

Sialic Acid Mutarotation Is Catalyzed by the *Escherichia coli* β -Propeller Protein YjhT^{*[S]}

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The acquisition of host-derived sialic acid is an important virulence factor for some bacterial pathogens, but *in vivo* this sugar acid is sequestered in sialoconjugates as the α -anomer. In solution, however, sialic acid is present mainly as the β -anomer, formed by a slow spontaneous mutarotation. We studied the *Escherichia coli* protein YjhT as a member of a family of uncharacterized proteins present in many sialic acid-utilizing pathogens. This protein is able to accelerate the equilibration of the α - and β -anomers of the sialic acid *N*-acetylneuraminic acid, thus describing a novel sialic acid mutarotase activity. The structure of this periplasmic protein, solved to 1.5 Å resolution, reveals a dimeric 6-bladed unclosed β -propeller, the first of a bacterial Kelch domain protein. Mutagenesis of conserved residues in YjhT demonstrated an important role for Glu-209 and Arg-215 in mutarotase activity. We also present data suggesting that the ability to utilize α -*N*-acetylneuraminic acid released from complex sialoconjugates *in vivo* provides a physiological advantage to bacteria containing YjhT.

Sialic acids are an important family of related 9-carbon sugars, present on the surface of many different cells and functioning in a wide range of different biological processes (1–3). The most common sialic acid, *N*-acetylneuraminic acid (Neu5Ac)³ is the predominant form present in humans and can be found as a terminal sugar on a wide range of surface glycoconjugates (2). A number of bacteria that can colonize humans make use of Neu5Ac as a nutrient source; for example,

the intestinal bacterium *Escherichia coli* can grow on Neu5Ac as a sole carbon source (4). The intestinal mucous layer is rich in sialic acids, and *E. coli* genes required for sialic acid catabolism are induced in this environment and are important for colonization (5). Additionally, uropathogenic *E. coli* express genes for sialic acid catabolism during growth in human urine (6). Other bacteria, like the respiratory pathogen *Haemophilus influenzae*, have an additional use for sialic acid in an immune evasion mechanism by adding Neu5Ac to their lipopolysaccharide (7, 8), which provides increased survival in human serum (9, 10). *H. influenzae* is unable to synthesize Neu5Ac *de novo* and hence must acquire it from the host (9, 11). This has recently been demonstrated to be dependent on a tripartite ATP-independent periplasmic (TRAP) transporter (12–14), which is encoded in a genomic region that also contains the genes for sialic acid catabolism (1, 12). Directly downstream of the transporter genes (*siaPQM*) is an uncharacterized gene, *HI0148* (Fig. 1), which encodes a predicted periplasmic protein that has been suggested to contain 7 Kelch motifs and be involved in sialic acid catabolism (15), but is not essential for Neu5Ac uptake (12). In *E. coli* the orthologue of this gene is *yjhT*, which is the second gene in a potential three gene operon, *yjhATS*, whose expression is induced by sialic acid (16). The first gene in this operon, *yjhA*, has already been demonstrated to encode a Neu5Ac-specific porin in the outer membrane of *E. coli* and has been renamed *nanC* (16). These data suggest a sialic acid-related function for the *yjhT/HI0148* gene products.

Here we describe the function of YjhT as a representative of this family of uncharacterized periplasmic proteins from pathogenic bacteria and demonstrate that this protein functions, unexpectedly, as a sialic acid mutarotase. We report the structure of YjhT and identify amino acid residues important for enzyme activity *in vitro*. In addition, we provide *in vivo* data consistent with our suggested function for this protein.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Native and Mutant YjhT-His₆ Proteins—The *yjhT* gene was amplified from genomic DNA of *E. coli* K12 MG1655 using KOD Hot Start polymerase (EMD Biosciences) and primers EcyjhT-NdeI and EcyjhT-XhoI (supplemental Table 1). The NdeI- and XhoI-cut PCR product was cloned into pET21b and confirmed by DNA sequencing. The resulting construct (pES5) encodes a C-terminally His₆-tagged variant of YjhT, in which the tag is spaced from the protein by a Leu-Glu dipeptide. *yjhT* mutant alleles were generated using a modified QuikChangeTM method (17) using pES5 as a template

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The atomic coordinates and structure factors (code 2uvk) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

[S] The on-line version of this article (available at <http://www.jbc.org/>) contains supplemental Experimental Procedures, additional references, Table 1, and Figs. 1–8.

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³ The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; TRAP transporter, tripartite ATP-independent periplasmic transporter; STD, saturation transfer difference; NOESY, nuclear Overhauser effect spectroscopy; BisTris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; SeMet, selenomethionine; MES, 4-morpholineethanesulfonic acid.

and KOD Hot Start polymerase. The mutagenic oligonucleotides used are listed in supplemental Table 1. Native and mutant YjhT proteins were expressed in BL21 (DE3) pLysS, which was grown at 25 °C in 1.5 liters of LB with antibiotics to an OD₆₅₀ of 0.4–0.5 before induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside overnight. Cells were harvested and incubated in SET buffer (0.5 M sucrose, 5 mM EDTA in 50 mM Tris-HCl, pH 8, 600 μg/ml lysozyme) at 30 °C for 1 h to release the periplasmic fraction, which was then clarified by centrifugation and dialyzed against 20 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, pH 7.5. All YjhT variants were purified by nickel-affinity chromatography on a 1-ml HisTrap HP column (GE Healthcare) and dialyzed against double distilled water containing 100 mM NaCl; proteins were ~95% pure as judged based on SDS-PAGE. Protein concentrations were determined from the absorbance at 280 nm (A_{280}) using a theoretical molar absorption coefficient of 73,540 (Y309A mutant) or 74,820 cm⁻¹ M⁻¹ (all other variants). A SeMet derivative of YjhT was prepared as described previously (18) by growing BL21 (DE3) pLysS pES5 cells in double-strength M9 (19) supplemented with selenomethionine; the expression and purification procedures for this protein were those used for native YjhT. Throughout this paper we will refer to the concentration of YjhT as that of the monomer. The masses of all the recombinant proteins were confirmed using electrospray mass spectrometry (see supplemental Experimental Procedures), which demonstrated that all had been correctly processed by removal of their signal peptide.

Crystallization Procedure—Purified SeMet-substituted YjhT was dialyzed against double distilled water and concentrated to 12 mg/ml. Crystallization experiments utilized the vapor diffusion method and a MOSQUITO nanoliter dispensing robot to set up sitting drops. Successful crystallization conditions were identified using the PACT screen (20). Crystals for data collection were grown from drops made up of 150 nl of SeMet-substituted YjhT and 150 nl of 200 mM sodium malonate, 0.1 M BisTris propane, pH 7.5, and 20% w/v polyethylene glycol 3350.

Data Collection and Structure Solution—A single wavelength data set extending to a resolution of 1.5 Å with a high redundancy was collected from a crystal of SeMet substituted YjhT at the ESRF, Grenoble, France, on beamline ID14-4 (Table 1). From the SAD signal with a resolution cutoff at 2.5 Å, the programs SHELXC and SHELXD (21) led to the location of all six selenium atoms in the asymmetric unit. Refinement of the selenium atom positions using the program MLPHARE provided experimental phase probability distributions in terms of the Hendrikson-Lattman coefficients. These served as restraints during the ARP/wARP-REFMAC model building process (22), which led to a model with 587 of 714 residues built and sequence coverage of 99%. The model was completed and refined in iterative cycles of REFMAC (23) and manual modeling in COOT (24). In subunit A there is clear electron density for residues 1–225 and 229–349, with a disordered surface loop from 226 to 228. In subunit B, all residues from 1 to 349 are visible. The C terminus, residues 350–357, is disordered in both subunits. Refinement statistics are given in Table 1. Coordinates and structure factors were deposited in the Pro-

TABLE 1
X-ray data collection and refinement statistics

Data collection	
X-ray source	ESRF ID14-4
Wavelength (Å)	0.9793
Collection temperature (K)	100
Resolution range (Å)	52.85 to 1.50
Space group	P2 ₁
Unit-cell parameters (Å)	$a = 44.8, b = 105.7, c = 74.2,$ $\beta = 99.95$
No. of unique reflections, overall/outer shell ^a	107,873/15,687
Completeness (%), overall/outer shell ^a	99.5/99.3
Redundancy, overall/outer shell ^a	8.1/8.2
$I/\sigma(I)$, overall/outer shell ^a	30.1/5.9
R_{merge}^b (%), overall/outer shell ^a	0.045/0.379
Refinement and model statistics	
R-factor ^c (R-free ^d)	0.182 (0.219)
Reflections (working/free)	102,206 (5376)
Outer shell R-factor ^c (R-free ^d)	0.220 (0.297)
Outer shell reflections (working/free) ^e	7508 (408)
Molecules/asymmetric unit	2
No. of non-hydrogen atoms	6001
No. of water molecules	729
R.m.s.d. mean square deviation from target ^f	
Bond lengths (Å)	0.011
Bond angles (°)	1.306
Average B-factor (Å ²)	16.0
Ramachandran plot ^g	97.8/2.2/0%

^a The outer shell corresponds to 1.58–1.50 Å.

^b $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_{hkl} \sum_i \langle I \rangle$, where I_i is the intensity of the i th measurement of a reflection with indexes hkl , and $\langle I \rangle$ is the statistically weighted average reflection intensity.

^c R-factor = $\sum |F_o| - |F_c| / \sum |F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively.

^d R-free is the R-factor calculated with 5% of the reflections chosen at random and omitted from refinement.

^e Outer shell for refinement corresponds to 1.539 to 1.500 Å.

^f r.m.s.d. indicates root mean square deviation of bond lengths and bond angles from ideal geometry.

^g Percent of residues in favored/allowed/disallowed regions of the Ramachandran plot, according to MolProbity (61).

tein Data Bank (code 2uvk). The protein was compared with proteins of known structure using the DALI software (25). The size of the dimer interface was calculated using the Pro-Face software (26).

NMR Spectroscopy—All NMR experiments were performed in 50 mM deuterated acetate buffer, pH 5.5, 100 mM NaCl, 10% (v/v) ²H₂O. ¹H NMR spectra were acquired on a Bruker 700-MHz spectrometer at 25 °C using 50 μM 3-(trimethylsilyl)propionic 2,2,3,3-*d*₄ acid sodium salt (Sigma) as a chemical shift (0 ppm) reference and using excitation sculpting (27) with gradients for suppression of the H₂O signal. Saturation transfer difference (STD) (28) spectra of Neu5Ac (1 mM) with YjhT (10 μM) were acquired with 8 scans and a 7-s saturation time with saturation at 0 ppm (saturation transfer) or at –20000 Hz (reference spectrum). A difference spectrum was obtained by subtracting the saturation transfer spectrum from the reference spectrum. One-dimensional spectra for the time courses were acquired with 128 scans and a 1-s delay between scans. Initially a “0” time point was acquired of 1 mM sialyllactose (α(2–3) and α(2–6) mixture from human milk; Sigma). Then 0.6 units of *Clostridium perfringens* sialidase (type V, Sigma) and 0.5 μM YjhT (or YjhT mutant) was added. Acquisition of the first spectrum took 5 min and was completed ~7 min after addition of the enzymes. Two-dimensional NOESY spectra were acquired with 1 mM Neu5Ac and 10 μM YjhT, 64 scans, and a mixing time of 100 ms. The addition of protein (sialidase and/or YjhT)

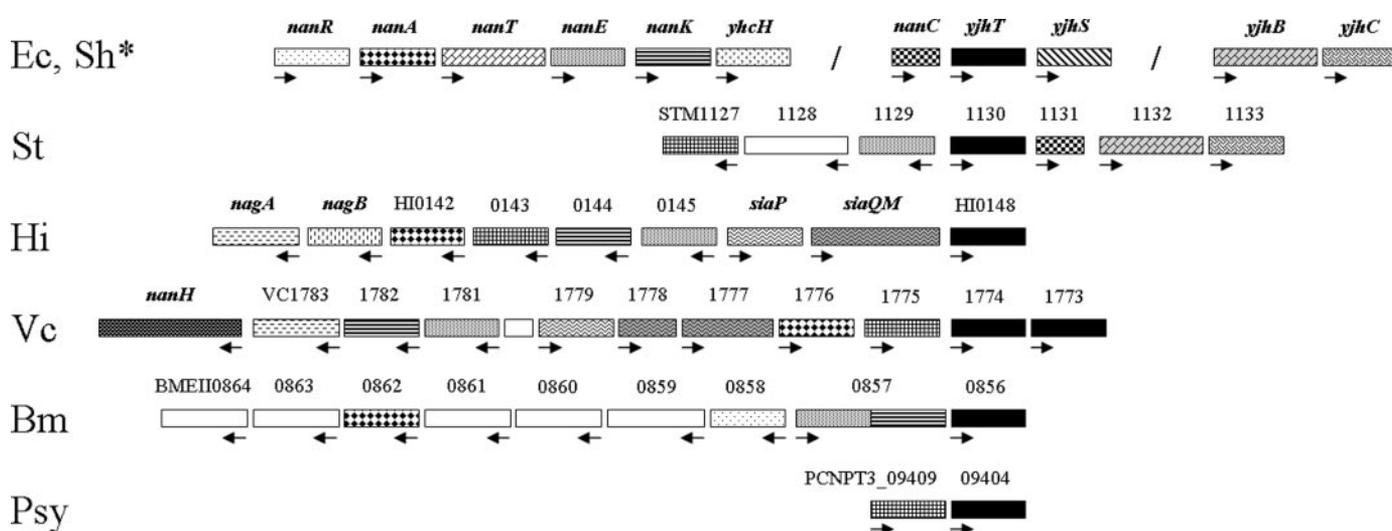


FIGURE 1. The genetic organization of *E. coli* *yjhT* and orthologues from pathogenic and other bacteria. *yjhT* and its orthologues are represented with black boxes, whereas sialometabolic and sialic acid-inducible genes are represented with various patterned boxes. The abbreviations used are as follows: *Ec*, *E. coli* K12; *Sh*, *Shigella* spp. (*flexneri*, *bodyii*, *somnei*); *St*, *Salmonella typhimurium* LT2; *Hi*, *H. influenzae*; *Vc*, *V. cholerae*; *Bm*, *Brucella melitensis* 16M; *Psy*, *Psychromonas* sp. CNPT3; *NanR*, sialic acid-responsive repressor of the FadR/GntR family of transcriptional regulators; *NanA*, sialic acid aldolase; *NanT*, Neu5Ac transporter; *NanE*, *N*-acetylmannosamine-6-P epimerase; *NanK*, *N*-acetylmannosamine kinase; *YhcH*, unknown function; *NanC*, Neu5Ac-specific porin; *YjhS*, unknown function; *YjhB*, *NanT*-like transporter of unknown function; *YjhC*, unknown function; *NagA*, *N*-acetylglucosamine-6-P deacetylase; *NagB*, glucosamine-6-P deaminase; *SiaPQM*, Neu5Ac TRAP transporter; *NanH*, sialidase. In *E. coli* the *yjhBC* genes belong to the *NanR* regulon as do the *nanATEKyhcH* operon and the putative *nanCyjhTS* operon (1). *, *Shigella* spp. and pathogenic *E. coli* strains display the same genetic arrangements as *E. coli* K12, except that *Shigella* spp. lack *yjhBC*. The genetic structure of the *Pasteurella multocida* sialometabolic locus is very similar to that of *H. influenzae* (1, 12).

and/or sugars did not alter the pH of the buffer. One-dimensional spectra were processed using Topspin (Bruker BioSpin) and SigmaPlot, whereas two-dimensional spectra were processed using NMRPipe (29) and viewed in NMRView (30).

Construction of Deletion Mutants—For this study we used three strains from the Keio collection (31) with Kan^R cassettes in the *nanC* (JW4274), *yjhT* (JW4273), and *yjhS* (JW4272) genes as well as the parental strain BW25113. We removed the Kan^R cassette by transformation with pCP20 followed by plasmid curing as described previously (32). Removal of the Kan^R cassette was confirmed by PCR, and the $\Delta yjhS$ and $\Delta yjhT$ regions were sequenced. Of note, the $\Delta yjhS$ strain contained an additional base in the Scar peptide-coding sequence, resulting in an extended Scar peptide about 60 amino acids long.

Neu5Ac Uptake Assay—For [¹⁴C]Neu5Ac uptake assays, single colonies from freshly streaked LB plates were inoculated in LB medium and grown for 8 h. Cells were harvested, washed once in M9 minimal medium, and inoculated into M9 with 0.4% v/v glycerol to an initial OD₆₅₀ of 0.05 and grown overnight. Cells were harvested, washed three times in M9, and then inoculated into M9 Neu5Ac (1 mg/ml) to an initial OD₆₅₀ of 0.1 and allowed to grow until mid-log phase (OD₆₅₀ ~0.4), when they were harvested, washed four times in M9, and finally resuspended to an OD₆₅₀ of 2 in 1 ml of M9 salts (no carbon source added), and stored on ice. To assay sialic acid uptake, such suspensions were diluted to an OD₆₅₀ of 0.3 in M9 salts pre-warmed at 37 °C and incubated with stirring at 37 °C for 2 min, when uptake was initiated by adding [¹⁴C]Neu5Ac (Sigma) to a final concentration of 0.25 μM. At the indicated times, 400-μl aliquots were taken, filtered through 0.45-μm pore-size nitrocellulose filters (Millipore), and washed with 3 ml of M9 salts, and the radioactivity within them was measured as described

(33). The protein concentration within each sample was determined by a modified Lowry assay (34).

Sequence Analysis and Bioinformatics—Sequence and genome contexts of *yjhT* orthologues were obtained from the SEED (35) with additional manual curation. Sequences were aligned using ClustalX (36).

RESULTS

YjhT Is a Periplasmic Protein That Interacts Weakly with Sialic Acid—To investigate the function of this family of uncharacterized proteins, we studied the protein from the model bacterium *E. coli* K12 MG1655 (37, 38), which, like other members of this family, is encoded within a sialic acid-induced operon known to contain other genes involved in sialic acid utilization (Fig. 1). The YjhT protein was overexpressed and extracted from *E. coli* periplasm as a C-terminally hexahistidine-tagged protein and then purified to apparent homogeneity in a single step using nickel affinity chromatography (supplemental Fig. 1, inset). The molecular weight of recombinant YjhT was examined using electrospray mass spectrometry (supplemental Fig. 1), which revealed two distinct sets of *m/z* peaks that were identified as having masses of 38,685 ± 4 and 77,371 ± 8. These are consistent with the monomeric (38,686) and dimeric (77,372) forms of the C-terminally His-tagged version of YjhT that had been processed to remove the 19-amino acid signal peptide upon export to the periplasm.

To examine whether YjhT was able to interact with Neu5Ac, we used ¹H NMR spectroscopy, a method used successfully to study other aspects of sialic acid biology (39). A ¹H NMR spectrum of Neu5Ac is shown in Fig. 2 (lower spectrum) where peaks have been assigned based on published spectra of Neu5Ac (40). The STD (28) spectrum of YjhT in the presence of Neu5Ac (Fig. 2, upper spectrum) demonstrates an interaction

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between YjhT and Neu5Ac. This STD signal was not observed with Neu5Ac alone, and STD signals were not observed in the presence of other sugars, specifically galactose, *N*-acetylglucosamine, and *N*-acetylmannosamine (data not shown).

The ability to interact with Neu5Ac, the periplasmic localization and the predicted β -propeller structure (15), suggested two potential functions for YjhT. The first, as a lectin-like multivalent sialic-binding protein, such as the *Psathyrella velutina* lectin (PVL) (41), was not supported by a ligand binding assay that detects stable complexes between protein and Neu5Ac (supplemental Fig. 2). The second, as a sialidase (42, 43), was tested using ^1H NMR spectroscopy and sialyllactose as a substrate. Only in the presence of a sialidase was there release of Neu5Ac from sialyllactose. In the presence of YjhT or with no added protein, sialyllactose was stable after overnight incubation (supplemental Fig. 3). These data suggest a novel function for this periplasmic protein related to its interaction with Neu5Ac.

YjhT Has a Neu5Ac Mutarotase Activity—In vertebrates, sialic acid is mostly present as part of larger sialoglycoconju-

gates, where it is linked to the rest of the macromolecule exclusively by α -linkages (2). Free sialic acid is released by sialidase action as the α -anomer, which mutarotates spontaneously into β -Neu5Ac, which is the most abundant anomer at equilibrium (over 90%) (39, 44). The spontaneous mutarotation rate of Neu5Ac is relatively slow, for example at pH 5.4 the $t_{0.5}$ is 80 min (45). Given that the doubling time of an *E. coli* cell (20 min) is faster than the spontaneous mutarotation rate, we reasoned that bacteria competing for a limited supply of sialic acid might gain a selective advantage by being able to mutarotate the α -anomer more quickly.

We investigated the possible Neu5Ac mutarotase activity of YjhT using ^1H NMR, a technique used previously to characterize other sugar mutarotases (46–48). Unlike other techniques traditionally used to characterize these enzymes (for example, polarimetry), ^1H NMR also allows the detection of chemical exchange between anomers in solutions at equilibrium (46–48), which is advantageous given the lack of commercially available α -Neu5Ac. Thus, we compared two-dimensional ^1H NOESY spectra of Neu5Ac acquired without (Fig. 3A) and with added YjhT (Fig. 3B); in these experiments anomeric exchange is detected only when this is quite fast, that is when it occurs within the mixing time of individual scans (100 ms). The spectrum in the presence of YjhT contains clear cross-peaks connecting the α and β frequencies that are not observed in its absence, indicating that in the presence of the protein the exchange between the two anomers has been accelerated so much that it is now within the detection limit of the ^1H NOESY experiment.

Next we wished to investigate YjhT-catalyzed Neu5Ac mutarotation in more *in vivo* like conditions where an excess of newly formed α -Neu5Ac mutarotates over time into the β -anomer. To overcome the limitation of the lack of commercial preparations of pure α -Neu5Ac, we used an NMR-based assay in which a sialidase is used to release α -Neu5Ac *in situ* from sialyllactose, the mutarotation of which is then followed over time in the absence (Fig. 4A) or in the presence of YjhT (Fig. 4B). The use of sialidasases to release α -Neu5Ac *in situ* from a sialoglycoconjugate is an established method to produce non-equilibrium populations of the two anomers that mimics the *in vivo* situation (39). In the absence of

YjhT, α -Neu5Ac accumulates as a consequence of sialidase action (Fig. 4A). This is particularly evident in the signal for the H3ax of α -Neu5Ac (H peak); the signal for the H3ax of α -Neu5Ac overlaps with those for the same hydrogen of the sialyllactose mixture (peak B), but it is still the most abundant peak. The intensities of the peaks for β -Neu5Ac (H3ax is peak E and H3eq is peak D) slowly increase during the time course of the assay because of spontaneous mutarotation, as observed in other studies (39), and only after 1 h is β -Neu5Ac the predominant species. When the experiment is

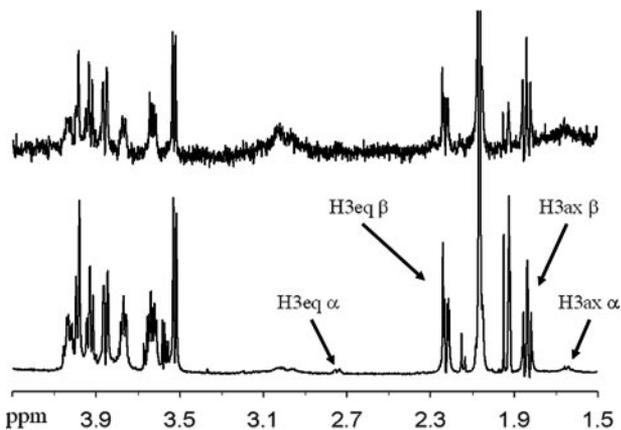


FIGURE 2. STD ^1H NMR analyses of the Neu5Ac-YjhT interaction. Lower spectrum, ^1H NMR reference spectrum of a mixture of 10 μM YjhT and 1 mM Neu5Ac. Upper spectrum, STD spectrum of the same mixture. The relevant peaks for the hydrogens at carbon 3 (H3ax/H3eq, where ax = axial, eq = equatorial) are indicated with arrows as reference for following figures. The peak at 1.92 ppm was present in the buffer and probably arises from residual ^1H acetate. The strong peak at 2.0 ppm is from the *N*-acetyl group.

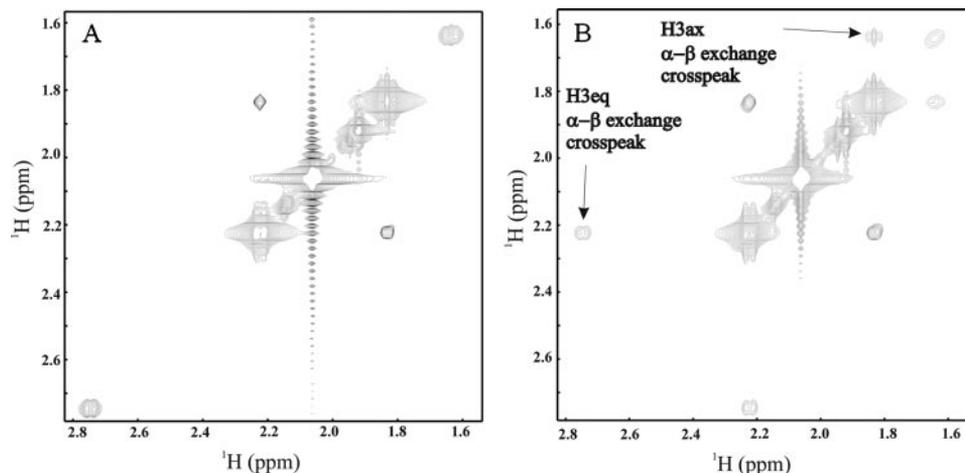


FIGURE 3. Two-dimensional ^1H - ^1H NOESY spectrum of 1 mM Neu5Ac in the absence (A) and presence (B) of 10 μM YjhT. Cross-peaks indicating the enzyme catalyzed mutarotation of Neu5Ac are labeled.

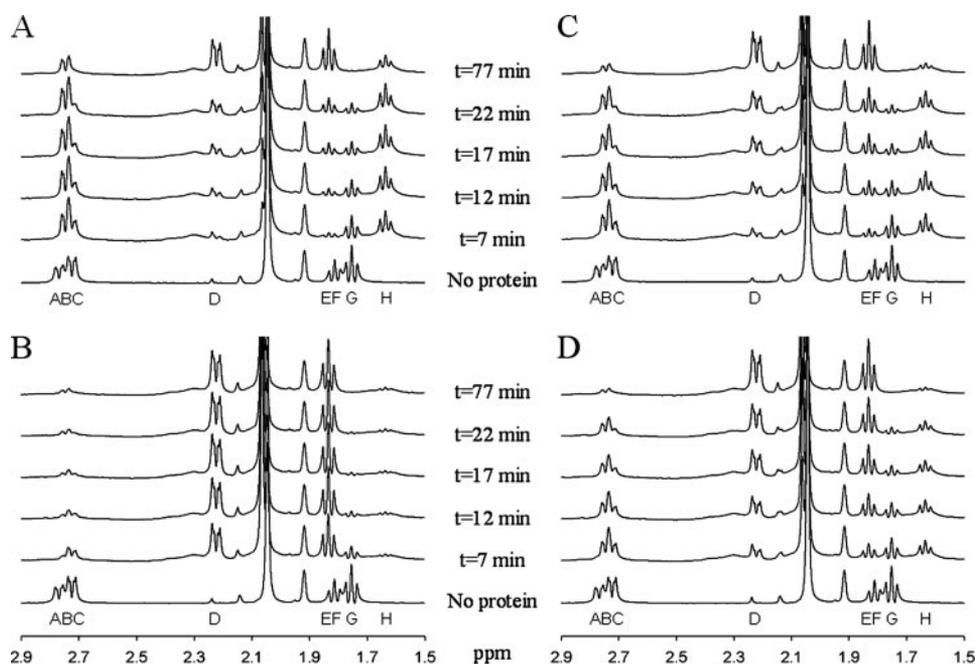


FIGURE 4. Time-resolved ^1H NMR analysis of spontaneous and YjhT-catalyzed mutarotation of α -Neu5Ac. *A*, time course of the production and mutarotation of α -Neu5Ac in the absence of YjhT. *B*, time course of the production and mutarotation of α -Neu5Ac in the presence of $0.5 \mu\text{M}$ YjhT. *C*, time course of the production and mutarotation of α -Neu5Ac in the presence of $0.5 \mu\text{M}$ YjhT^{E209A}. *D*, time course of the production and mutarotation of α -Neu5Ac in the presence of $0.5 \mu\text{M}$ YjhT^{R215A}. *A* and *C*, H3eq peaks of $\alpha(2-3)$ - and $\alpha(2-6)$ -sialyllactose, respectively; *F* and *G*, H3ax peaks of $\alpha(2-3)$ - and $\alpha(2-6)$ -sialyllactose, respectively; *B* and *H*, H3eq and H3ax peaks of α -Neu5Ac, respectively; *D* and *E*, H3eq and H3ax peaks of β -Neu5Ac, respectively. The sialidase exhibits higher activity toward $\alpha(2-3)$ -linkages, which can be seen as the H3 peaks of $\alpha(2-3)$ -sialyllactose disappear much faster than those of the $\alpha(2-6)$ species do (compare in either time course peaks *A*, *F* with peaks *C* and *G*).

repeated in the presence of $0.5 \mu\text{M}$ YjhT (Fig. 4*B*), strikingly α -Neu5Ac does not accumulate, and β -Neu5Ac is already the most abundant species after 7 min. These spectra are identical to the ^1H NMR spectrum observed for a solution of Neu5Ac at chemical equilibrium (for example in Fig. 2, lower spectrum). Because α -Neu5Ac is the primary product of sialidase activity (and we have shown that YjhT is not a sialidase), this indicates that the α -anomer is being rapidly mutarotated into the β -anomer (see scheme in Fig. 5). Addition of YjhT also resulted in faster hydrolysis of sialyllactose, which is consistent with YjhT scavenging the sialidase primary product and thus pulling the reaction forward. These data demonstrate that YjhT is able to catalyze the exchange of the α - and β -anomers at equilibrium and also can accelerate mutarotation in the direction which is likely to occur *in vivo* in nonequilibrium solutions.

Investigation of the *in Vivo* Function of YjhT—Given the biochemical data demonstrating the mutarotase activity of YjhT, we wished to identify an *in vivo* phenotype for *E. coli* lacking a functional *yjhT* gene. Using a [^{14}C]Neu5Ac uptake assay, we were able to measure a 20% drop in the rate of Neu5Ac uptake using subsaturating amounts of substrate (Fig. 6). Significantly, neither a $\Delta yjhS$ mutant (Fig. 6) nor a $\Delta nanC$ mutant (data not shown) showed any uptake defect in these conditions demonstrating the $\Delta yjhT$ specificity of this phenotype. The small phenotype is best explained by assuming that the *E. coli* Neu5Ac transporter NanT recognizes the abundant β -anomer so that only in the presence of the mutarotase is the α -Neu5Ac pool available to NanT during the time course of the assay. This

assumption is consistent with the observation of a similar phenotype when these assays are repeated at pH 5.5 in MES minimal medium (data now shown), where the spontaneous mutarotation rate is decreased by at least 3-fold (45). In these conditions, the amount of total Neu5Ac taken up during the assay (20%) is twice that of the available α -Neu5Ac pool (<10%), and given that spontaneous mutarotation is slow ($t_{0.5}$ of 80 min at pH 5.5), this is not compatible with the linear uptake we observe.

We then investigated whether the $\Delta yjhT$ mutant displayed any noticeable growth phenotype on Neu5Ac. We were able to grow *E. coli* cells on minimal medium supplemented with colominic acid, an $\alpha(2-8)$ -homopolymer of Neu5Ac that *per se* does not serve as a carbon source for *E. coli* but can be utilized if sialidase is supplied to release monomeric α -Neu5Ac (supplemental Fig. 4). Under these conditions a very small but highly reproducible growth delay was observed during growth of the $\Delta yjhT$ mutant over the first

few hours of incubation (supplemental Fig. 4*A*). This result is consistent with a transient period in which α -Neu5Ac is present in excess over its concentration at equilibrium allowing cells expressing *yjhT* to have a growth advantage. This defect was not observed for the $\Delta yjhS$ mutant thus ruling out any polarity effects of the *yjhT* mutant. When free Neu5Ac (over 90% β) was used as sole carbon source, the $\Delta yjhT$ mutant showed no growth defect even when the sugar was provided at concentrations sustaining growth rates comparable with those observed in colominic acid medium (supplemental Fig. 4*B*). No growth defect was observed also when cells were pregrown with glycerol rather than Neu5Ac (data not shown), indicating that expression of *yjhT* is required for this small growth advantage. Taken together these results are supportive of a function for YjhT in the efficient use of exogenous α -Neu5Ac.

YjhT Is a Kelch Domain Containing Protein That Forms a 6-Bladed β -Propeller—To further study the YjhT protein, we crystallized full-length protein containing a C-terminal hexahistidine tag and solved the structure to 1.5 Å (Table 1 and Fig. 7*A*). The structure is a dimer in the crystal, with each subunit consisting of a 6-bladed β -propeller that contains Kelch motifs (49), defined by residues 14–47, 60–100, 112–185, 195–233, 247–302, and 311–348 (Fig. 7 and supplemental Fig. 5). The dimer interface is formed by a large continuous patch with a buried surface area of $\sim 2500 \text{ \AA}^2$ and contains multiple hydrogen bonds between the two subunits. The dimeric species of YjhT was visible in electrospray mass spectrometry analysis of the protein (supplemental Fig. 1), so we used analytical ultra-

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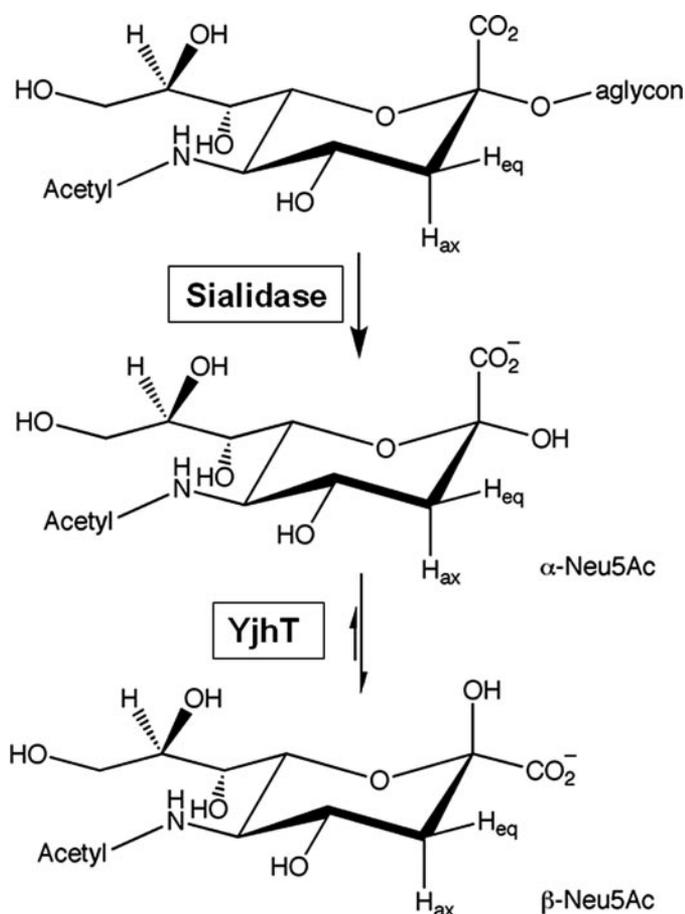


FIGURE 5. Biochemical interconversion of conjugated Neu5Ac (top) to free α -Neu5Ac, the majority of which mutarotates to β -Neu5Ac at equilibrium. The former reaction is catalyzed by a sialidase and the latter by YjhT. The positions of the H3 hydrogens are indicated.

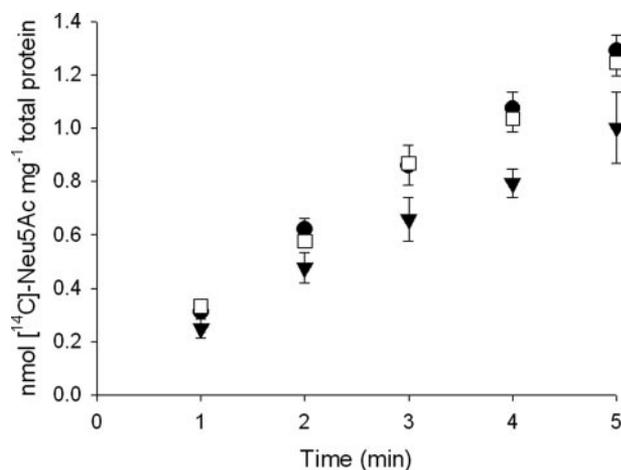


FIGURE 6. Uptake of $[^{14}\text{C}]\text{Neu5Ac}$ by *E. coli* BW25113 and by isogenic $\Delta yjhT$ and $\Delta yjhS$ strains. These results are the average of three biological replicas \pm S.D. and are shown for the WT (●), the $\Delta yjhT$ (▼), and $\Delta yjhS$ (□) mutants. The uptake assay was carried out also using higher concentrations of substrate (0.5, 1, and 2 μM) giving comparable results (not shown).

centrifugation to confirm that YjhT is a dimer in solution. Data from sedimentation equilibrium experiments fit well to a single species of the dimeric molecular mass of 73.9 kDa (supplemental Fig. 6A), which does not change with protein concentration (supplemental Fig. 6B) implying that the dimer is highly stable.

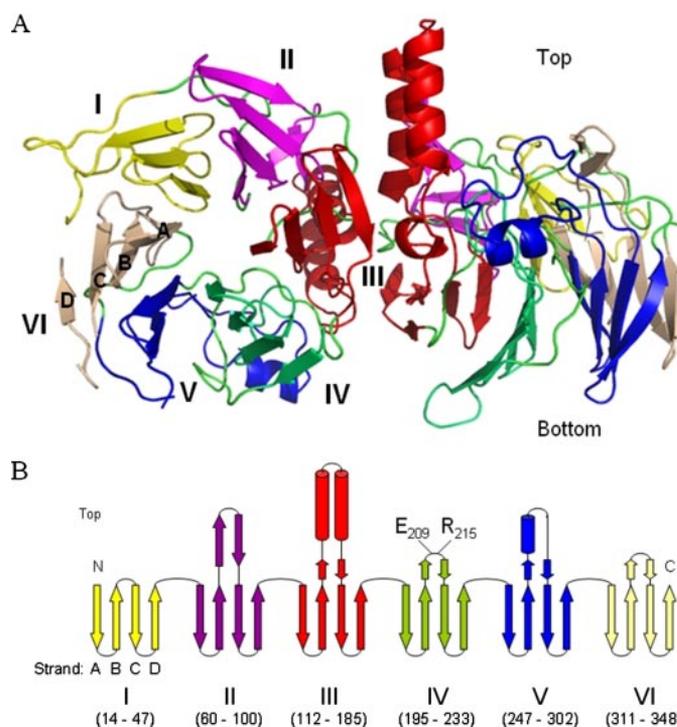


FIGURE 7. Structure of YjhT. A, dimeric structure showing the arrangement of the two Kelch domains in the dimer and the dimerization interface. Each blade is colored differently, and the blade order used throughout is highlighted in blade I. B, schematic of the topology of YjhT with conserved residues Glu-209 and Arg-215 highlighted. The color scheme is the same as for A.

Extensions from blades II and III extend upwards from the top of the β -propeller and form a surface against which the second subunit packs at about a 90° angle to the first subunit, providing a larger interaction face than simply packing the two β -propellers against one another (43). The β -propeller is not closed using the typical “Velcro” mechanism seen in most other β -propellers (50) but is rather “unclosed” as seen first in the prolyl oligopeptidase (51). A DALI search revealed the best hit to be human Keap1 (52) with the second hit to the fungal galactose oxidase (53). Although these eukaryotic proteins have quite different functions, they both contain a Kelch motif β -propeller structure. This motif was first observed in the *Drosophila* Kelch ORF1 protein as a repeating element of around 50 amino acids, which is present in a range of other organisms performing a wide range of functions (49, 54, 55). The motif is characterized by a run of four hydrophobic residues followed by conserved twin glycines, which are followed by two conserved aromatic residues at a particular spacing (49). The Keap1 protein has an almost perfect set of 6 Kelch domains with all elements present in all 6 blades, but in YjhT there are some slight variations in a number of the blades (supplemental Fig. 5). Of note is a conserved pair of small/polar amino acids (N/D or G) found between strands a and b in all 6 blades, which are exposed to solvent in the structure.

Site-directed Mutagenesis of Conserved Amino Acids within YjhT Reveals a Potential Active Site—To investigate the enzymatic function of YjhT further, we used a sequence-based site-directed mutagenesis strategy to identify residues potentially important for mutarotase activity. Selected YjhT homologues from other bacteria (see Fig. 1) were collected and aligned with

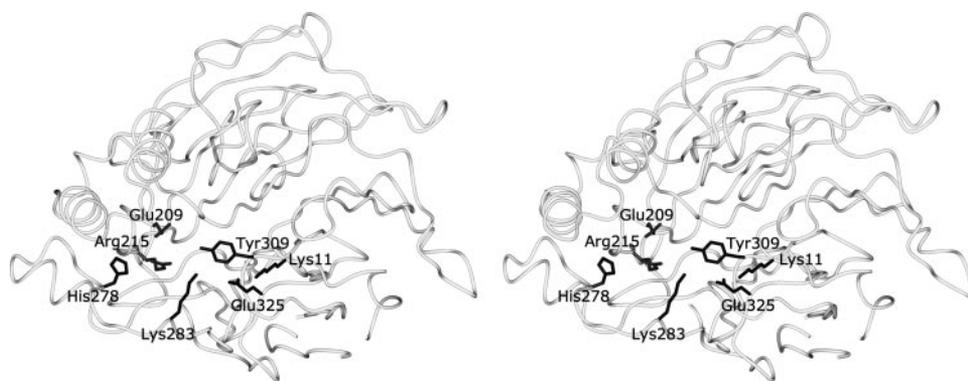


FIGURE 8. Stereo image of YjhT highlighting the amino acid side chains of conserved residues mutagenized in this study.

the secondary structural elements of YjhT (supplemental Fig. 7). There are a small number of conserved residues besides those that are part of the Kelch motifs, which all sit in the same region on the surface of the β -propeller (Fig. 8). We mutated these seven conserved residues (indicated on the alignment in supplemental Fig. 7) to alanine and expressed and purified the resulting recombinant proteins. We investigated their ability to enhance the mutarotation rate using the *in vitro* time course assay of sialic acid release and mutarotation. Significantly a E209A mutant of YjhT is severely impaired in its ability to enhance the mutarotation rate above the spontaneous rate (Fig. 4C), whereas an R215A mutant gives a decreased but observable activity (Fig. 4D). Replacement of Lys-11, His-278, Lys-283, Tyr-309, or Glu-325 with alanine residues has no effect on the activity of the protein in this assay (data not shown). The circular dichroism spectra of the E209A and R215A mutant are indistinguishable from that of the wild-type protein, suggesting that the phenotype was not because of misfolding of YjhT (data not shown). To further characterize the E209A mutant we acquired a ^1H NOESY spectrum of this protein in the presence of Neu5Ac and were unable to detect cross-peaks indicative of fast mutarotation at equilibrium as observed in the presence of the wild-type protein (data not shown). Interestingly the Glu-209 residue sits at the bottom of a deep cleft on the upper surface of YjhT and is very close to Arg-215 (supplemental Fig. 8), suggesting that this region of the protein is important for the activity of the enzyme. The mechanism of this enzyme is currently under further investigation.

DISCUSSION

We here report the *in vitro* and preliminary *in vivo* characterization of the product of the *E. coli* *yjhT* gene. Our *in vitro* analyses demonstrated that this β -propeller protein has a function as a mutarotase that accelerates the equilibration between the α - and β -anomers of Neu5Ac. This is both a novel enzymatic activity related to bacterial sialic acid utilization and represents a new fold for a mutarotase. In accordance with the nomenclature of sialic acid metabolic genes in *E. coli*, we propose to rename the *yjhT* gene *nanM* (for *N*-acetylneuraminase mutarotase).

The existence of a sialic acid mutarotase has not been anticipated despite many years of work on the biology of this important cell surface sugar, presumably as this reaction does occur

spontaneously. After release of the α -anomer from sialoglycoconjugates by sialidase action, Neu5Ac spontaneously mutarotates to reach an equilibrium position with over 90% in the β -anomer (see Fig. 4A) (39, 44). *In vitro* NanM accelerates the conversion of newly released α -Neu5Ac into β -Neu5Ac so quickly that the α -anomer never accumulates after its release; thus it seems reasonable that the physiological role of NanM might be to facilitate a sialidase-negative bacterium such as *E. coli* to compete successfully for limited amounts of extracellular Neu5Ac, which is likely to be taken up as β -Neu5Ac. Also, given that endogenously released sialic acid is an inflammatory indicator in the host (56), its rapid removal from solution might be advantageous to the bacterium to damp down host responses. Sialic acid is an important molecule in the life-style of both pathogenic and commensal strains of *E. coli*, and although its significance *in vivo* has been normally associated with the synthesis of a polysialic acid capsule by *E. coli* K1 and K92 strains, there is now evidence demonstrating that unencapsulated strains of *E. coli* can also use sialic acid to their advantage as a carbon and nitrogen source *in vivo*. Mucosal surfaces that are colonized by *E. coli* contain high levels of sialylated mucins, and free sialic acid can be made available to *E. coli* by the action of sialidases expressed by other bacteria in the same niche or by host sialidases in the course of inflammation (56). A number of genes for sialic acid utilization are induced specifically during growth in mucus, including genes in both the catabolic *nanATEK* operon and the *nanCMyjhS* operon containing *nanM* (5). Remarkably, a *nanAT* mutant was impaired in the colonization of the mouse intestine (5), providing direct evidence for an important role of sialic acid metabolism in this environment. In *E. coli* sialic acid also inhibits the expression of type 1 fimbriae, which are well known virulence factors in uropathogenic strains of *E. coli* (56), thus reducing the number of antigenic structures exposed on the bacterial cell surface. In this context *E. coli* cells might use sialic acid as an indicator of inflammation (56), and expressing a mutarotase might allow the cell to perceive the immune response of the host at earlier stages.

Our characterization of the role(s) of NanM *in vivo* has been complicated by the very small percentage (below 10%) of α -Neu5Ac in solution. We did observe a highly reproducible growth defect when a *nanM* mutant was grown on α -Neu5Ac generated *in situ* from colominic acid (poly- $\alpha(2-8)$ -sialic acid); however this defect is transient, which can probably be ascribed to the gradual accumulation of β -Neu5Ac in the experiment through sialidase activity and spontaneous mutarotation. The *nanM* mutant grew normally on Neu5Ac provided as monomeric substrate, consistent with the preferential utilization of the β -anomer by the cells. Our attempts to increase this growth defect by changing the composition of the minimal medium (*i.e.* pH and ionic strength) were unsuccessful (data not shown). A similar lack of phenotype for growth on Neu5Ac was

observed in an *E. coli nanC* mutant (16), and together these observations suggest that the function of the *nanCMyjhS* operon is unlikely to be for utilization of normal equilibrium solutions of Neu5Ac, but rather that they might allow utilization of host-derived sialic acids being released slowly from a mucosal surface. Also, *in vivo* phenotypes for other mutarotases have not been observed in wild-type cells and have required the use of artificial substrates (57, 58). In contrast, the *nanM* mutant displayed a clear sialic acid uptake phenotype with a rate about 80% that of the WT. As sialic acid uptake rates were determined using concentrations of [¹⁴C]Neu5Ac well below the reported *K_s* of 30 μM for sialic acid uptake in *E. coli* (1), these results are consistent with the *nanM* mutant being unable to use efficiently all the available Neu5Ac. By extrapolation, it is conceivable that in the presence of an excess of α-Neu5Ac, immediately after sialic acid cleavage from sialoglycoconjugates, the contribution of NanM to sialic acid uptake may be more significant.

Orthologues of *nanM* from various pathogens cluster with other sialic acid utilization/acquisition genes (Fig. 1) in a variety of different arrangements. Proteomic data from *H. influenzae* revealed that HI0148 is a relatively abundant periplasmic protein (59), although it is not essential for uptake of free Neu5Ac (12). Interestingly, *Vibrio cholerae* and *Vibrio vulnificus* encode two NanM orthologues (Fig. 1), only one of which has a signal peptide, indicating that the second is located in the cytoplasm. This suggests that these organisms have a requirement for a cytoplasmic as well as periplasmic mutarotase. There is also a clear *nanM* orthologue in the free-living marine bacterium *Psychromonas* sp. CNPT3, suggesting that sialic acid mutarotation might be required in other environments.

The structure of the β-propeller in NanM is interesting as this is the first prokaryotic example of a Kelch domain protein to be solved. Kelch domains are rare in prokaryotes (currently about 200 from all sequenced bacteria in InterPro (IPR006652)), and they are scattered in a range of organisms and contained within a wide range of different protein types, none of which have been characterized functionally. Kelch domains are much more abundant in eukaryotic organisms, with humans possessing 71 Kelch domain proteins with many cellular functions (55), and it is possible that prokaryotic Kelch domain proteins may have been acquired by horizontal gene transfer from eukaryotic systems.

We identified Glu-209 and Arg-215 as being important for full activity of NanM in our assay conditions. Although the mechanism of this enzyme is currently under investigation, it is of note that a Glu residue is essential for the activity of the galactose mutarotase (60). In this enzyme, the Glu and a His function in a general acid/base mechanism initiated by abstraction of a proton by the conserved Glu-304 from the C1 hydroxyl group of galactose followed by protonation of the ring oxygen by the conserved His-170 residue, which leads to ring opening. Mutarotation can then occur, and the ring closes again by reversal of the ring opening steps (60). Therefore it is possible that Glu-209 might function to abstract a proton from the C2 hydroxyl in Neu5Ac; however, the complementary functional group for this mechanism does not appear to be the conserved His-278, as mutation of this amino acid results in wild-type

activity in our assay. The role of the Arg-215 might relate to modulation of the function of Glu-209 to which it is hydrogen bonded, or it might have a direct function in coordinating the Neu5Ac via a salt bridge to the C1 carboxylate group.

In conclusion, we have identified a novel and unexpected mutarotase enzyme that acts on sialic acid, an important cell surface molecule, which may have a function *in vivo* to aid in the acquisition of host-derived sialic acid.

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