

# Sialic Acid (*N*-Acetyl Neuraminic Acid) Utilization by *Bacteroides fragilis* Requires a Novel *N*-Acetyl Mannosamine Epimerase<sup>∇†</sup>

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**We characterized the *nanLET* operon in *Bacteroides fragilis*, whose products are required for the utilization of the sialic acid *N*-acetyl neuraminic acid (NANA) as a carbon and energy source. The first gene of the operon is *nanL*, which codes for an aldolase that cleaves NANA into *N*-acetyl mannosamine (manNAc) and pyruvate. The next gene, *nanE*, codes for a manNAc/*N*-acetylglucosamine (NAG) epimerase, which, intriguingly, possesses more similarity to eukaryotic renin binding proteins than to other bacterial NanE epimerase proteins. Unphosphorylated manNAc is the substrate of NanE, while ATP is a cofactor in the epimerase reaction. The third gene of the operon is *nanT*, which shows similarity to the major transporter facilitator superfamily and is most likely to be a NANA transporter. Deletion of any of these genes eliminates the ability of *B. fragilis* to grow on NANA. Although *B. fragilis* does not normally grow with manNAc as the sole carbon source, we isolated a *B. fragilis* mutant strain that can grow on this substrate, likely due to a mutation in a NAG transporter; both manNAc transport and NAG transport are affected in this strain. Deletion of the *nanE* epimerase gene or the *rokA* hexokinase gene, whose product phosphorylates NAG, in the manNAc-enabled strain abolishes growth on manNAc. Thus, *B. fragilis* possesses a new pathway of NANA utilization, which we show is also found in other *Bacteroides* species.**

Many bacteria have the ability to release sialic acids from complex glycoproteins and oligosaccharides present in the media or on cell surfaces at sites of colonization or infection. To use the released sialic acids as a rich source of carbon and nitrogen for growth, bacteria must have the ability to transport these compounds into the cell and convert the nine carbon sugars into intermediates that enter the central glycolytic pathways. The utilization of *N*-acetyl neuraminic acid (NANA), one of the sialic acids, has been well studied in *Escherichia coli* (36, 37), *Haemophilus* spp. (1, 35), and *Clostridium* spp. (38), to name a few.

In many microorganisms, the genes for NANA utilization are arranged in an operon that may be regulated by a repressor protein, termed NanR. A comprehensive review of the organization and composition of several prokaryotic operons involved in NANA utilization has been published (36). Many of these operons share common components, including a transport gene for NANA (*nanT*), a gene encoding an aldolase (*nanA*) that splits NANA into pyruvate and *N*-acetyl man-

nosamine (manNAc), a gene encoding a kinase activity (*nanK*) that phosphorylates manNAc to form manNAc 6-P and, finally, an epimerase gene (*nanE*) whose product converts manNAc 6-P to *N*-acetylglucosamine 6-P (NAG 6-P). NAG 6-P then enters the common pathway of aminosugar utilization (21). For a schematic of the NANA utilization pathway in *E. coli*, the current paradigm of prokaryotic NANA utilization, see Fig. 7A.

*Bacteroides fragilis* possesses a neuraminidase activity, which can liberate free NANA from complex glycoproteins and oligosaccharides. Godoy et al. (11) have established a role for this activity in the ability of *B. fragilis* to proliferate in two in vivo model systems of infection. In the present study, we establish the NANA catabolic pathway in *B. fragilis*, define a three-gene *nanLET*, NANA utilization operon (Fig. 1), and demonstrate the regulation of this operon by the *B. fragilis* NanR protein. Moreover, we demonstrate a new pathway for manNAc utilization in *B. fragilis*.

## MATERIALS AND METHODS

**Reagents.** All chemicals used in the present study were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Agawam, MA) unless otherwise specified. Oligonucleotide primers were synthesized by IDT (Coralville, IA). NANA was purchased from Nacalai Tesque (Kyoto, Japan). The radiolabeled amino sugar [<sup>3</sup>H]manNAc (specific activity, 20 Ci/mmol) was purchased from American Radiolabeled Chemical Co. (ARC Co., St. Louis, MO). [<sup>14</sup>C]manNAc (specific activity, 15 Ci/mmol) and [<sup>14</sup>C]NANA (specific activity, 55 mCi/mmol) were generous gifts from Eric Vimr (University of Illinois, Champaign-Urbana).

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains and plasmids used in the present study are described in Table 1. *B. fragilis* cells were grown in brain heart infusion broth (BHIS) supplemented with 0.5% yeast extract and 15 μg of hematin/ml (33) in an anaerobic chamber (Coy Laboratory Products) at 37°C with an atmosphere of 85% N<sub>2</sub>, 10% H<sub>2</sub>, and 5% CO<sub>2</sub> (Airgas East). Strains deficient in glucose utilization were grown on BHIS with the

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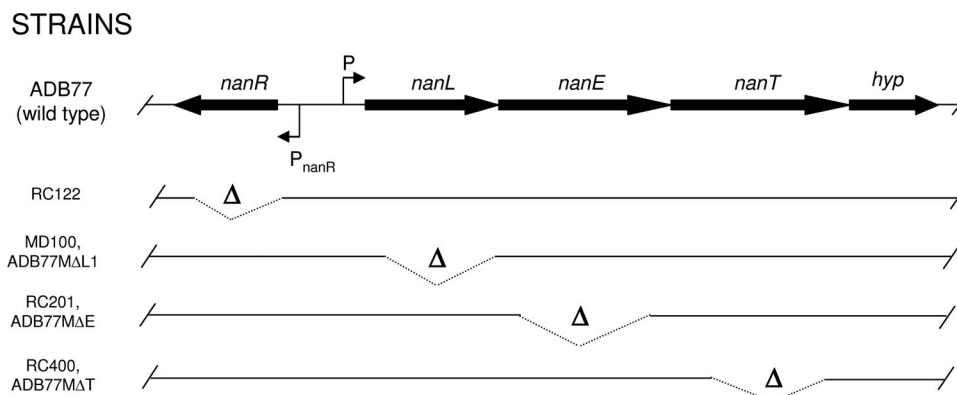


FIG. 1. Schematic of the *nanLET/nanR* operon. The *nanR* gene product is a ROK family repressor protein. The *nanL* gene product is a NANA lyase (aldolase). The *nanE* gene product possesses similarity to the mammalian RnBP, also known to be an *N*-acetylglucosamine 2-epimerase. The *nanT* gene product is a transport protein of the major facilitator superfamily. The *hyp* gene encodes a hypothetical protein. Relevant deletion constructs and the schematics of the deletions are listed.

addition of 0.5% (wt/vol) galactose. Thymine (200  $\mu\text{g/ml}$ ) was added for growth of *thyA* mutant strains. *E. coli* strain DH5 $\alpha$  (39) was used for cloning, and HB101/RK231 (12) was used for mobilization of plasmids from DH5 $\alpha$  to *B. fragilis*, as previously described (33). *E. coli* cells were grown in Luria broth (Difco) at 37°C. Ampicillin (100  $\mu\text{g/ml}$ ), tetracycline (2  $\mu\text{g/ml}$  for *B. fragilis*, 10  $\mu\text{g/ml}$  for *E. coli*), chloramphenicol (25  $\mu\text{g/ml}$ ), rifampin (50  $\mu\text{g/ml}$ ), gentamicin (50  $\mu\text{g/ml}$ ), kanamycin (50  $\mu\text{g/ml}$ ), trimethoprim (80  $\mu\text{g/ml}$ ), and erythromycin (8  $\mu\text{g/ml}$ ) were used as indicated.

For *B. fragilis* growth experiments, overnight cultures in BHIS broth containing thymine, were harvested by centrifugation, washed twice with modified phosphate-buffered saline (MPBS), resuspended in MPBS and used to inoculate SAMP broth, a defined minimal medium (32) to a starting  $A_{600}$  of  $\sim 0.05$ . Growth was monitored by monitoring the absorbance at 600 nm. All minimal medium cultures containing NANA as the main carbon source were supplemented with 0.02% glucose or galactose to allow for adaptation to growth on NANA.

**Sugar accumulation studies.** (i) [ $^3\text{H}$ ]manNAc accumulation. Cells were grown anaerobically at 37°C in SAMP broth containing thymine and 0.5% manNAc or containing manNAc and 0.5% xylose and then harvested at an  $A_{600}$  of 0.5, washed twice with MPBS, and resuspended in fresh SAMP broth with thymine and 0.5% xylose. [ $^3\text{H}$ ]manNAc was added to a final concentration of 4 mM (specific activity, 0.25  $\mu\text{Ci/mmol}$ ), followed by incubation of the cells anaerobically at 37°C for 15 min with agitation. A 1-ml aliquot was harvested on nitrocellulose filters (0.22- $\mu\text{m}$  pore size; Millipore, Inc., Bedford, MA) and washed with 3 ml of fresh SAMP broth with no additions. Cell-associated radioactivity was determined with a Beckman model LS5000TD scintillation counter. To determine the protein concentration, a separate aliquot was removed before the addition of labeled substrate, sonicated for 45 s using a Branson model 250 sonifier, and then used with Bio-Rad (Hercules, CA) protein assay reagent according to the manufacturer's instructions. The total accumulation of [ $^3\text{H}$ ]manNAc by each strain was expressed as nmol/mg of whole-cell protein.

(ii) [ $^3\text{H}$ ]NAG accumulation. [ $^3\text{H}$ ]NAG accumulation was measured as described above, except that cells were grown in SAMP broth containing thymine and 0.5% NAG and then incubated with 4 mM [ $^3\text{H}$ ]NAG (specific activity, 0.25  $\mu\text{Ci/mmol}$ ). The inhibition of [ $^3\text{H}$ ]NAG accumulation by unlabeled manNAc and the inhibition of [ $^3\text{H}$ ]manNAc accumulation by unlabeled NAG were performed as described above, except a range of inhibitor concentrations (4 to 40 mM) were examined. The kinetics of inhibition were determined by analysis of Lineweaver-Burke plots or Dixon plots (10).

(iii) [ $^{14}\text{C}$ ]NANA accumulation. Cells grown in SAMP broth with the addition of thymine and 0.5% xylose were prepared as described above. [ $^{14}\text{C}$ ]NANA was added to a final concentration of 10  $\mu\text{M}$  (specific activity, 10  $\mu\text{Ci/mmol}$ ), and the cells were incubated anaerobically at 37°C for up to 30 min with agitation. At several time points, 0.2-ml aliquots of cells were harvested on nitrocellulose filters, and the cell-associated radioactivity was determined. The total accumulation of [ $^{14}\text{C}$ ]NANA by each strain was expressed as nmol/mg of whole-cell protein.

**Cloning, DNA sequencing and analysis, and strain manipulation.** Details of all cloning and strain manipulations can be found in the supplemental material.

**Purification of an oligohistidine-tagged NanE protein.** A description of the purification procedures can be found in the supplemental material.

**Enzyme assays.** A description of the NANA lyase assay as used in the present study can be found in the supplemental material (7).

The NanE activity in *B. fragilis* cell extracts (50 to 100  $\mu\text{g}$  of total protein per reaction) was detected by chromatographic separation of the substrate manNAc from reaction products. Assays were performed with 25 mM unlabeled manNAc in 0.05 M Tris (pH 8.0) and 10 mM  $\text{MgCl}_2$  in the presence or absence of ATP (30 mM final concentration) in a final volume of 15  $\mu\text{l}$ . Assay mixtures were incubated at 37°C for 1 h and then heat inactivated at 70°C for 20 min. A 10- $\mu\text{l}$  aliquot of the reaction mixture was spotted onto 1% sodium borate-treated Whatman 3MM filter paper and allowed to dry. Descending paper chromatography was then performed as described previously (26), using butanol-pyridine- $\text{H}_2\text{O}$  (6:4:3) as the solvent. Chromatograms were baked at 95°C for 5 min, developed using Ehrlich's reagent, and then baked at 50°C for 10 min or until colored spots appeared. Standards of NAG6-P, NAG, and manNAc were run in parallel.

The activity of the purified NanE<sub>6HIS</sub> was measured as described above. To each reaction, a total of 5  $\mu\text{g}$  of NanE<sub>6HIS</sub> was added to 5 mM unlabeled manNAc and 0.1  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]manNAc (final specific activity, 0.8  $\text{mCi/mmol}$ ) in 0.05 M Tris-10 mM  $\text{MgCl}_2$  (pH 8.0), in the presence or absence of ATP (30 mM final concentration), or the nonhydrolyzable adenosine 5'-*O*-3-thiotriphosphate (ATP- $\gamma$ -S) (20 mM final concentration). Descending paper chromatography was performed as described above. After drying, the chromatograms were exposed on a Kodak MD146-931 phosphor screen for 20 to 30 min and scanned on a Storm 850 PhosphorImager. Spot intensities were quantified by using ImageQuant 1.2 imaging software (Molecular Dynamics).

To measure kinetic parameters of the NanE enzymatic reaction, a coupled assay that included purified Roka kinase, pyruvate kinase, and lactate dehydrogenase was used. The reaction mixture contained ATP (2 mM), NADH (0.6 mM), phosphoenolpyruvate (5 mM), 1  $\mu\text{l}$  of pyruvate kinase (from rabbit muscle [EC 2.7.1.40], 1,351 U of activity/ml), 5  $\mu\text{l}$  of lactate dehydrogenase (12,500 U/ml), and 1  $\mu\text{l}$  of purified Roka protein (0.75  $\mu\text{g}$  [6]) in an assay buffer consisting of 0.1 M Tris-HCl (pH 7.0) and 0.01 M  $\text{MgCl}_2$ . Purified NanE<sub>6HIS</sub> protein and manNAc (final concentrations ranged from 33.5  $\mu\text{M}$  to 13.4 mM from a 0.67 M stock solution) were then added, and the reaction was monitored by measuring the decrease in the  $A_{340}$  as  $\text{NAD}^+$  was formed from NADH at 25°C. All auxiliary enzymes were present in excess. The  $K_m$  and  $V_{\text{max}}$  of NanE were determined by analysis of a double reciprocal (Lineweaver-Burk) plot.

**Phylogenetic analyses.** Sequences of the *nanL*, *nanA*, and its homologs and *nanE* and its homologs were aligned by using the CLUSTAL W method. Phylogenetic trees were calculated by using MEGA 3.1 or MEGA 4.0 (16) using the neighbor-joining algorithm. The confidence limits were estimated by using 500 bootstrapping replicates. Phylogenetic trees were also calculated by using the minimum evolution algorithm, and the confidence limits were estimated by using 500 bootstrapping replicates.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description <sup>a</sup>	Source or reference <sup>b</sup>
<b>Strains</b>		
<i>B. fragilis</i>		
ADB77	TM4000, $\Delta thyA$ ; Tp <sup>r</sup>	3
CJB100	ADB77: $\Delta rokA1$	6
ADB77M	ADB77:manNAc growth enabled <sup>+</sup>	This study
MD100	ADB77: $\Delta nanL1$	This study
RC201	ADB77: $\Delta nanE1$	This study
RC140	ADB77: $\Delta nanT1$	This study
RC122	ADB77: $\Delta nanR1$	This study
ADB77M $\Delta L1$	ADB77M: $\Delta nanL1$	This study
ADB77M $\Delta E$	ADB77M: $\Delta nanE1$	This study
ADB77M $\Delta T$	ADB77M: $\Delta nanT1$	This study
ADB77M $\Delta rok$	ADB77M: $\Delta rokA1$	This study
RC122T	RC122: $\Delta nanT$	This study
ADB77::pRAG210	ADB77, disruption of the <i>nanL</i> open reading frame with pRAG210	This study
<i>B. thetaiotaomicron</i>		
VPI 5482	$\Delta nanL nanE^+ \Delta nanT rokA^+$	VPI
TAL21586	Clinical <i>B. thetaiotaomicron</i> isolate	TAL
<i>E. coli</i>		
DH5 $\alpha$	$\lambda$ nonlysogen; Cm <sup>s</sup> Amp <sup>s</sup>	39
HB101	<i>rpsL20</i> ; Tet <sup>s</sup> Amp <sup>s</sup>	33
BL21	F <sup>-</sup> <i>hsdS<sub>B</sub></i> ( <i>r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup></i> ) <i>gal dem me131</i>	Invitrogen
<b>Plasmids</b>		
RK231	RP4 derivative, Tet <sup>r</sup> Tra <sup>+</sup> Amp <sup>s</sup>	12
pYT102	p15A <i>ori</i> ; Cm <sup>r</sup> ; RP4 <i>oriT</i> , <i>B. fragilis</i> suicide vector containing <i>B. fragilis thyA<sup>+</sup></i> ; Tet <sup>r</sup>	32
pLITMUS29	pUC19 <i>ori</i> ; M13 <i>ori</i> ; Amp <sup>r</sup> ; <i>lacZ<math>\alpha</math></i>	NEB
pKDe112	pYT102 $\Delta rokA$	6
pMBD1	pLITMUS29 containing the 3' fragment of $\Delta nanL1$	This study
pMBD2	pLITMUS29 containing the 5' fragment of $\Delta nanL1$	This study
pMBD4	pLITMUS29 $\Delta nanL1$	This study
pMBD5	pYT102 $\Delta nanL1$	This study
pRC150	pYT102 $\Delta nanT1$	This study
pRC200	pYT102 $\Delta nanE1$	This study
pRC120	pYT102 $\Delta nanR1$	This study
pRAG210	pYT102 containing a 852-bp internal fragment of the <i>nanL</i> gene	This study
pET24	pBR322 <i>ori lacI</i> ; Kan <sup>r</sup>	Invitrogen
pRC4-3	pET24 containing <i>nanE<sub>His6</sub></i>	This study

<sup>a</sup> Cm, chloramphenicol; Tet, tetracycline; Amp, ampicillin; Tp, trimethoprim; Kan, kanamycin; s, sensitive; r, resistant.

<sup>b</sup> VPI, Virginia Polytechnic Institute, Blacksburg, VA; TAL, Tufts Anaerobic Laboratory, Boston, MA; NEB, New England Biolabs, Beverly, MA.

## RESULTS

**Identification of an operon encoding genes required for NANA utilization.** The action of *B. fragilis* NanH on sialic acid-containing oligosaccharides and glycoproteins produces free NANA which *B. fragilis* can use as the main carbon and energy source in minimal medium (Fig. 2A). We used a genomics approach to find additional genes required for NANA catabolism. We hypothesized that these genes would be regulated by the presence of NANA in the medium and might share upstream regulatory sequences. Thus, we used the DNA sequences upstream of the *nanH* gene (25) to search the *B. fragilis* NCTC 9343 and the unfinished *B. fragilis* 638R genome databases for matches. Indeed, a matching region was found just upstream of a gene cluster whose protein products showed similarities to proteins involved in NANA catabolism in other organisms. The *B. fragilis* gene cluster contains a gene encoding a NANA lyase homolog (*nanL*), followed by *nanE*, encoding a homolog of the mammalian renin binding protein (RnBP); a *nanT* homolog, based on its similarity to a trans-

porter of the major facilitator superfamily; and a 444-bp open reading frame that encodes a hypothetical protein.

***B. fragilis* NanR is a negative regulator of NANA utilization gene expression.** A gene encoding a predicted regulatory protein, with similarities to many ROK family regulators (Fig. 1), is located 297 bp upstream of and divergent to this *nanLET* cluster. We refer to this gene as *nanR* (R. Caughlan et al., unpublished data). An in-frame deletion of the *nanR* gene was used to examine whether NanR plays a role in NANA utilization, as well as in the regulation of the *nanLET* operon. Since the *nanR* deletion strain RC122 grows in minimal medium with NANA as the principal carbon source (Fig. 2A), the *B. fragilis* NanR protein, like the *E. coli* NanR protein (15), is not a positive regulator of the NANA utilization genes. Extracts of RC122 grown with different carbon sources were assayed for NANA lyase expression by coupling the removal of pyruvate produced during the lyase reaction to a lactate dehydrogenase reaction in the presence of NADH. Figure 3 shows that RC122 extracts from any of the growth conditions tested exhibited

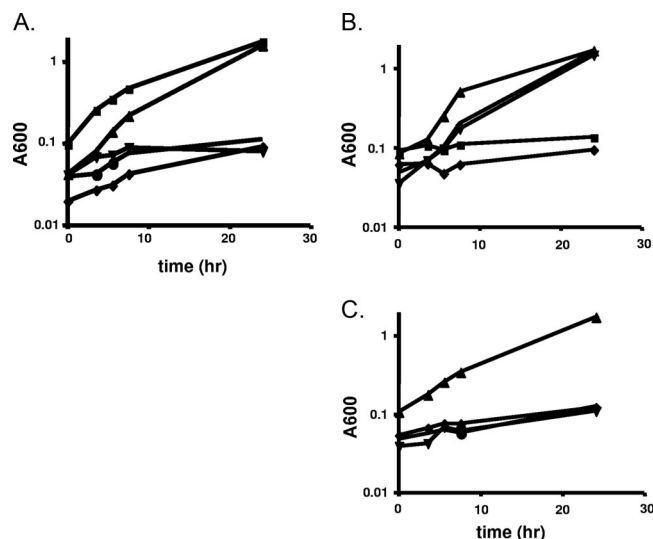


FIG. 2. Growth phenotypes of *nanR* and *nanLET* gene knockout strains, and manNAc-utilizing strains. (A) Strains were grown in SAMM broth containing thymine, 0.5% NANA, and 0.02% glucose as described in Materials and Methods. Strains: ADB77 (wild type, ■) and RC122 ( $\Delta nanR$ , ▲),  $\Delta L1$  ( $\Delta nanL$ , ▼), RC201 ( $\Delta nanE$ , ◆), RC140 ( $\Delta nanT$ , ●). (B) Growth on manNAc. Strains were inoculated in SAMM with thymine and 0.5% manNAc as described in Materials and Methods. Strains: ADB77 (■), ADB77M (▲), ADB77M $\Delta L1$  (▼), ADB77M $\Delta T$  (●), ADB77M $\Delta E$  (◆). (C) Growth of manNAc-utilizing strain and derivatives on NANA. Symbols represent the same strains as in panel B. Strains were inoculated into SAMM with thymine, 0.5% NANA, and 0.02% glucose as described in Materials and Methods. All curves shown here are representative of three separate growth experiments.

constitutive levels of lyase activity; compare this to extracts from wild-type cells that show elevated activity only if the strain is grown in the presence of NANA. This result establishes that NanR is a negative regulator of the *nanLET* operon.

**Deletions of *nanL*, *nanE*, or *nanT* affect the growth of *B. fragilis* on NANA as a main carbon source.** To establish the role of each of the *nanLET* genes in NANA utilization, we created strains bearing in-frame deletions of each gene as

described in the supplemental material and tested these strains for growth with NANA as the main carbon and energy source. The *nanL* in-frame deletion strain MD100, the *nanE* in-frame deletion strain RC200, and the *nanT* in-frame deletion strain RC140 all grew poorly, if at all, on NANA (Fig. 2A). Thus, NanL, NanE, and NanT are directly involved in NANA utilization in *B. fragilis*. Enzymatic and functional assays were then performed to establish the specific role of each gene in NANA utilization.

**NanL is an *N*-acetyl neuraminidase lyase.** *B. fragilis* wild-type strain ADB77 was grown in minimal medium containing xylose, glucose, or NANA. As shown in Fig. 3, extracts of NANA-grown cells possess 15 times more NANA lyase activity than extracts of cells grown in the presence of glucose or xylose. Interestingly, the lyase activity in extracts of cells grown in glucose is similar to the activity of extracts from cells grown in xylose, suggesting that there is no “glucose effect” on the expression of the *nanL* gene. Extracts prepared from the *nanL* deletion strain MD100 were devoid of lyase activity, as expected, under all growth conditions tested (data not shown).

**NanE is a *N*-acetylglucosamine 2-epimerase.** The *nanE* gene product is annotated in the NCTC genome project as having homology to the porcine RnBP, a polypeptide first characterized by its ability to tightly bind and inactivate the peptidase renin (20, 34). RnBPs from human and porcine kidney have been shown to possess *N*-acetylglucosamine 2-epimerase activity (18, 28, 30). We tested for this activity in extracts of the wild-type ADB77 and RC201 ( $\Delta nanE$ ) using manNAc and ATP as described in Materials and Methods. If *B. fragilis* NanE utilizes manNAc6-P as does the NanE from *E. coli* (22), we would expect this assay to yield NAG6-P. However, the NanE reaction might produce nonphosphorylated NAG since this is the product of the RnBP reaction, i.e., manNAc  $\leftrightarrow$  NAG. Indeed, *B. fragilis* extracts containing NanE catalyzed the formation of NAG from manNAc (Fig. 4A) and only formed NAG6-P if the extracts also contained the Roka kinase, which can phosphorylate NAG (6). In  $\Delta rokA$  extracts, only NAG could be detected; there was no production of NAG6-P (data not shown). That there is no activity in *B. fragilis* capable of phosphorylating manNAc to produce manNAc6-P can be de-

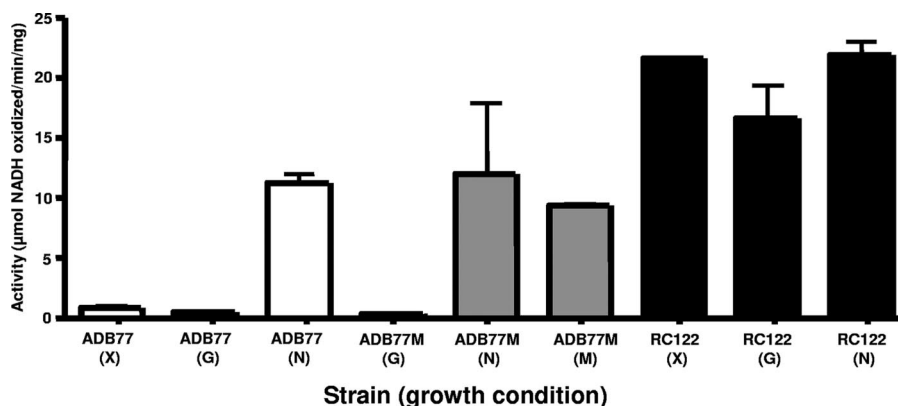


FIG. 3. NANA lyase (aldolase) activities of *B. fragilis* strains. All strains were grown in SAMM broth with thymine and 0.5% of the indicated sugar. Extracts were prepared from cells and NANA lyase (aldolase) activity was measured as indicated in the supplemental material. ADB77 (wild type) cells were grown in xylose (X), glucose (G), and NANA (N). ADB77M cells were grown in glucose, NANA, and manNAc (M). RC122 ( $\Delta nanR$ ) cells were grown in xylose, glucose, and NANA.

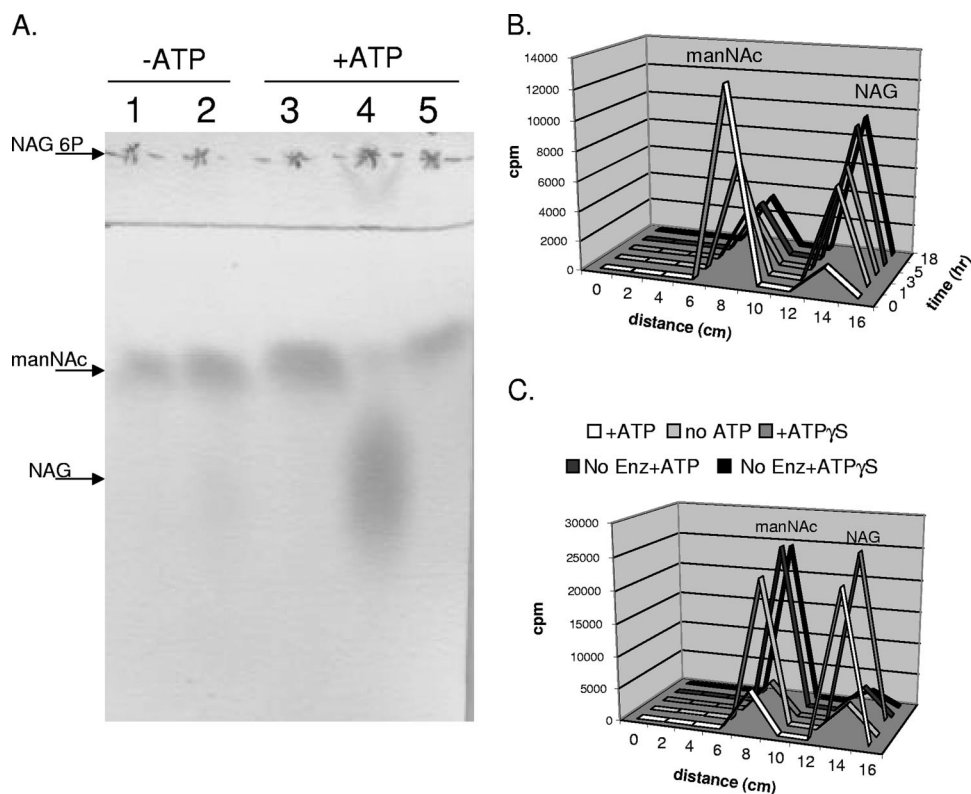


FIG. 4. NanE enzymatic activity. (A) manNac is converted to NAG in an ATP-dependent manner. Extracts of *B. fragilis* cells with (ADB77, lanes 2 and 4) or without (RC201, lanes 1 and 3) an intact *nanE* gene were incubated with manNac with or without ATP as described in Materials and Methods. Lane 5 is manNac incubated in buffer without added extract. Arrows indicate the positions of NAG, manNac, and NAG6-P standards, as determined in separate experiments. (B) manNac→NAG steady-state ratio is 1:3. Purified NanE<sub>His6</sub> was incubated with ATP and [<sup>14</sup>C]manNac (specific activity, 20 μCi/mmol) for the times indicated. (C) ATP, but not ATP<sub>γ</sub>S, hydrolysis is required for manNac epimerase activity. Purified NanE<sub>His6</sub> was incubated with or without ATP or ATP<sub>γ</sub>-P and [<sup>14</sup>C]manNac (specific activity, 20 μCi/mmol) as indicated. Each reaction in panel C was incubated for 18 h at 37°C. In panels B and C, the positions of manNac and NAG were determined in separate experiments.

duced by the failure to detect this product when extracts of the  $\Delta nanE$  strain are incubated with manNac and ATP (Fig. 4A). These observations, along with the inability of *rokA* mutants to utilize NANA (6), lend support to the conclusions that *B. fragilis* lacks any NanK activity that would phosphorylate manNac to form manNac6-P (as seen in *E. coli*) and that the RokA protein phosphorylates the NAG which is produced by NanE acting on manNac, which is produced by NanL action on NANA.

Further characterization of NanE kinetics was performed with an oligohistidine-tagged version of the protein, NanE<sub>His6</sub> that had been expressed in *E. coli* and purified by Ni<sup>2+</sup> affinity chromatography. The activity of purified NanE<sub>His6</sub> was tested in the coupled assay using manNac, purified RokA kinase (6), pyruvate kinase, and lactate dehydrogenase. The  $K_m$  for manNac was determined to be 4.63 mM, which is slightly lower than the  $K_m$  of the human, rat, and porcine NAG 2-epimerases (13.2, 7.6, and 13.7 mM, respectively) as reported by Takahashi et al. (27, 30). The  $V_{max}$  of the manNac→NAG conversion was determined to be 9.61 μmol/min/mg and, as shown in Fig. 4B, the manNac→NAG conversion reached steady state after 5 h at a manNac/NAG ratio of 1:3. For the NAG→manNac reaction, after 18 h the conversion of NAG to manNac was poor, and the NAG/manNac ratio was only 10:1, indicating that the favored reaction is to convert manNac to NAG. To determine

whether ATP hydrolysis was necessary for NanE activity, NanE<sub>His6</sub> was incubated with manNac and ATP<sub>γ</sub>-S, a non-hydrolyzable ATP analog (Fig. 4C). We could still detect epimerase activity in the assays with ATP<sub>γ</sub>-S, indicating that NanE activity does not require ATP hydrolysis. This result is similar to the finding of Takahashi et al. (27, 29), which showed that the eukaryotic *N*-acetylglucosamine 2-epimerases required ATP as a cofactor and not as an energy source.

**The *nanT* deletion mutant is deficient in NANA accumulation.** Martinez et al. (17) identified a NANA transporter protein in *E. coli*. However, the putative *B. fragilis* NanT protein (412 amino acids) is shorter in length than the *E. coli* NanT protein (496 amino acids) and is only 21% identical to the *E. coli* NanT. Having previously shown that the deletion of *nanT* abolishes NANA utilization in *B. fragilis*, we tested whether or not the deletion affected NANA accumulation. We incubated strains RC122 ( $\Delta nanR$ ) and RC122T ( $\Delta nanR\Delta nanT$ ) with [<sup>14</sup>C]NANA as described in Materials and Methods. The *nanR* deletion strains were used since they express the NANA utilization genes constitutively (see Fig. 3 for results with NanL activity) and would express the *nanLET* gene cluster even in the absence of NANA. Thus, as shown in Fig. 5, the strain that is lacking the NanR repressor (RC122) accumulated 10 times more NANA over the course of a 30-min time period than the  $\Delta nanR \Delta nanT$  double-deletion strain (RC122T). We also ex-

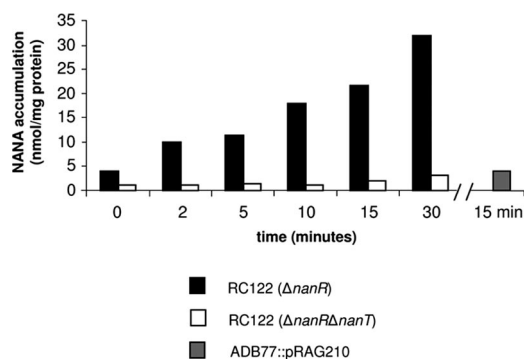


FIG. 5. NANA accumulation in RC122, RC122T, and the *nanL* disruption strain ADB77::pRAG210. Cells were incubated with labeled sugar (specific activity, 10  $\mu\text{Ci}/\text{mmol}$ ) for a time course of 30 min as described in Materials and Methods. Aliquots were harvested at the times indicated on the x axis. The [ $^{14}\text{C}$ ]NANA specific activity was 0.1  $\mu\text{Ci}/\text{mmol}$ . The experiment with ADB77::pRAG210 cells was incubated for 15 min as described in Materials and Methods.

aminated NANA accumulation in strain ADB77::pRAG210, which has a large insertion in the *nanL* gene and is unable to grow on NANA (Fig. 5). Strain ADB77::pRAG210 accumulated four- to fivefold less labeled NANA than the wild-type strain after 15 min, suggesting that the insertion in *nanL* exerts a polar effect on the expression of *nanT* and, thus, the *nanLET* gene cluster is expressed as an operon.

**Isolation of a manNAc utilizing strain of *B. fragilis*.** Wild-type *B. fragilis* strains do not grow on manNAc as the main carbon and energy source (Fig. 2B). However, since wild-type *B. fragilis* grows on NANA through a pathway that generates intracellular manNAc, it could be that the block in the utilization of external manNAc results from an inability to transport this substrate into the cytoplasm. We therefore attempted to isolate a mutant strain of *B. fragilis* that could now utilize extracellular manNAc. We used the method of Plumbridge and Vimr (21), which allowed them to isolate mutants of *E. coli* K-12 that grew better than the wild-type strain on manNAc as a carbon source. For details of the method used to isolate this strain, see the supplemental material. One such manNAc-utilizing strain of *B. fragilis* was designated ADB77M. It grows on manNAc as the main carbon and energy source with a doubling time of 3 h; the parental strain failed to grow on this medium (Fig. 2B). Strain ADB77M was tested for NANA lyase activity. Assays of extracts from cells of ADB77 and ADB77M grown in glucose showed the same low level of expression, while extracts from the same cells grown in NANA showed the fully induced level of activity (Fig. 3). However, extracts of ADB77M grown on manNAc as the main carbon source showed high levels of lyase activity, similar to what was observed in the fully induced, NANA-grown wild-type cells. This suggests that either manNAc is capable of inducing expression of the genes in the *nanLET* operon (at least *nanL*) or that manNAc is altered in the cell to create the true inducer of *nanL* (or *nanLET*) expression.

**ADB77M is able to accumulate manNAc.** We postulate that the enabling mutation in strain ADB77M alters a transporter, or the expression of a transporter, that imports manNAc into the cell. To test this hypothesis, we performed [ $^3\text{H}$ ]manNAc

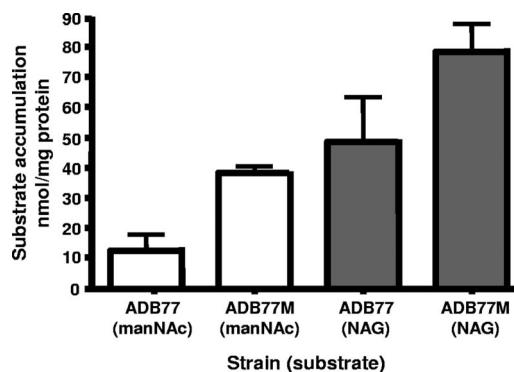


FIG. 6. manNAc and NAG uptake in ADB77 (wild type) and ADB77M. Cells were incubated with labeled sugar (specific activity, 2.5  $\mu\text{Ci}/\text{mmol}$ ) as described in Materials and Methods. Accumulation experiments were performed in triplicate.

accumulation experiments in strain ADB77M and compared these results with the wild-type strain, ADB77. As shown in Fig. 6, the enabled mutant strain ADB77M accumulates three times more manNAc in a 15-min time period than does wild-type ADB77. This might result from changes in the NanT transporter, however, the *nanT* deletion derivative of ADB77M, ADB77M $\Delta$ T, still grows on manNAc as the main carbon source (Fig. 2B), eliminating a role for NanT in manNAc uptake. In contrast, strain ADB77M exhibits increased uptake of NAG compared to the wild-type ADB77 (Fig. 6). Although the exact genetic alteration(s) in ADB77M, the manNAc-utilizing strain, are not known, we suggest that alteration of an existing NAG transporter may be responsible for manNAc uptake in this strain.

This hypothesis is also supported by a determination of the affinities of wild-type ADB77 and the mutant ADB77M strains for manNAc in an accumulation assay. Each strain was incubated with various concentrations of manNAc or NAG, and the accumulation of amino sugar over time was analyzed by double reciprocal plots. Interestingly, the affinities for NAG in the wild-type ADB77 and the mutant ADB77M are similar with  $K_m$ s of 30.95 and 47 mM, respectively. However, there is a marked difference between the two strains in the affinity for manNAc. The  $K_m$  of manNAc in mutant strain ADB77M is 55.4 mM, similar to the  $K_m$  for NAG. However, the  $K_m$  for manNAc in wild-type ADB77 is 60 times greater than that of ADB77M at 337.75 mM. Using different manNAc concentrations, we measured the [NAG] required to inhibit manNAc uptake. We found that the  $K_{i, \text{NAG}}$  of the wild-type strain ADB77 was approximately 122 mM, whereas the  $K_{i, \text{NAG}}$  of the enabled mutant ADB77M was 61 mM. We also measured accumulation of radiolabeled NAG in the presence of unlabeled manNAc, and determined a  $K_{i, \text{manNAc}}$  for wild-type ADB77 of 225 mM, and a  $K_{i, \text{manNAc}}$  for the enabled mutant ADB77M was 60 mM.

**Role of *nanLET* operon expression in growth of ADB77M on manNAc.** To test whether the *nanLET* operon plays a role in manNAc utilization in the enabled mutant ADB77M, we constructed a series of strains with deletions in each of the *nanLET* genes in the ADB77M background (see the supplemental material) and tested their ability to grow with

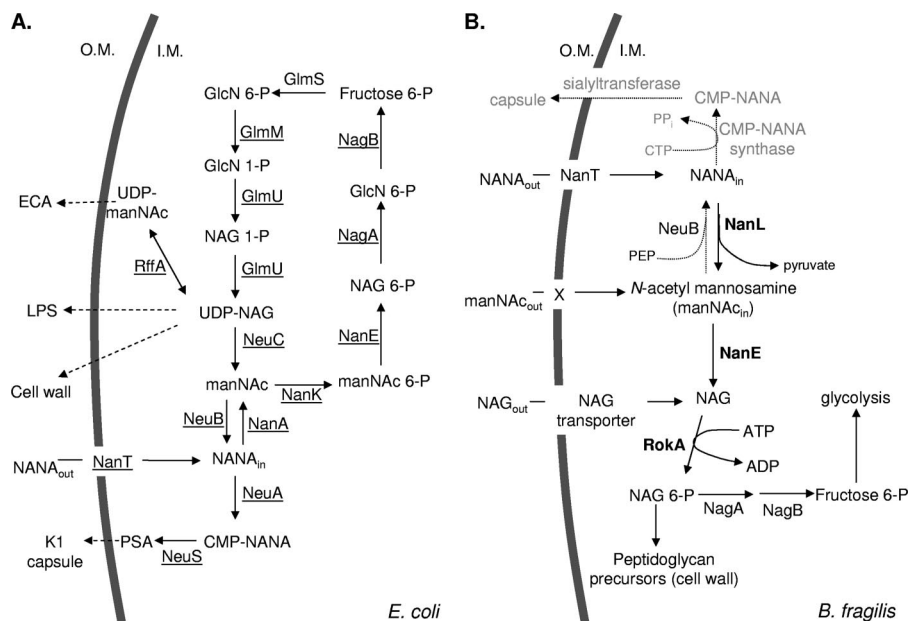


FIG. 7. Comparison of the NANA and amino sugar utilization pathways of *E. coli* (A) and *B. fragilis* (B). (A) Pathway adapted from Vimr et al. (36). GlcN, D-glucosamine. (B) Enzymes in boldface have been assayed by our laboratory. The NagA reaction has not been experimentally verified by us; however, there is an annotated sequence of such a gene in the *B. fragilis* 638R partial genome sequence. X designates the transporter, probably a NAG transporter, that contains the enabling mutation present in the strain ADB77M.

manNAc as carbon source. The *nanL* derivative of ADB77M (ADB77M $\Delta$ L1, Table 1) grows on manNAc, as expected, although it cannot grow on NANA (Fig. 2B and 2C). These data suggest that the NanL gene product is not necessary for utilization of manNAc. A similar result was found with the *nanT* deletion derivative of ADB77M (data not shown). However, a *nanE* deletion derivative of ADB77M (ADB77M $\Delta$ E, Table 1) is defective for growth on either manNAc or NANA as the main carbon and energy source (Fig. 2B and C) underscoring its importance in manNAc utilization.

**Role of the Roka kinase in NANA and manNAc utilization.** We have previously shown that the *B. fragilis rokA* deletion strain, CJB100, cannot utilize NANA as the main carbon and energy source. We have also shown that the purified RokaA protein does not phosphorylate NANA or manNAc in vitro (6). To examine whether a mutation in the *rokA* gene in the ADB77M background would similarly affect manNAc utilization, we constructed the ADB77M $\Delta$ rok strain, with an internal deletion within the *rokA* gene. Indeed, this strain no longer grows with manNAc as a carbon source (data not shown). Taken together, this evidence establishes that the RokaA kinase is required for NANA and manNAc utilization in *B. fragilis*. However, given previous results, it is unlikely that RokaA phosphorylates manNAc but instead phosphorylates NAG, the product of NanE action on manNAc in the *B. fragilis* cell.

## DISCUSSION

**Comparison of NANA utilization pathways in *B. fragilis* and *E. coli*.** The NANA utilization pathway in *B. fragilis* differs significantly from the previously described pathway in *E. coli* (22) and other bacteria (35, 36). Figure 7 presents a comparison of these pathways. Differences occur only after NANA is

cleaved by the lyase, NanL or NanA, to form manNAc and pyruvate. In *E. coli* and many other bacteria, including well-studied pathogens, manNAc is immediately phosphorylated by a NanK kinase to produce manNAc 6-phosphate. The *E. coli* NanE epimerase then converts manNAc 6-P to NAG 6-P, which then enters the amino-sugar utilization pathway (22). In contrast, in *B. fragilis* the NanE epimerase enzyme converts unphosphorylated manNAc to NAG, using ATP as a cofactor. In this respect the *B. fragilis* NanE catalytic function is similar to the mammalian RnBP/NAG 2-epimerases. In *B. fragilis*, NAG is now phosphorylated by the RokaA kinase to yield NAG 6-P, which is further metabolized. RokaA-deficient mutants of *B. fragilis* are unable to utilize NANA (or manNAc when the *rokA* deletion is introduced into the manNAc enabled strain ADB77M). This implies that *B. fragilis* does not contain a specific manNAc kinase (NanK) or a hexokinase other than RokaA that can phosphorylate manNAc.

Phylogenetic comparisons between the *nanE* gene products from *B. fragilis*, other *Bacteroides* species, and other bacterial NAG 2-epimerases involved in NANA utilization suggests that they may have independent origins. A phylogenetic tree was constructed from a multiple-sequence alignment of eukaryotic RnBP/NAG 2-epimerases and bacterial NanE homologs. Clearly, the manNAc 6-P 2-epimerases, such as those from *E. coli*, *C. perfringens*, and *P. multocida*, form their own distinct clade, as do all RnBPs including that of *B. fragilis* (Fig. 8). Indeed, the *B. fragilis* NanE is closely related to the mammalian RnBPs/NAG 2-epimerases, and it groups into a larger clade that includes all RnBPs, clearly excluding *E. coli* NanE and the other manNAc 6-P 2-epimerases (Fig. 8). This suggests two distinct origins of the NanE epimerase groups. Surprisingly, NanE homologs from *Bacteroides thetaiotaomicron* and

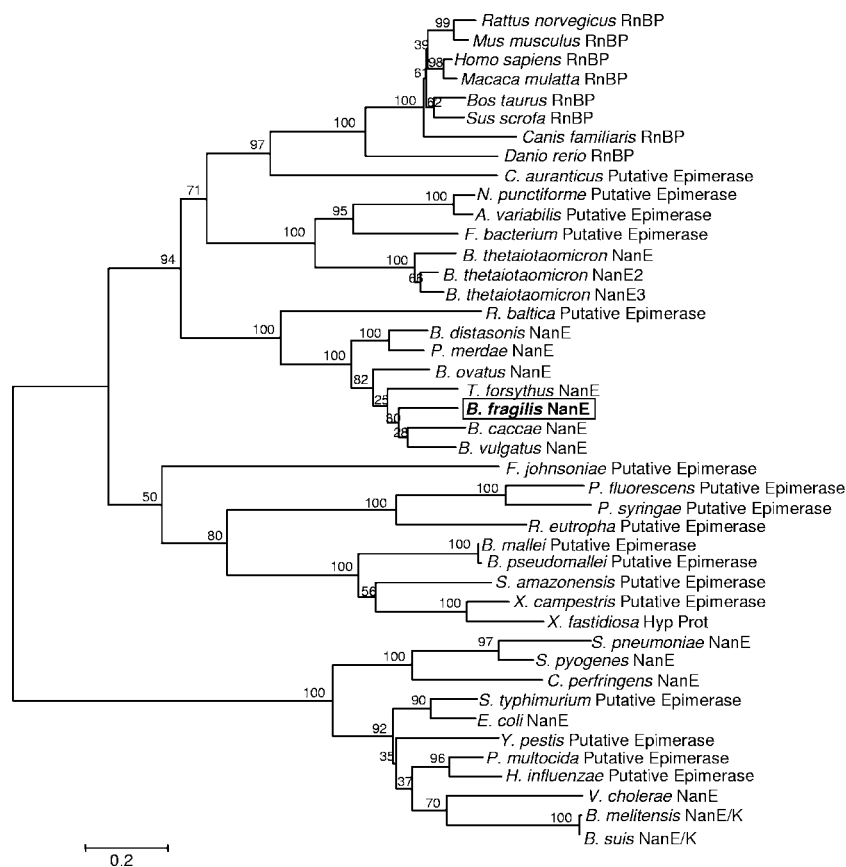


FIG. 8. Phylogenetic tree of NanE proteins and RnBPs, including *B. fragilis* NanE (boxed). The tree was constructed by using the neighbor-joining method and was visualized with MEGA 3.1. hyp prot, hypothetical protein.

the *Bacteroidetes* group member *Flavobacteriales* strain ALC-1 do not group as closely as might be expected with the enzymes of other *Bacteroidetes*, such as *Tannerella forsythia*, *B. fragilis*, *Bacteroides ovatus*, *Parabacteroides distasonis*, and several other intestinal *Bacteroidetes* (Fig. 8). This could represent different degrees of divergence of the *nanE* sequence since its acquisition by each of these organisms.

Sequence alignment of *B. fragilis* NanE and eukaryotic RnBP's indicates marked differences, as well as similarities, between the *B. fragilis* NanE and the RnBPs (see Fig. S2 in the supplemental material). Takahashi, et al. describe several cysteine residues that are important for the function of the human epimerase enzyme (known as C1 to C10) (31). Only one of these cysteines appears to be conserved between *B. fragilis* NanE and RnBPs (C1, see Fig. S2 in the supplemental material). Obviously, the absence of the other cysteine residues in *B. fragilis* NanE does not affect the epimerase activity. Furthermore, Itoh et al. (14) have produced a crystal structure of the porcine kidney NAG 2-epimerase, which identified active site residues in the protein, many of which are aromatic side chains such as tryptophan and phenylalanine. These residues are conserved in the other mammalian RnBPs/NAG 2-epimerases and strikingly, most of these residues are conserved in *B. fragilis* NanE (see Fig. S2 in the supplemental material). In a study by Itoh et al. (14), the NAG 2-epimerase crystals were saturated with NAG, and conserved residues that might play a role in

sugar contacts were determined. These same residues are present not only in *B. fragilis* NanE but also in the epimerases of *T. forsythia* and *B. thetaiotaomicron* (see Fig. S2 in the supplemental material). The genome sequences of several intestinal *Bacteroidetes*, such as *B. vulgatus* (40), *B. caccae*, *B. ovatus*, *Parabacteroides distasonis*, and *Parabacteroides* contain *nanE* homologs, with all of the aforementioned conserved residues (data not shown). There are several other enzymes in *B. fragilis* that show greater similarity to "eukaryotic" enzymes than to bacterial enzymes. The *B. fragilis* aconitase shares striking similarity to the eukaryotic mitochondrial aconitase (3). This has been interpreted to suggest that a *Bacteroides* species may have been the symbiont that eventually became the mitochondrion in eukaryotic cells.

In contrast to the similarities of *B. fragilis* NanE and the eukaryotic RnBP, there does not appear to be a close eukaryotic homolog of *B. fragilis* NanL. A phylogenetic tree was constructed using a multiple-sequence alignment of NANA lyase proteins (see Fig. S1 in the supplemental material), including NanL homologs from other *Bacteroidetes*, the *E. coli* NanA protein and its homologs, and putative NANA lyase proteins from other commensal or pathogenic bacteria. NanL proteins from the *Bacteroidetes* form a distinct clade, with the exception of the putative NanL of *F. bacterium*. This clade is clearly distinct from the *E. coli*/*Salmonella*/*Shigella* clade of NanA proteins, suggesting either two separate origins of



NANA lyase or a large divergence between NanL homologs and NanA homologs. Notably absent from the phylogenetic tree shown in Fig. S1 in the supplemental material is a NanL homolog from *B. thetaiotaomicron* (see below).

As was demonstrated by the phylogenetic tree in Fig. 8, *B. fragilis* and *E. coli* NanE proteins are not similar to each other on the primary sequence level. The same conclusion can also be drawn when comparing *B. fragilis* NanL to *E. coli* NanA (see Fig. S1 in the supplemental material). The degree of difference, however, between NanE proteins is much greater than the degree of difference between NanL and NanA. NanL is 34% identical (56% positive) at the primary sequence level compared to NanA, while *E. coli* and *B. fragilis* NanE proteins share no significant similarity. Furthermore, *B. fragilis* NanE (377 amino acids) is significantly larger than *E. coli* NanE (229 amino acids). In contrast, the NanL (302 amino acids) and NanA (297 amino acids) proteins are similar in size. This suggests two very different origins for the *B. fragilis* NanL and NanE proteins.

**Sialic acid utilization systems in other *Bacteroidetes*.** Sialic acids are available in large quantities in the human colon (23, 24). It is not surprising that *B. fragilis* can use NANA and other sialic acid-containing substrates for growth. Indeed, *B. fragilis* has been shown to express a variety of glycoside hydrolases (4), including neuraminidase (8, 11, 19), that can convert mucins into usable carbon sources. The ability to metabolize NANA gives *B. fragilis* a competitive advantage in the niche of the colonic mucosa and also at sites of infection, since it is capable of cleaving and utilizing sialic acid-rich components of intestinal mucins and epithelial cell glycoproteins. Based on the availability of sialic acids in the colon, we might expect other colonic residents, including other *Bacteroides* species, to possess a NANA utilization system similar to that in *B. fragilis*. Surprisingly, *E. coli* strains lack neuraminidase activity, although neuraminidase activity has been detected in many other colonic *Bacteroides* species (23). Recently, genome sequences of several colonic *Bacteroides* have become available. Many of these genomes, such as that of *Bacteroides vulgatus* and *Parabacteroides distasonis*, contain *nanLET* gene clusters with close sequence similarity to *B. fragilis nanLET*. These genomes also contain a *nanH* (neuraminidase) homolog and a *rokA* homolog. The genomes of two *Bacteroidetes*, *Bacteroides uniformis* and *Porphyromonas gingivalis*, appear to contain neuraminidase homologs but do not contain *nanLET* genes. The genome of one *Bacteroides* species, *Bacteroides capillosus*, does not appear to have a neuraminidase gene or any NANA utilization genes. *B. capillosus* also does not appear to have a *rokA* homolog in its genome, making it a unique *Bacteroides* species among the species whose genome sequences are available.

Analysis of the published *B. thetaiotaomicron* VPI 5482 genome reveals the presence of a putative neuraminidase gene but not a NANA lyase (*nanL*) gene. This suggests that *B. thetaiotaomicron* should not be able to use NANA for growth. Furthermore, we found that *B. thetaiotaomicron* strains VPI 5482 and TAL21586 do not grow in minimal medium supplemented with NANA (data not shown). Interestingly, the *B. thetaiotaomicron* VPI 5482 genome seems to possess three separate manNAc 2-epimerase genes, whose products are similar to the *B. fragilis* NanE. The role of these epimerase candidates in *B. thetaiotaomicron* metabolism is as yet unknown.

Neuraminidase activity has also been detected in oral *Bacteroidetes* (19), including, but not limited to, *P. gingivalis*, *T. forsythia*, and *Prevotella* sp. The sialidase gene of *T. forsythia*, *siaHI*, has been isolated and characterized (13). This sialidase does not share sequence homology with any other bacterial sialidase/neuraminidase, including *B. fragilis* NanH or the neuraminidase candidates from *B. thetaiotaomicron* or *P. gingivalis*. A search of the *T. forsythia* genome database (<http://www.oralgen.lanl.gov/oralgen/bacteria/tfor/>) reveals the presence of a *nanLET*-like gene cluster, suggesting that *T. forsythia* should be capable of utilizing NANA as a carbon and energy source. The gene arrangement and sequences of the putative protein products of the *nanLET*-like cluster in *T. forsythia* are similar to the products of the *B. fragilis nanLET* operon, although there is no *nanR*-like gene present upstream of and divergent to the *nanLET* cluster. A search of the *P. gingivalis* and *Prevotella intermedia* sequences reveals possible neuraminidase genes but no other genes whose protein products could be involved in NANA utilization.

There is considerable interest in the question of when sialic acids first appeared in living systems (see references 2, 5, 9, and 36 for recent discussions). Until recently, it had been accepted that sialic acids were absent until the appearance of the deuterostomes, perhaps 500 million years ago. With the discovery of sialic acid biosynthesis capabilities in several bacteria, especially human pathogens, it is now considered possible that sialic acids appeared billions of years before the split. Complicating the issue is the likelihood that lateral gene transfer between bacteria and eukaryotes has played an important role in the current genetic makeup of sialic acid-synthesizing and sialic acid-utilizing organisms (5). No matter what the actual explanation turns out to be, the NANA utilization system of *B. fragilis* represents another solution to sialic acid metabolism. *B. fragilis* and all other bacteria that metabolize NANA cleave NANA into manNAc and pyruvate. In *B. fragilis* and presumably in other *Bacteroides* strains that contain a similar NanE protein, manNAc is epimerized to NAG, which is then phosphorylated by the Roka hexokinase to form NAG 6-P, an intermediate in the common pathway for aminosugar utilization. In *E. coli* and other related bacteria, another pathway-specific enzyme is required to convert manNAc to manNAc 6-P, specifically the kinase, NanK. manNAc6-P is then epimerized to NAG6-P by an enzyme specific for the phosphorylated sugar derivatives. It is not clear which pathway came first in time and, indeed, the subject becomes even more complicated with the realization that the *B. fragilis* pathway contains two proteins, Roka (6) and NanE, with close similarity to eukaryotic proteins. If it can be shown that sialic acid biosynthesis and degradation systems were present in bacteria long before the appearance of eukaryotes capable of sialic acid biosynthesis, it may be possible to conclude that the bacteria were the ultimate source of the enzymes required for these pathways. Without such proof, it remains impossible to decide the question.

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