended on a functional sialic acid synthase (*neuB*), as shown by the inability of a strain lacking this enzyme to accumulate a detectable endogenous sialate pool. The *neuB* mutant concentrated trace sialate from the medium, indicating its potential value for quantitative analysis of free sialic acids in complex biological samples. The function of the sialate aldolase (encoded by *nanA*) in limiting intermediate flux through the synthetic pathway was determined by analyzing free sialate accumulation in *neuA* (CMP–sialic acid synthetase) *nanA* double mutants. The combined results demonstrate how *E. coli* avoids a futile cycle in which biosynthetic sialate induces the system for its own degradation and indicate the feasibility of generating sialooligosaccharide precursors through targeted manipulation of sialate metabolism.

Key words: sialic acid/metabolic engineering/*Escherichia coli* K1/pathway analysis/carbohydrate flux

Introduction

The oligosaccharide chains on glycoconjugates play important roles in cell adhesion and signaling events, and, though an exact understanding of how carbohydrates modulate these processes is frequently unavailable, the subversion of animal glycoconjugates by microorganisms for the purposes of adhesion, invasion, and propagation is a central feature of most host–pathogen interactions (Karlsson, 1998). Thus, ability to block these interactions with carbohydrate-based therapeutics would provide new avenues to the treatment or prevention of infectious diseases. Blocking carbohydrate-based recognition events may also be useful for treating a variety of apparently noninfectious diseases requiring cell–cell or cell–molecule interactions for their progression. Although the chemoenzymatic syntheses of oligosaccharides offers an attractive route to the production of therapeutic blocking reagents (for example, see Gilbert *et al.*, 1998), the commercial scarcity of required glycosyltransferases and expense of precursors impedes progress in the design or large-scale production of certain oligosaccharides.

The difficulty in producing some oligosaccharide precursors is especially evident with members of the family of nonulosonates known as the sialic acids. These unique N- or O-substituted derivatives of *N*-acetylneuraminic acid (Neu5Ac) are ubiquitous in animals of the deuterostome lineage (starfish to humans) but do not seem to be synthesized by most plants, protostomia, protists, *Archaea*, or eubacteria (Warren, 1994). Despite the limited distribution of sialates in nature, a few (mostly pathogenic) bacterial strains synthesize Neu5Ac *de novo* and incorporate it into cell surface glycolipids (Vimr *et al.*, 1995). Of medical importance is *Escherichia coli* K1, the leading cause of Gram-negative neonatal meningitis in humans, in addition to a frequent etiologic agent of childhood and adult urinary tract infections and septicemia (Silver and Vimr, 1990). The polysialic acid (PSA) capsule of *E. coli* K1 inhibits host innate defense mechanisms and provides molecular mimicry of the PSA chains on the neural cell adhesion molecule (NCAM). The discovery of PSA as both virulence factor and regulator of central nervous system development prompted extensive investigations into sialate biosynthesis.

In the mammalian liver, Neu5Ac is synthesized and activated for transfer to glycoconjugate acceptors (R-OH) in five sequential steps involving phosphorylated intermediates (Warren, 1994), beginning with UDP-*N*-acetylglucosamine (UDP-GlcNAc):

$$\underset{}{\text{UDP-GlcNAc}} \xrightarrow[1]{} \text{ManNAc + ATP} \xrightarrow[2]{} \text{ManNAc-6-P + PEP} \xrightarrow[3]{} \text{Neu5Ac-9-P} \xrightarrow[4]{} \text{Neu5Ac}$$

$$\text{Neu5Ac + CTP} \xrightarrow[5]{} \text{CMP-Neu5Ac + HO-R} \xrightarrow[6]{} \text{Neu5Ac-O-R.}$$

The specificity of sialyltransferases for a given acceptor (step 6) dictates the pattern of sialoglycoconjugates (Neu5Ac-O-R) synthesized by various cells. The bifunctional enzyme, UDP-GlcNAc 2-epimerase/*N*-acetylmannosamine (ManNAc) kinase

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(Stasche *et al.*, 1997), catalyzes the first two steps of the pathway, yielding ManNAc-6-phosphate (step 2). Production of CMP-Neu5Ac in step 5 is tightly regulated through allostery, with increasing CMP-Neu5Ac concentrations inhibiting the epimerase that produces ManNAc in step 1 (Kornfeld *et al.*, 1964). In step 3, ManNAc-6-P is condensed with phosphoenolpyruvate (PEP) to yield Neu5Ac-9-P, with subsequent dephosphorylation by specific or nonspecific phosphatase(s) in step 4 yielding free Neu5Ac. Alternative mechanisms for synthesizing Neu5Ac may involve the direct epimerization of free GlcNAc to ManNAc (Maru *et al.*, 1996), Neu5Ac recycling from surface glycoconjugates through a potential salvage pathway involving turnover by lysosomal sialidase and the direct enzymatic condensation of ManNAc with pyruvate by Neu5Ac aldolase (Rodriguez-Aparico *et al.*, 1995).

Irrespective of the exact mechanism(s) used by different eukarya to synthesize sialic acids, synthesis of the activated sialyl donor, CMP-Neu5Ac, by *E. coli* K1 does not appear to be tightly regulated by allosteric inhibition (Steenbergen and Vimr, 1990; Vimr and Troy, 1985b). Because *E. coli* lacks CMP-Neu5Ac hydrolase (Masson and Holbein, 1983), accumulation of CMP-Neu5Ac in a polymerase-defective genetic background is a physiological dead-end. From a commercial viewpoint, exploitation of this metabolic blockade for targeted overproduction of free Neu5Ac or CMP-Neu5Ac offers alternatives to the chemoenzymatic syntheses of these compounds. A solely fermentative source of CMP-Neu5Ac would obviate the need for expensive starting materials, such as Neu5Ac, PEP, nucleoside phosphates, or recycling enzymes currently required for efficient *in vitro* syntheses of sialooligosaccharides. Our current results contribute to the understanding of regulation of sialic acid metabolism by the microbial metabolic engineering of intermediate flux through the synthetic pathway.

Results

Bacterial sialometabolism

Recent advances in understanding sialate and PSA synthesis and degradation (Vimr *et al.*, 1995; Plumbridge and Vimr, 1999), and the results presented in this communication support our metabolic network model in Figure 1. Although the potential reversibility of most of the individual reactions has not been formally investigated, genetic and biochemical analyses of Neu5Ac flux in mutant and wild-type strains indicate that the enzymes of the catabolic pathway (*nan* gene products) do not function biosynthetically (*neu* gene products) under balanced growth conditions (Vimr and Troy, 1985b). This conclusion is challenged by the suggestion that sialate aldolase (encoded by *nanA*) is the sole biosynthetic source of free Neu5Ac in *E. coli* and other Gram-negative bacteria (Rodriguez-Aparico *et al.*, 1995; Ferrero *et al.*, 1996; Barrallo *et al.*, 1999).

Table I lists the bacterial sialate synthetic and catabolic gene products depicted in Figure 1 compared with known or suspected mammalian homologs. This comparison highlights two likely mechanistic differences between the bacterial and mammalian modes of Neu5Ac synthesis: (1) organization of the bacterial genes for UDP-GlcNAc epimerase and putative ManNAc kinase into distinct synthetic and catabolic operons, respectively, and (2) use of free ManNAc rather than its 6-phosphate derivative by the bacterial synthase to produce free Neu5Ac. These differences have important implications for designed redirection of sialometabolism in bacteria.

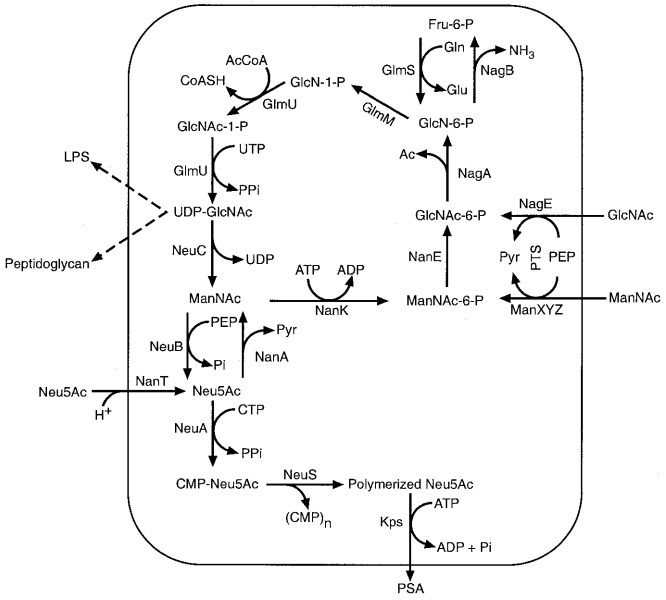

Fig. 1. Proposed model of sialate synthesis and degradation in *Escherichia coli*. Intracellular reactions are shown within the rectangle representing a hybrid *E. coli* K1 cell. Broken arrows indicate that synthesis of LPS and peptidoglycan ultimately depends on the availability of UDP-GlcNAc. See text for description of enzymes or reactants.

Effect of nan or neu genotype on sialate overproduction

To better understand how mutations in *nan* or *neu* influence sialate synthesis, intracellular Neu5Ac was determined by high pH anion exchange chromatography equipped with pulsed amperometric detection (HPAEC/PAD) after hydrolysis to convert CMP-Neu5Ac that accumulated intracellularly to the free sugar. Figure 2 shows that the wild-type strain, EV36, did not accumulate a detectable sialate pool, estimating the limits of sialate detection at approximately 0.1 nmol (Table II). Because EV36 synthesizes PSA and expresses K1 capsule (Figure 1), we expected that the intermediary metabolites Neu5Ac and CMP-Neu5Ac would be rapidly converted to polysialate end product and hence not detected by our assay (Figure 2). In contrast, sialate accumulation was dramatically increased in strains carrying a null mutation in *neuS* (strain EV136 in Figure 2).

Compared to the virtually undetectable Neu5Ac concentration in the wild type (Figure 2), polysialyltransferase-deficient mutant EV136 produced about 30 times more sialate than EV36 when the strains were grown in Luria Bertani (LB) medium (Figure 2 and Table II), results consistent with our previous observations (Steenbergen and Vimr, 1990; Vimr and Troy, 1985b). The two peaks eluting prior to Neu5Ac were independent of medium composition or a given strain's ability to synthesize Neu5Ac or PSA (compare Figure 2 with Figure 3); these peaks were not investigated further. When a mutation in *nanA* was simultaneously present in the *neuS* background, as in strain EV240, there was little increase in sialate concentration (Table II). Four independent repetitions of this comparison

Table I. Known or proposed functions of *neu* and *nan* gene products in *Escherichia coli* and comparison with their mammalian homologs or functional equivalents

Protein	Function in *E. coli*	Homolog[a] (E value)[b]	Accession no.	Reference
NanA	Sialate aldolase	F[c]	NA[f]	Vimr and Troy, 1985a
NanT	Sialate permease	F	NA	Martinez *et al.*, 1995
NanE	ManNAc-6-P epimerase	None	NA	Plumbridge and Vimr, 1999
NanK	ManNAc kinase	H[d] (0.08)	NP005467	Stasche *et al.*, 1997
NanR	Sialate repressor	None	NA	Plumbridge and Vimr, 1999
NeuA	CMP-sialate synthase	H ($4e^{-14}$)	CAA06915	Munster *et al.*, 1998
NeuB	Sialate synthase	H ($8e^{-50}$)	AAF75261	Lawrence *et al.*, 2000
NeuC	UDP-GlcNAc epimerase	H ($2e^{-20}$)	NP005467	Stasche *et al.*, 1997
NeuS	Polysialyltransferase	FS[e]	NA	Steenbergen and Vimr, 1990

[a]Only mammalian homologs are indicated.
[b]E values indicate the likelihood that a match is not due to chance.
[c]F, functional counterpart of the indicated *E. coli* protein; no sequence available and thus gene product may or may not be homologous.
[d]H, homologous with its *E. coli* counterpart.
[e]FS, sequenced gene encoding a protein with the same function as its *E. coli* counterpart, but not homologous.
[f]NA, not applicable

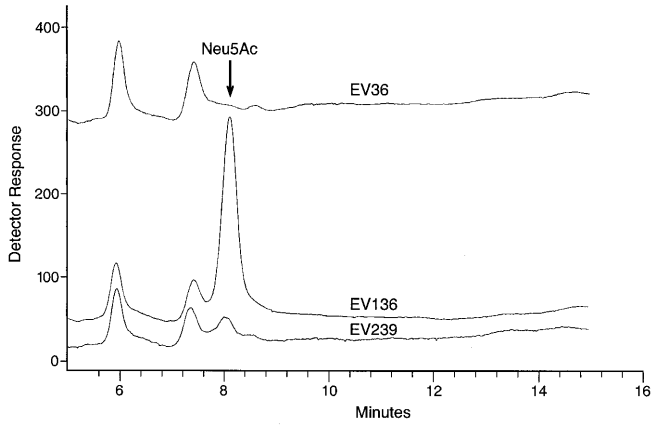

Fig. 2. Accumulation of intracellular sialate in wild-type or mutant *E. coli* strains. The indicated strains were grown in LB prior to analysis by HPAEC/PAD.

indicated no difference ($P > 0.05$) in sialate concentration between EV136 and EV240. This result suggests that aldolase (NanA) does not normally compete with CMP-Neu5Ac synthetase (NeuA) for the common substrate, Neu5Ac. That most of the sialate detected in extracts of the strains examined in Table II is in the form of CMP-Neu5Ac is confirmed below.

Effect of medium composition on sialate overproduction

LB is an unbuffered medium composed of peptides, amino acids, vitamins or other cofactors, and trace amounts of carbohydrates, including sialic acid (Steenbergen *et al.*, 1992; Vimr, 1992). We wished to test whether sialate production could be optimized by simple alterations to a defined minimal growth medium. As shown in Table II, basal medium supported the overproduction of Neu5Ac by EV136 to an amount comparable with that of this strain on LB. Sialate was not dramatically increased by supplementing the basal medium with CMP

Table II. Effects of growth medium and genotype on sialate pool size in genetically engineered *E. coli*

Experiment	Strain	Relevant genotype	Medium	Neu5Ac (nmols/ A_{600}/ml)
1	EV36	Wild type	LB	ND[b]
	EV136	*neuS*	LB	4.5
	EV239	*nanA neuB neuS*	LB	0.5
	EV240	*nanA neuS*	LB	4.4
	EV136	*neuS*	B[a]	7.8
	EV136	*neuS*	B+Glc	17.8
	EV136	*neuS*	B+CMP	8.1
	EV136	*neuS*	B+Glc+CMP	13.0
	EV239	*nanA neuB neuS*	B+Glc	ND
2	EV136	*neuS*	B+GlcNAc	8.8
	EV136	*neuS*	B+Fru	9.7[c]
	EV136	*neuS*	B+Gro	8.8
	EV240	*nanA neuS*	B+Fru	22.4[c]
	EV240	*nan*A *neu*S	B+Gro	10.9

[a]B, basal medium, composed of M63 salts with 1% (w/v) casamino acids (CAA) and, where indicated, 0.2% (w/v) sugar or nucleotide supplements.
[b]ND, not detectable above the minimal detection limit of 0.1 nmol.
[c]The difference between these strains was not significant (see text).

(Table II). In contrast, supplementing basal medium with 0.2% glucose (Glc) resulted in a nearly fourfold increase in Neu5Ac relative to EV136 grown in LB (Table II). The apparent stimulatory effect of Glc could have resulted from the presence of this preferred carbon source in the medium, thus augmenting UDP-GlcNAc pool size for synthesis of essential lipopolysaccharide (LPS) and peptidoglycan cell wall components

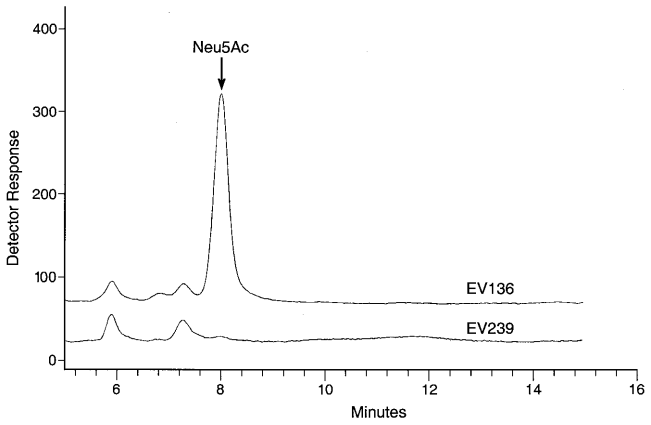

Fig. 3. Accumulation of intracellular sialate requires NeuB. The indicated strains were grown in basal salts medium supplemented with Glc and CAA. Note the absence of a sialate peak from the *neuB* strain, EV239, compared to the peak in the EV136 extract.

(Figure 1). Alternatively, or in addition to the effect of carbon source on cell wall–precursor synthesis, Glc induces catabolite repression of the *nan* operon (Vimr and Troy, 1985a). This response would be expected to reduce expression of *nanA* and *nanK* potentially shunting metabolite flux toward the synthetic pathway (Figure 1). In an attempt to distinguish between these possibilities, EV136 was grown in medium B supplemented with fructose (Fru), GlcNAc, or glycerol (Gro).

As indicated in Figure 1, sugars such as GlcNAc, ManNAc, or Fru (not shown) are transported as phosphorylated derivatives by the phosphotransferase uptake system (PTS). Gro (not shown) enters cells by a facilitated diffusion process and therefore would not influence PTS-regulated operons. The modest increases in sialate pool size of EV136 or EV240, when grown in basal medium containing GlcNAc or Fru, but not Gro (Table II), suggest that catabolite repression is not critical for sialate overproduction. Furthermore, there was no difference ($P > 0.05$) between sialate pools from cells grown with Fru or Glc (data not shown), suggesting that the type of PTS sugar was unimportant to sialate overproduction. We infer from these results that the effect of medium composition on *E. coli* sialate pool size is largely a function of final cell density, which for basal medium supplemented with Glc or Fru is two to three times greater than that of LB. Therefore, when the carbon supply is plentiful, there appears to be adequate UDP-GlcNAc synthesized to support the essential production of peptidoglycan and LPS while simultaneously allowing overproduction of sialate (Table II). We have not systematically investigated whether sialate pool size is affected by leakage or efflux mechanisms; however, previous results suggest such losses are probably minor (Vimr and Troy, 1985b). We conclude that fermentation in a defined medium produces approximately 20 nmols of CMP-Neu5Ac per A_{600} per ml. Because growth yields of 30 absorbance units are routinely attainable (Hoffman *et al.*, 1995), gram quantities of CMP-Neu5Ac should be readily available through simple fermentation of appropriate bacterial strains in defined or complex media.

Mechanism of sialate overproduction

To determine if the overproduction of Neu5Ac in *neuS* mutants is dependent on a functionally intact biosynthetic system (Figure 1), we analyzed the sialate pool in the triple mutant EV239. This mutant has defects in NanA, NeuS, and sialate synthase (NeuB), suggesting either that the Neu5Ac peak detected in this strain (Figure 2) arose from an alternate synthetic pathway or, less likely, a compound unrelated to Neu5Ac eluted at the same time as authentic Neu5Ac. The first possibility was considered more likely for two reasons. First, EV36 extracts contained no detectable sialate (Figure 2), suggesting that the peak in EV239 was actually Neu5Ac. Second, it was possible we were observing a phenotype of the synthase mutant that was being magnified by the simultaneous polysialyltransferase and aldolase deficiencies in EV239. Others (Rodriguez-Aparico *et al.*, 1995) concluded that *E. coli* K1 relies on NanA to synthesize Neu5Ac by catalyzing the aldol condensation of pyruvate with ManNAc, suggesting that the NeuB-independent peak detected in extracts of EV239 may have arisen from residual *nanA* expression. However, when EV239 was grown in defined medium, Neu5Ac was no longer detected (Figure 3 and Table II). Because LB contains trace sialate (Steenbergen *et al.*, 1992), we concluded that the peak detected in EV239 grown on LB (Figure 2) resulted from intracellular concentration of exogenous Neu5Ac by the sialic acid–specific permease, NanT (Figure 1).

NanA limits the accumulation of free Neu5Ac

Assuming our proposed model of sialate biosynthesis in Figure 1 is correct, it should be possible to overproduce free Neu5Ac by truncating the biosynthetic pathway at *neuA*. Although the absence of NanA appeared to have little effect on increasing the sialate pools in *neuS* strains (Table II), a defective *neuA* gene should allow increased sialate accumulation until full induction of *nanA* is reached. The metabolic consequence of this phenotype (aldolase induction) is predicted to limit the endogenous sialate pool size. As shown in Figure 4, NanR negatively regulates the *nan* operon by acting as a repressor that is inactivated on binding free Neu5Ac (Plumbridge and Vimr, 1999). Therefore, on full induction of the *nan* operon, NanA would convert the available sialate pool to ManNAc and, ultimately, back to Fru-6-P (Figure 1). However, in a *nanA neuA* double mutant, active aldolase would not exist and the sialate pool should be stabilized.

To determine the role of *nanA* in regulating sialate flux, we created isogenic *nanA neuA* and *nanA*$^+$ *neuA* strains by transducing the *nanA4* allele from EV52 into the *neuA* recipient, EV5. As shown in Figure 5, *nanA* transductants (numbers 1 and 6) accumulated significantly more sialate ($P < 0.0001$) than EV5 or its *nanA*$^+$ *neuA* transductant (number 12). The increased sialate pool sizes of the doubly mutated transductants over those of EV5 or transductant 12 confirm that NanA regulates the biosynthetic pathway when free Neu5Ac concentrations are permitted to rise. The K_D (unknown) for Neu5Ac of NanR presumably defines the regulatory signal, above which point full induction of the *nan* operon is achieved. These results also confirm that the sialate pools detected in the different *neuS* mutants described above are in the form of CMP-Neu5Ac, consistent with direct chemical and functional analyses showing that this nucleotide sugar accumulates in

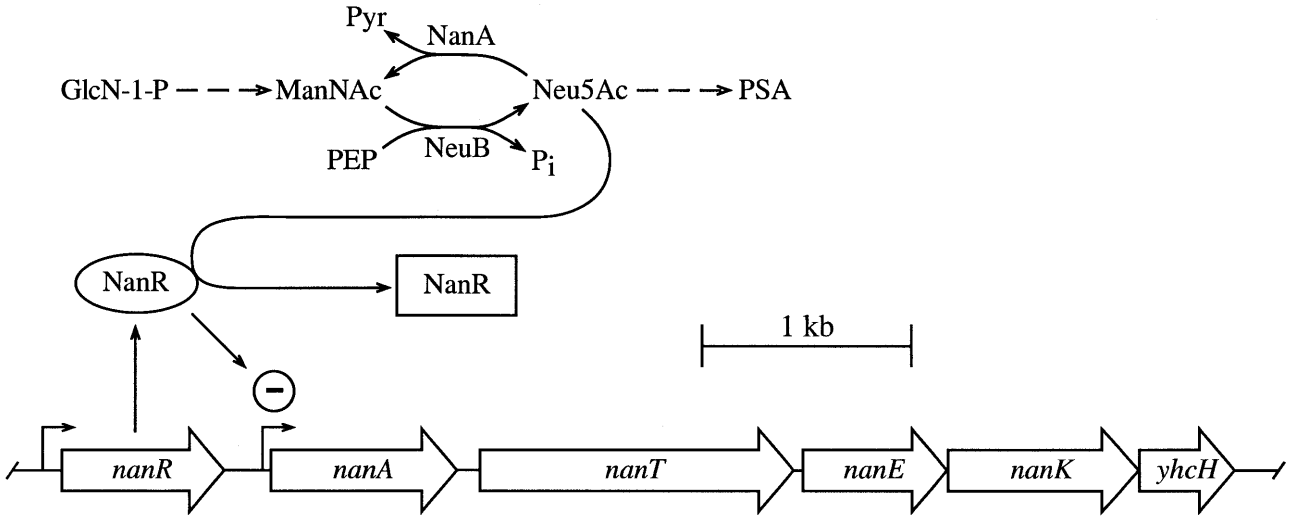

Fig. 4. Regulation of *nanA* expression in *E. coli*. Open arrows indicate the genes comprising the *nan* operon (*nanATEKyhcH*), and the upstream gene encoding this operon's repressor, NanR. Bent arrows indicate putative transcriptional start sites, with the *nanA* promoter negatively regulated by NanR (circle with internal negative sign). Endogenously produced Neu5Ac (curved arrows) is the precursor of PSA and functions as the probable inducer of the *nan* operon by converting NanR to an inactive state (rectangle). Broken arrows indicate that more than one step is required to synthesize ManNAc or PSA, as shown in Figure 1.

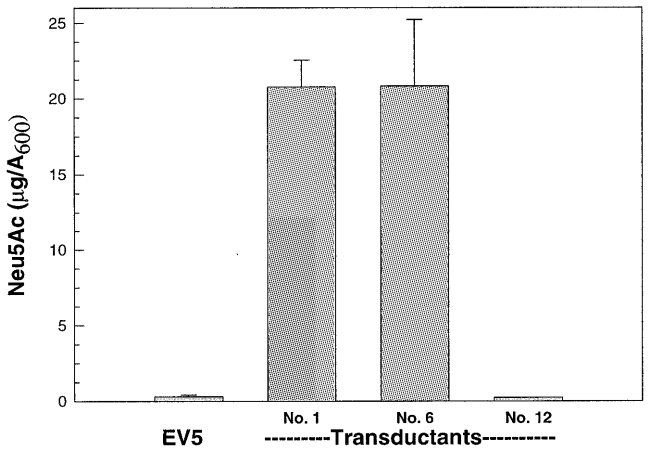

Fig. 5. Effect of sialic acid aldolase on Neu5Ac accumulation in *neuA* mutants. Cells were grown in basal salts medium supplemented with Glc and CAA prior to analyzing extracts for free Neu5Ac. Results are the concentrations of Neu5Ac normalized per unit of A_{600} ± the standard deviation of three independent measurements of each strain.

NeuS-deficient strains (Vimr and Troy, 1985b; Steenbergen and Vimr, 1990). When taken together, our results demonstrate the feasibility of fermentation for targeted biosynthesis of sialooligosaccharide precursors and suggest additional approaches for metabolic engineering of sialate pathways.

Discussion

Metabolic engineering of sialate metabolism

The ability to overproduce CMP-Neu5Ac is necessary for metabolic engineering of sialooligosaccharide biosynthesis in *E. coli*. Although we had previously shown that *E. coli neuS* strains overproduce CMP-Neu5Ac (Vimr and Troy, 1985b; Steenbergen and Vimr, 1990), these studies used complex (undefined) growth medium and the relatively insensitive and nonspecific thiobarbituric acid assay to quantify intracellular sialate pools. Using HPAEC/PAD under conditions established for anionic carbohydrate analysis, the current results both confirm and extend our previous studies, demonstrating that approximately 20 nmols of CMP-Neu5Ac are obtained per unit of A_{600} per ml by fermentation of the appropriate strain in defined medium. Further increases in yields of targeted compounds may be achieved by overproducing CTP synthetase (encoded by *pyrG*), if the pool size of this nucleotide is limiting for CMP-Neu5Ac synthesis. Alternatively, eliminating NanK (as demonstrated for NanA in this study) is also likely to increase flux of sialate intermediates through the synthetic pathway. Therefore, creating mutants simultaneously defective in *nanA* and *nanK* is predicted to complete the separation of sialic acid degradation from biosynthesis, potentially maximizing the yield of CMP-Neu5Ac obtainable from a *nanAK neuS* triple mutant.

Current methods for the chemoenzymatic synthesis of sialooligosaccharides require specific precursors and enzymes to generate CMP-Neu5Ac (Liu *et al.*, 1992; Gilbert *et al.*, 1998). Fermentation has obvious advantages of simplicity and nominal cost in comparison to traditional organic synthetic methods and potentially obviates the need for CMP-Neu5Ac regeneration *in vitro*. More important, by expressing the appropriate sialyltransferase in tandem with CMP-Neu5Ac overproduction, it should be possible to engineer *E. coli* that synthesize virtually any sialylated oligosaccharide by coexpression of the relevant acceptor. The large number of completely sequenced microbial genomes and availability of *E. coli* with modified sialometabolic pathways should greatly facilitate engineering sialooligosaccharide synthesis in bacteria.

Mechanism of sialic acid biosynthesis

Our current results demonstrate the obligatory requirement of NeuB for sialate biosynthesis in *E. coli*. Others failed to detect this activity *in vitro*, suggesting that NanA instead of NeuB

was the sole biosynthetic source of sialate in *E. coli* (Rodriguez-Aparico *et al.*, 1995; Ferrero *et al.*, 1996). Although earlier studies contradicted this suggestion (Vimr and Troy, 1985b; Steenbergen *et al.*, 1992; Vimr, 1992), our current results (Figure 3) allow unambiguous rejection of the hypothesis that NanA normally functions biosynthetically in *E. coli*. This conclusion, together with direct biochemical support (Vann *et al.*, 1997), demonstrates the versatility and power of experimentally coupling the relevant physiology of amino sugar metabolism to systematic genetic and biochemical analyses of sialic acid synthesis.

In mammals, the bifunctional UDP-GlcNAc 2-epimerase/ManNAc kinase is likely the pivotal biosynthetic enzyme for synthesis of ManNAc-6-P *in vivo*. It is noteworthy that in *E. coli*, the putative ManNAc kinase (NanK) is genetically and functionally separate from the UDP-GlcNAc epimerase (NeuC). NanK would thus appear to function exclusively in sialic acid degradation as a product of the *nan* operon, whereas its mammalian counterpart appears to be a purely biosynthetic enzyme. The low yet potentially significant similarity between the bacterial and mammalian ManNAc kinases (Table I) may support an ancient origin of sialate metabolic enzymes. However, because sialate biosynthesis is a sporadic trait in microbes, the possibility that biosynthesis of this sugar evolved late remains tenable. Entertaining the speculation that microbes may have acquired some of their biosynthetic and catabolic machinery from eukaryotes (Hoyer *et al.*, 1992), it will be interesting to compare whether select unicellular eukaryotes such as *Candida albicans*, which reportedly synthesizes sialic acid (Soares *et al.*, 2000), utilizes the prokaryotic or mammalian synthetic mechanism.

In *Neisseria meningitidis* group B, the ortholog of *neuC* (*siaA*) is reported to be a GlcNAc-6-P to ManNAc-6-P epimerase (Petersen *et al.*, 2000). Though biosynthesis of Neu5Ac in meningococci (as well as in *E. coli*) appears to require unphosphorylated ManNAc as the hexosamine sialate precursor (Figure 1), it has been postulated that *N. meningitidis* uses specific or nonspecific phosphatase(s) to produce ManNAc (Petersen *et al.*, 2000). Despite the homology between *neuC* and *siaA*, *neuC* and its mammalian homolog use UDP-GlcNAc as ManNAc precursor, whereas SiaA may recognize GlcNAc-6-P. However, GlmS produces GlcN-6-P instead of GlcNAc-6-P, with GlcN-6-P subsequently isomerized by GlmM and converted to UDP-GlcNAc via the bifunctional GlmU (Figure 1). This microbial pathway for the production of UDP-GlcNAc implies that there is little intracellular GlcNAc-6-P in the absence of an exogenous supply of free GlcNAc. If the proposed function of SiaA is correct, there must be an alternative source of GlcNAc-6-P in *N. meningitidis*. Pending purification of NanK, it should become straightforward to chemoenzymatically synthesize radiolabeled ManNAc-6-P for detection of the predicted specific or nonspecific phosphatase(s) in *N. meningitidis* or other organisms.

Conclusion

We have quantified the overproduction of free Neu5Ac and CMP-Neu5Ac under defined growth conditions and shown how sialate aldolase diverts the flux of intermediates away from the synthetic pathway. Recently, Betenbaugh and his associates cloned and expressed the mammalian equivalent of *neuB* in insect cells (Lawrence *et al.*, 2000). The transformed cells were shown to synthesize Neu5Ac when provided with an exogenous source of ManNAc, but did not produce CMP-Neu5Ac. By capitalizing on the ability of genetically engineered *E. coli* to synthesize PSA, we have shown that simply growing the appropriate nonpathogenic strain in defined medium attains overproduction of sialoglycoconjugate precursor synthesis. It should be possible to further engineer this system for synthesis of more complex sialosides.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are described in Table III. Transductants 1, 6, and 12 were constructed by infecting strain EV5 with bacteriophage P1*vir* grown on strain EV52, with selection for a tetracycline resistance element (*zgj-791*::Tn*10*) linked to the *nanA4* mutation (Table III). Drug-resistant transductants were screened for aldolase phenotype by assessing toxicity to exogenous sialic acid as described (Vimr and Troy, 1985a).

Bacteria were routinely propagated in LB broth purchased from Fisher Chemical Co. Defined media were composed of M63 salts (Miller, 1972) supplemented with 1% CAA from Sigma and sugars or nucleotides at 0.2% final concentration. Cultures were grown aerobically in a rotary water bath at 200 r.p.m.. Bacteria were harvested by centrifugation when cultures reached early stationary phase.

Sample preparation

After harvesting cells (2.5–5.0 ml) by centrifugation, culture supernatants were discarded and the tube sides wiped to remove as much residual medium as possible. Water was added to give a tenfold concentration of the starting culture, and the A_{600} of samples determined spectrophotometrically after appropriate dilution. With our instrument (Beckman DU 640), an A_{600} of 1.0 corresponds to 6.25×10^8 cells/ml. Cultures were frozen at –20°C, thawed, and then sonicated briefly to disrupt bacteria. After cell disruption, TCA was added to 10% final concentration and samples chilled on ice for 1 h. The copious precipitate that formed after incubation was removed by centrifugation; the supernatant was dried under vacuum in a Savant SpeedVac.

Table III. Bacterial strains used in this study

Strain	Relevant genotype	Reference or source
EV5	*neuA22*	Vimr *et al.*, 1989
EV36	Wild type	Vimr and Troy, 1985a
EV52	*nanA4 zgj-791*::Tn*10*	Vimr and Troy, 1985a
EV136	*neuS*::Tn*10*	Steenbergen and Vimr, 1990
EV239	*nanA4 neuS*::Tn*10*	Steenbergen *et al.*, 1992
EV240	*nanA4 neuB25 neuS*::Tn*10*	Steenbergen *et al.*, 1992
Transductant 1	*nanA4 neuA22*	Present study
Transductant 6	*nanA4 neuA22*	Present study
Transductant 12	*neuA22*	Present study

Sialic acid analysis

For quantitation of Neu5Ac, lyophilized samples of hydrolyzed culture products of known volume were reconstituted in 250 µl of deionized water and subjected to ultrafiltration (Ultrafree-MC 100,000 NMWL Filter Unit, Millipore). Neu5Ac analysis was carried out with a Dionex model DX-300 high-performance liquid chromatography system equipped with pulsed amperometric detection. Isocratic runs were partitioned using a Carbopac PA-1 column in 0.1 M NaOH–0.05 M sodium acetate at a flow rate of 1.0 ml/min. Elution times and concentrations were compared with a standard curve generated from commercial Neu5Ac (Sigma). Relative detector responses shown in Figure 2 and 3 are in nA. Unless indicated otherwise, data were normalized to express the concentration of sialate per unit of A_{600} per ml of culture.

Statistical analyses

Differences in sialate accumulation between *E. coli* strains EV136 and EV240 were analyzed by the *t*-test. Differences in sialate accumulation among *E. coli* EV5 and three isogenic transductants (Table III) were analyzed by analysis of variance using contrasts to compare the transductants to EV5. *P*-values < 0.05 were considered significant.

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Abbreviations

CAA, casamino acids; Fru, fructose; Glc, glucose; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; Gro, glycerol; HPAEC/PAD, high pH anion exchange chromatography with pulsed amperometric detection; LB, Luria Bertani medium; LPS, lipopolysaccharide; ManNAc, *N*-acetylmannosamine; NCAM, neural cell adhesion molecule; Neu5Ac, *N*-acetylneuraminic acid; PEP, phosphoenolpyruvate; PSA, polysialic acid; PTS, phosphotransferase system; Pyr, pyruvate; TCA, trichloroacetic acid.

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