Biosynthesis of Sialylated Lipooligosaccharides in *Haemophilus ducreyi* Is Dependent on Exogenous Sialic Acid and Not Mannosamine. Incorporation Studies Using *N*-Acylmannosamine Analogues, *N*-Glycolylneuraminic Acid, and ^{13}C-Labeled *N*-Acetylneuraminic Acid†

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ABSTRACT: *Haemophilus ducreyi* is a Gram-negative bacterium that causes chancroid, a sexually transmitted disease. Cell surface lipooligosaccharides (LOS) of *H. ducreyi* are thought to play important biological roles in host infection. The vast majority of *H. ducreyi* strains contain high levels of sialic acid (*N*-acetylneuraminic acid, NeuAc) in their LOS. Here we investigate the biosynthetic origin of *H. ducreyi* sialosides by metabolic incorporation studies using a panel of *N*-acylmannosamine and sialic acid analogues. Incorporation of sialosides into LOS was assessed by matrix-assisted laser desorption and electrospray ionization mass spectrometry. A Fourier transform ion cyclotron resonance mass spectrometer provided accurate mass measurements, and a quadrupole time-of-flight instrument was used to obtain characteristic fragment ions and partial carbohydrate sequences. Exogenously supplied *N*-acetylmannosamine analogues were not converted to LOS-associated sialosides at a detectable level. In contrast, exogenous ^{13}C-labeled *N*-acetylneuraminic acid ([^{13}C]NeuAc) and *N*-glycolylneuraminic acid (NeuGc) were efficiently incorporated into LOS in a dose-dependent fashion. Moreover, approximately 1.3 μM total exogenous sialic acid was sufficient to obtain about 50% of the maximum production of sialic acid-containing glycoforms observed under in vitro growth conditions. Together, these data suggest that the expressed levels of sialylated LOS glycoforms observed in *H. ducreyi* are in large part controlled by the exogenous concentrations of sialic acid and at levels one might expect in vivo. Moreover, these studies show that to properly exploit the sialic acid biosynthetic pathway for metabolic oligosaccharide engineering in *H. ducreyi* and possibly other prokaryotes that share similar pathways, precursors based on sialic acid and not mannosamine must be used.

The Gram-negative human mucosal pathogen *Haemophilus ducreyi* is one of the principal causes of genital ulcer disease (chancroid). Although this sexually transmitted infection is now uncommon in the United States, outbreaks continue in some urban and regional areas and may be systematically underreported (*1*). In contrast, it is prevalent in many developing countries worldwide (*2*) where it has also been shown to be a significant risk factor for transmission of human immunodeficiency virus (HIV) (*3*−*5*). Recent evidence has also pointed to the emergence of antibiotic resistant strains (*2*, *6*).

Outer-membrane lipooligosaccharides (LOS) are considered to be a major virulence factor for *H. ducreyi* and have been implicated in the adherence and invasion of human foreskin fibroblasts and keratinocytes (*7*, *8*). The structures of LOS from several *H. ducreyi* strains have been reported in recent years (*9*−*11*), revealing that the principal cell surface glycoforms expressed by the majority of wild-type strains terminate in the disaccharide *N*-acetyllactosamine (LacNAc),[1] and to a lesser extent lactose. In addition, the terminating galactose residues of these disaccharides are often modified with sialic acid (*N*-acetylneuraminic acid, NeuAc),

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[1] Abbreviations: ESI-MS, electrospray ionization mass spectrometry; LacNAc, *N*-acetyllactosamine; O-LOS, O-deacylated lipooligosaccharide; MS/MS, tandem mass spectrometry; P, phosphate; PEA, phosphoethanolamine; PEP, phosphoenolpyruvate; MALDI, matrix-assisted laser desorption/ionization; ManNAc, *N*-acetylmannosamine; d_3-ManNAc, d_3-*N*-acetylmannosamine; ManPent, *N*-pentanoylmannosamine; ManLev, *N*-levulinoylmannosamine; $(OAc)_4$-d_3-ManNAc, 1,3,4,6-tetra-*O*-acetyl-*N*-acetyl-d_3-D-mannosamine; $(OAc)_4$-ManPent, 1,3,4,6-tetra-*O*-acetyl-*N*-pentanoyl-D-mannosamine; $(OAc)_4$-ManLev, 1,3,4,6-tetra-*O*-acetyl-*N*-levulinoyl-D-mannosamine; [^{12}C]NeuAc, natural *N*-acetylneuraminic acid; [^{13}C]NeuAc, ^{13}C-labeled *N*-acetylneuraminic acid (C1 position); NeuGc, *N*-glycolylneuraminic acid.

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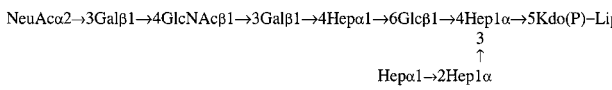
forming sialyl-*N*-acetyllactosamine or sialyllactose (*12*). Although recent evidence has identified a competing biosynthetic pathway that adds additional repeating units of *N*-acetyllactosamine instead of sialic acid (*13*), the major route observed during growth in vitro is the addition of sialic acid. Indeed, a comparative study among several strains that contain LOS glycoforms with *N*-acetyllactosamine has shown that between 30 and 50% are modified with sialic acid (*12*):



Sialylated glycoconjugates have attracted significant attention as targets for biosynthetic engineering. In mammalian cells, unnatural *N*-acylmannosamine derivatives have been shown to undergo metabolic conversion into their corresponding unnatural sialic acids, which are then incorporated into cell surface sialosides (*14*−*17*). Even functionalized mannosamine derivatives bearing an unnatural ketone moiety such as *N*-levulinoylmannosamine (ManLev) have been found to successfully undergo this uptake and metabolic process (*18*−*20*). Once introduced, appropriately functionalized sialic acid derivatives have been shown to serve as chemoselective reaction sites. For example, if these derivatives contain a reactive moiety not normally found on the cell surface, such as a keto (ManLev) or azido group (*21*), they can be selectively modified with exogenous reagents. Moreover, unnatural sialic acids in the context of polysaccharides can be highly immunogenic (*22*). Thus, incorporation of unnatural sialic acids into bacterial LOS might promote immunological recognition and bacterial destruction.

In bacteria, a different biosynthetic pathway exists for the synthesis of sialic acid prior to its introduction into various excreted exopolysaccharides or cell surface lipopolysaccharides. As shown in Figure 1 for *Escherichia coli*, *N*-acetylmannosamine (ManNAc) and phosphoethanol pyruvate (PEP) are condensed by NeuAc synthetase to yield sialic acid (NeuAc) (*23*). Subsequently, the enzyme cytidine 5′-monophosphate-*N*-acetylneuraminic acid (CMP-NeuAc) synthetase catalyzes the formation of the nucleoside sugar CMP-NeuAc from sialic acid and CTP. Finally, a sialyltransferase is needed to transfer the sialic acid residue from CMP-NeuAc onto a hydroxyl group of a carbohydrate acceptor molecule.

In mammals, a sialic acid biosynthesis involves the condensation of *N*-acetylmannosamine 6-phosphate (ManNAc-6-P) with phosphoethanolpyruvate (PEP) to give *N*-acetylneuraminic acid 9-phosphate (NeuAc-9-P), which is then dephosphorylated (*24*). Recently, an alternative to the pathway for *E. coli* was proposed by Vimr et al. (*25*) for *Haemophilus influenzae*. In these studies, the sialylation of LOS in *H. influenzae* was shown to require an exogenous source of sialic acid. In a separate study on human UDP-GlcNAc 2-epimerase-deficient cells, Keppler et al. (*26*) discussed evidence for direct uptake of sialic acid from serum. Therefore, it seems possible that at least two independent mechanisms exist for the synthesis of sialosides: one involving mannosamine as a precursor and the other involving the direct transport of exogenous sialic acid into the organism, but both involving activation to the nucleoside sugar by CMP-NeuAc synthetase.

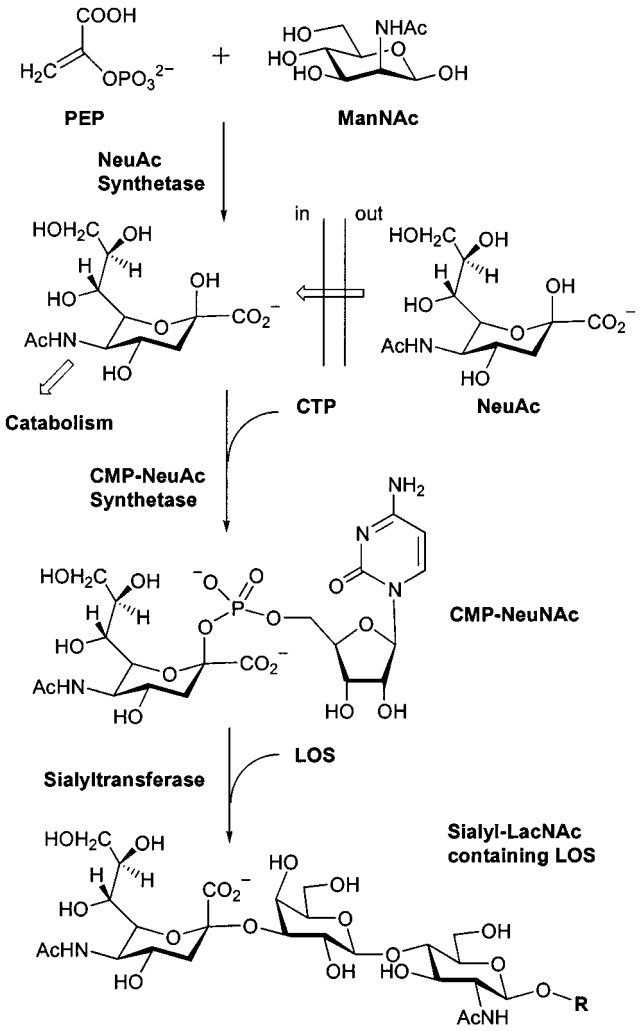


FIGURE 1: Proposed biosynthetic pathways leading to the formation of sialylated lipooligosaccharides in bacteria. In *E. coli*, sialic acid is synthesized endogenously from ManNAc and PEP. Alternatively, a pathway has been proposed for *H. influenzae* involving the direct uptake of sialic acid via a permease (*25*, *41*). Once formed, sialic acid (NeuAc) is converted to its corresponding nucleoside sugar donor (CMP-NeuAc) via CMP-NeuAc synthetase, and then finally transferred to the appropriate acceptor sugar by a specific sialyltransferase. In this case, sialic acid is shown to form the trisaccharide, α2−3-sialyl-*N*-acetyllactosamine, the terminal portion of the major sialylated LOS glycoform of *H. ducreyi*. The R group attached to this trisaccharide refers to the remainder of the LOS molecule.

In this work, we have investigated the ability of *H. ducreyi* to transport, metabolically convert, and incorporate several exogenously supplied mannosamine derivatives and/or sialic acid analogues into sialylated LOS products. We have taken advantage of the high expression levels of these sialylated LOS glycoforms to examine their structures directly using mass spectrometry. Deuterium-labeled d_3-*N*-acetylmannosamine and its more hydrophobic tetra-*O*-acetyl analogue were employed to investigate their utility as substrates and their transport mechanism into cells. Similarly, we used ^{13}C-labeled sialic acid ([^{13}C]NeuAc) and the *N*-glycolylneuraminic acid (NeuGc) to determine if direct importation of these sugars was possible. In both cases, LOS were isolated from these bacteria under various growth conditions (with and without added sugars) and subjected to direct composition analysis and a series of mass spectrometric techniques

capable of accurate mass assignment and sequence determination. Taken together, these studies indicate that *H. ducreyi* directly imports sialic acid (or the structurally related *N*-glycolylneuraminic acid) for incorporation into LOS, and does not utilize *N*-acetylmannosamine even when supplied in a form that would not require a permease. Moreover, the concentration required for an efficient sialylation process was similar to that expected in a human host.

EXPERIMENTAL PROCEDURES

Materials

N-Acetylneuraminic acid, *N*-glycolylneuraminic acid, anhydrous hydrazine, and hemin chloride were obtained from Sigma (St. Louis, MO). *N*-Acetyl[1-^{13}C]neuraminic acid was purchased from Omicron Biochemicals Inc. (South Bend, IN). GC Medium Base, brain heart infusion, and hemoglobin were obtained from Difco (Detroit, MI). Inactivated fetal bovine serum (FBS) was obtained from the Cell Culture Facility at the University of California, San Francisco. IsoVitaleX and Petri dishes were purchased from Becton Dickinson (Franklin Lakes, NJ). All reagents used in chemical syntheses were obtained from commercial suppliers and used without further purification unless otherwise noted. Mannosamine hydrochloride was purchased from Pfansteihl Laboratories (Waukegan, IL). Acetic anhydride-d_6 was obtained from Cambridge Isotope Laboratories (Andover, MA). Flash chromatography was performed using 230−400 mesh silica gel 60. Tetrahydrofuran was dried over sodium benzophenone. Distilled H_2O was used in all manipulations. *J* values are given in hertz.

Methods

Characterization of Synthetic Substrates. The ^{1}H and ^{13}C NMR spectra for the synthetic sugars were obtained on a Bruker DRX-500 spectrometer. High-resolution fast atom bombardment (FAB^+) mass spectra were obtained from the Mass Spectrometry Facility, at the University of California, Berkeley. In some cases, lithium salts were added to increase ionization efficiencies.

Synthesis of N-Acylmannosamine Derivatives. (1) N-Acetyl-d_3-D-mannosamine (d_3-ManNAc). To a solution of mannosamine hydrochloride (0.24 g, 1.1 mmol) in 10 mL of H_2O was added 0.2 mL of triethylamine (1.4 mmol). After the solution had been stirred for 15 min, 0.13 mL of d_6-Ac_2O (1.3 mmol) was added and the reaction mixture was stirred at room temperature for 12 h under a N_2 atmosphere. The solution was concentrated in vacuo and subsequently purified by silica gel chromatography, eluting with a gradient from a 20:1 to 5:1 $CHCl_3$/MeOH mixture to give 0.19 g (75%) of a white solid: ^{1}H NMR (500 MHz, D_2O) δ 3.27 (ddd, 1H, J = 2.2, 4.9, 9.9), 3.37 (app t, 1H, J = 9.8), 3.47 (app t, 1H, J = 9.5), 3.64−3.76 (m, 6H), 3.90 (dd, 1H, J = 4.7, 9.9), 4.17 (dd, 1H, J = 1.4, 4.6), 4.30 (dd, 1H, J = 1.4, 4.4), 4.87 (d, 1H, J = 1.6), 4.97 (d, 1H, J = 1.4); ^{13}C NMR (125 MHz) δ 8.12, 16.68, 53.07, 53.94, 57.33, 60.28, 66.39, 66.66, 68.74, 71.88, 71.94, 76.22, 92.87, 93.00, 174.71, 175.63; HRMS (FAB^+) calcd for $C_8H_{13}D_3NO_6$ $(M + H)^+$ 225.1166, found 225.1170.

(2) 1,3,4,6-Tetra-O-acetyl-N-acetyl-d_3-D-mannosamine [(OAc)$_4$-d_3-ManNAc]. To a solution of mannosamine hydrochloride (1.0 g, 4.8 mmol) in 25 mL of pyridine was added 0.5 mL of d_6-Ac_2O (5.3 mmol). After the reaction mixture had been stirred at room temperature for 12 h under a N_2 atmosphere, 15 mL of Ac_2O was added and the reaction mixture was stirred for an additional 12 h. The solution was concentrated in vacuo to give a clear yellow syrup. Purification of the crude product by silica gel chromatography eluting with a 1:2 hexanes/ethyl acetate mixture yielded 1.3 g (68%) of a white foam. α-Anomer: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.95 (s, 3H), 2.00 (s, 3H), 2.04 (s, 3H), 2.05 (s, 3H), 3.76 (ddd, 1H, J = 2.5, 5.48, 9.3), 4.03 (dd, 1H, J = 2.4, 12.4), 4.22 (dd, 1H, J = 5.5, 12.4), 4.72 (ddd, 1H, J = 1.7, 3.9, 9.2), 5.00−5.09 (m, 2H), 5.81 (d, 1H, J = 1.7), 6.02 (d, 1H, J = 9.2); ^{13}C NMR (125 MHz) δ 20.75, 20.80, 20.84, 49.54, 62.18, 65.49, 71.36, 73.47, 90.78, 168.50, 169.86, 170.17, 170.65, 170.86. β-Anomer: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.95 (s, 3H), 2.00 (s, 3H), 2.03 (s, 3H), 2.12 (s, 3H), 3.95−4.01 (m, 2H), 4.21 (dd, 1H, J = 5.3, 12.3), 4.59 (ddd, 1H, J = 1.7, 4.5, 9.2), 5.13 (app t, 1H, J = 10.2), 5.27 (dd, 1H, J = 4.5, 10.2), 5.95 (d, 1H, J = 1.5), 6.43 (d, 1H, J = 9.2); ^{13}C NMR (125 MHz) δ 20.72, 20.79, 20.93, 49.34, 62.40, 65.76, 68.92, 70.19, 91.85, 168.37, 169.94, 170.16, 170.67, 170.77; HRMS (FAB^+) calcd for $C_{16}H_{20}D_3LiNO_{10}$ $(M + Li)^+$ 399.1670, found 399.1674.

(3) N-Pentanoyl-D-mannosamine (ManPent). To 0.5 g (2.3 mmol) of mannosamine hydrochloride was added 2.3 mL of a 1 M solution of NaOMe in MeOH. The reaction mixture was stirred for 15 min at room temperature after which it was diluted with 2.3 mL of MeOH followed by the addition of 0.55 mL (2.8 mmol) of valeric anhydride. After the solution had been stirred for 6 h, the reaction mixture was concentrated in vacuo to give a dark yellow solid. Purification of the crude product by silica gel chromatography eluting with a gradient from a 20:1 to 10:1 $CHCl_3$/MeOH mixture yielded 0.42 g (69%) of a white solid: ^{1}H NMR (500 MHz, D_2O) δ 0.84 (t, 3H, J = 7.4), 0.85 (t, 3H, J = 7.4), 1.28 (m, 4H), 1.53 (m, 4H), 2.27 (app t, 2H, J = 7.5), 2.31 (app t, 2H, J = 7.6), 3.36 (ddd, 1H, J = 2.2, 4.9, 9.9), 3.47 (app t, 1H, J = 9.8), 3.58 (app t, 1H, J = 9.4), 3.74−3.86 (m, 6H), 4.01 (dd, 1H, J = 4.7, 9.8), 4.28 (dd, 1H, J = 1.3, 4.6), 4.40 (dd, 1H, J = 1.3, 4.4), 4.98 (d, 1H, J = 1.6), 5.06 (d, 1H, J = 1.3); ^{13}C NMR (125 MHz) δ 12.93, 12.95, 21.51, 21.60, 27.49, 35.29, 35.48, 52.99, 53.83, 60.26, 66.36, 66.61, 68.67, 71.89, 76.23, 92.86, 93.11, 177.99, 178.84; HRMS (FAB^+) calcd for $C_{11}H_{22}NO_6$ $(M + H)^+$ 264.1447, found 264.1452.

(4) 1,3,4,6-Tetra-O-acetyl-N-pentanoyl-D-mannosamine [(OAc)$_4$-ManPent]. Following the procedure described above, 1.5 g (7.0 mmol) of mannosamine hydrochloride was converted into ManPent without purification. The crude compound was then treated with 30 mL of a 2:1 Pyr/Ac_2O mixture and stirred at room temperature for 12 h. The reaction mixture was concentrated in vacuo, washed with 1 M HCl (2 × 30 mL) and saturated $NaHCO_3$ (1 × 30 mL), and dried over Na_2SO_4. Purification by silica gel chromatography eluting with a 3:2 hexanes/ethyl acetate mixture provided 2.0 g (66%) of a white foam: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.92 (m, 6H), 1.35−1.41 (m, 4H), 1.59−1.66 (m, 4H), 1.98 (s, 3H), 1.99 (s, 3H), 2.5 (app s, 6H), 2.08 (s, 3H), 2.09 (s, 3H), 2.16 (s, 3H), 2.23−2.30 (m, 4H), 3.79 (ddd, 1H, J = 2.4, 5.1, 9.6), 4.01−4.05 (m, 2H), 4.08 (dd, 1H, J = 2.3, 12.5), 4.25 (app t, 1H, J = 5.2), 4.28 (app t, 1H, J = 5.3), 4.65 (ddd, 1H, J = 1.7, 4.4, 9.3), 4.77 (ddd,

1H, $J = 1.6, 3.9, 9.0$), 5.03 (dd, 1H, $J = 4.0, 9.9$), 5.11 (app t, 1H, $J = 9.9$), 5.16 (app t, 1H, $J = 10.2$), 5.31 (dd, 1H, $J = 4.4, 10.2$), 5.77 (dd, 1H, $J = 9.3, 12.1$), 5.84 (d, 1H, $J = 1.6$), 6.00 (d, 1H, $J = 1.6$); ^{13}C NMR (125 MHz) δ 13.94, 13.95, 20.83, 20.85, 20.87, 20.89, 20.92, 21.04, 22.30, 22.39, 27.89, 28.06, 36.49, 36.69, 49.22, 49.53, 62.08, 62.23, 65.39, 65.59, 69.09, 70.27, 71.57, 73.59, 90.85, 91.94, 168.36, 168.49, 169.86, 170.19, 170.28, 170.70, 173.37, 173.96; HRMS (FAB$^+$) calcd for $C_{19}H_{29}LiNO_{10}$ $(M + Li)^+$ 438.1952, found 438.1943.

(5) N-Levulinoyl-D-mannosamine (ManLev). To a solution of 2.6 mL of triethylamine (18.5 mmol) in 56 mL of anhydrous THF was added 1.9 mL of levulinic acid (18.5 mmol). After the solution had been stirred for 15 min at room temperature under a N_2 atmosphere, 2.4 mL of isobutyl chloroformate (18.5 mmol) was added dropwise by a syringe. The reaction mixture was stirred for 3 h during which time a white precipitate formed. The levulinic acid isobutyl carbonic anhydride was used in the next step without further purification. To a solution of 3.6 g of mannosamine hydrochloride (16.7 mmol) in 112 mL of a 1:1 H_2O/THF mixture was added 3.1 mL of triethylamine (21.8 mmol). The solution was stirred for 15 min at room temperature after which the levulinic acid isobutyl carbonic anhydride was added dropwise by an addition funnel. After being stirred for 36 h at room temperature under a N_2 atmosphere, the reaction mixture was concentrated in vacuo followed by cation and anion exchange (Bio-Rad model AG501-X8) chromatography eluting with H_2O. Further purification by silica gel chromatography eluting with a gradient from a 20:1 to 5:1 $CHCl_3$/MeOH mixture provided 3.6 g (77%) of a light yellow solid: ^{1}H NMR (500 MHz, D_2O) δ 2.17 (s, 3H), 2.18 (s, 3H), 2.49−2.52 (m, 2H), 2.57 (app t, 2H, $J = 6.7$), 2.79−2.86 (m, 4H), 3.35 (ddd, 1H, $J = 2.3, 5.0, 9.9$), 3.47 (app t, 1H, $J = 9.8$), 3.57 (app t, 1H, $J = 9.5$), 3.73−3.85 (m, 6H), 3.98 (dd, 1H, $J = 4.7, 9.8$), 4.25 (dd, 1H, $J = 1.5, 4.7$), 4.39 (app d, 1H, $J = 1.4, 4.4$), 4.96 (d, 1H, $J = 1.6$), 5.05 (d, 1H, $J = 1.4$); ^{13}C NMR (125 MHz) δ 29.05, 29.09, 29.20, 38.01, 38.08, 53.05, 53.94, 60.29, 60.30, 66.39, 66.66, 68.69, 71.87, 71.95, 76.22, 92.89, 93.01, 175.69, 176.48, 213.91, 214.11; HRMS (FAB$^+$) calcd for $C_{11}H_{20}NO_7$ $(M + H)^+$ 278.1240, found 278.1241.

(6) 1,3,4,6-Tetra-O-acetyl-N-levulinoyl-D-mannosamine [(OAc)$_4$-ManLev]. ManLev was synthesized from 1.5 g of mannosamine hydrochloride (7.0 mmol) following the procedure described above. Acetylation of the crude compound was accomplished by adding 40 mL of a 2:1 Pyr/Ac_2O mixture and stirring for 12 h at room temperature. The reaction mixture was concentrated in vacuo, washed with 1 M HCl (2 × 30 mL) followed by saturated $NaHCO_3$ (1 × 30 mL), and dried over Na_2SO_4. Purification of the crude compound by silica gel chromatography eluting with a 2:1 hexanes/ethyl acetate mixture yielded 2.1 g (66%) of a faint yellow foam: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.98 (s, 3H), 1.99 (s, 3H), 2.49 (s, 3H), 2.05 (s, 3H), 2.12 (s, 3H), 2.13 (s, 3H), 2.14 (s, 3H), 2.16 (s, 3H), 2.20 (s, 3H), 2.21 (s, 3H), 2.40−2.45 (m, 2H), 2.58−2.64 (m, 2H), 2.75−2.86 (m, 4H), 3.79 (ddd, 1H, $J = 2.7, 4.9, 9.3$), 4.01−4.04 (m, 2H), 4.06 (dd, 1H, $J = 2.2, 12.4$), 4.12 (dd, 1H, $J = 2.4, 12.2$), 4.29 (dd, 1H, $J = 4.6, 12.4$), 4.59 (ddd, 1H, $J = 1.7, 4.3, 9.3$), 4.73 (ddd, 1H, $J = 1.6, 3.9, 9.3$), 5.01 (dd, 1H, $J = 4.1, 9.6$), 5.10 (app t, 1H, $J = 9.5$), 5.16 (app t, 1H, $J = 10.1$), 5.28 (dd, 1H, $J = 4.4, 10.1$), 5.85 (d, 1H, $J = 1.4$), 6.03 (d, 1H, $J = 1.5$), 6.21 (d, 1H, $J = 9.1$), 6.27 (d, 1H, $J = 9.3$); ^{13}C NMR (125 MHz) δ 20.85, 20.88, 20.90, 20.94, 20.99, 21.07, 30.01, 30.19, 30.25, 38.98, 49.36, 62.05, 62.10, 65.40, 65.54, 69.27, 70.34, 71.47, 73.54, 90.77, 91.94, 168.37, 169.74, 170.22, 170.94, 172.55, 208.02; HRMS (FAB$^+$) calcd for $C_{19}H_{27}LiNO_{11}$ $(M + Li)^+$ 452.1744, found 452.1743.

Determination of the Free N-Acetylneuraminic Acid Concentration. The concentration of NeuAc in the medium was determined by a fluorometric assay using a highly sensitive reagent 1,2-diamino-4,5-methylenedioxybenzene (DMB) that is specific for α-keto acids (*27*, *28*). Fluorescence spectra were recorded with a model LS50B luminescence spectrometer (Perkin-Elmer); emission maxima were recorded at 448 nm (excitation at 373 nm). A calibration curve was obtained using commercial NeuAc as the standard (linear range of 62.50−833.35 pmol/mL, coefficient $R = 0.99918$).

Metabolic Incorporation Studies. To investigate the incorporation efficiencies of exogenous sugars into sialylated LOS glycoforms, *H. ducreyi* strain 35000 was grown in liquid and/or solid media in the presence and absence of various sugar substrates. The medium for solid chocolate agar plates contained GC Medium Base, 1% (w/v) hemoglobin, and 1% (v/v) IsoVitaleX (*29*). This medium was first autoclaved, cooled to 45 °C, and subsequently poured into Petri dishes to obtain regular chocolate agar plates. To add exogenous sugars to the medium, the various neuraminic acid substrates were dissolved in water, sterile filtered, and added to the autoclaved and cooled medium (45 °C), just prior to pouring the Petri plates. Usually, a total volume of 10 mL of medium containing the test sugar was prepared, yielding substrate concentrations ranging from 0.0001 to 5 mM. Bacteria were grown directly on these chocolate agar plates containing the various sugars in a candle jar apparatus at 34 °C for 2 days. As controls, bacteria were also cultivated without adding sugar substrates. The medium for liquid cultures contained brain heart infusion broth supplemented with 5% inactivated fetal bovine serum (FBS) and 0.0025% (w/v) hemin chloride solution (predissolved in 20 mM NaOH), and 1% IsoVitaleX was used for growth of *H. ducreyi* in liquid media (*29*) (as will be noted later, this growth medium contains approximately 0.5 μM NeuAc, presumably originating from the IsoVitaleX supplement). NeuAc and mannosamine derivatives, respectively, were dissolved in water, sterile filtered, and added to the prepared liquid medium to yield a total volume of 10 mL (substrate concentrations of 0.1−10 mM). *H. ducreyi* were grown in broth containing mannosamine or sialic acid substrates using an incubator shaker at 34 °C and 250 rpm (2 days). OD values were monitored at λ values of 600 and 650 nm.

Extraction of Lipooligosaccharides (LOS). To prepare a partially purified LOS fraction, a phenol extraction micromethod was used. In brief, harvested bacterial cells obtained from either broth or plates were first washed with 0.5−1 mL of saline PBS (pH 7.4) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The washed cells were resuspended in 0.5 mL of H_2O and transferred into a heat-resistant Eppendorf tube. An equal volume of a 90% phenol/H_2O solution (w/v) was added, and the tube was heated at 65 °C for 35 min (vigorous stirring every 10 min) (*30*). Subsequently, the mixture was centrifuged at 4 °C (8500*g*) for 30 min. Both

phases were separated, and the phenol phase was extracted with 0.5 mL of H_2O. The combined water extracts were concentrated to a volume of 100 μL. The LOS were precipitated by addition of 900 μL of ethanol and cooling at −20 °C overnight. The LOS pellet was obtained by centrifugation at 4 °C (8500*g*) for 45 min.

Preparation of O-Deacylated and HF-Treated LOS. O-Deacylated LOS are generally more amenable to mass spectrometric analysis (*9*). The dried LOS were O-deacylated by incubation with 50 μL of anhydrous hydrazine at 37 °C for 35 min (*31*). The mixture was cooled, and chilled acetone (200 μL) was slowly added. The solution was kept at −20 °C for 2 h and then centrifuged at 4 °C (8500*g*) for 45 min. The O-deacylated LOS (O-LOS) precipitate was separated from the supernatant, dried, and finally redissolved in 20 μL of H_2O. Aliquots of the sample were desalted by drop dialysis using nitrocellulose membranes (20 μm, Millipore, Bedford, MA). To remove phosphate and phosphoesters, O-deacylated LOS were treated with cold 48% aqueous HF (10 μg/μL solution) for 13 h at 4 °C. Details of this procedure can be found elsewhere (*10*).

Mass Spectrometric Characterization of LOS. Mass spectra were obtained for all LOS samples by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on a Voyager DE spectrometer (PE Biosystems, Framingham, MA). The instrument was equipped with a nitrogen laser (337 nm) and run under delayed extraction conditions (*9*): delay times of 100 ns (Voyager DE) and 200−350 ns (Voyager DESTR) and grid voltage that was 92−94% of the full acceleration voltage (20−30 kV). Mass spectra were run in the negative-ionization mode. The obtained mass spectra were externally calibrated with an equimolar mixture of angiotensin II, bradykinin, LHRH, bombesin, α-MSH, and ACTH 1-24 (CZE mixture, Bio-Rad). All samples were prepared using a 320 mM 2,5-dihydroxybenzoic acid (DHB) solution in a 4:1 (v/v) acetone/water mixture containing 175 mM 1-hydroxyisoquinoline (HIC) as first reported for underivatized oligosaccharides (*32*). In all cases, 1 μL of analyte (0.1−1 μg of material) was mixed with 1 μL of matrix solution, desalted with cation exchange resin beads (DOWEX 50X, NH_4^+) (*33*), and then air-dried at room temperature on a stainless steel target. Typically, 20−50 laser shots were used to record each linear spectrum.

For high-resolution exact mass measurements of selected LOS and to better determine the level of the incorporation of [^{13}C]NeuAc, an APEX II Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, MA), equipped with a 7 T actively shielded superconducting magnet, was used (*34*). For these experiments, solutions of the O-deacylated LOS were prepared for MS analysis at a concentration of 1−10 pmol/μL in a 60:40 acetonitrile/water mixture, and ions were generated with a home-built nanospray source using metal-coated microtip pipets (World Precision Instruments, Sarasota, FL). The ions externally accumulated for 2 s in an RF-only hexapole ion guide before being transferred to the ICR cell for mass analysis. Before Fourier transformation, a Gaussian function was applied to the transient, which was composed of 30−70 scans each containing 512K data points. These spectra were acquired in the negative-ion mode using a Bruker Daltonics data station operating XMASS version 5.0.6 and were internally calibrated using well-characterized glycoforms within the LOS mixture.

To determine the terminal sequences of modified LOS structures, samples were further analyzed under collision activation conditions on a quadrupole orthogonal time-of-flight mass spectrometer (QSTAR, PE Sciex, Concorde, ON) equipped with a Protana nanospray ion source. The QSTAR analyzer consists of a high-pressure RF-only ion guide followed by a quadrupole mass filter, a high-pressure quadrupole collision cell, and a reflectron TOF mass analyzer with an effective flight path of 2.5 m. Samples were dissolved in a 1:1 solution of 100 mM NH_4OAc (pH 4.5) and acetonitrile and loaded into a Protana nanospray tip. The nanospray needle voltage was typically 1200−1300 V. Mass spectra (ESI-MS) and tandem mass spectra (ESI-MS/MS) of O-deacylated LOS and HF-treated O-deacylated LOS were recorded in the positive-ion mode. In the MS mode, a resolution of 8000−10000 fwhm was achieved which allowed unambiguous determination of the charge state and isotope distribution pattern. For CID-MS/MS studies, the mass window of the quadrupole mass analyzer was set to ±1 Da to select precursor ions for fragmentation. For studies that relied on the isotope distribution pattern of MS/MS fragment ions, the mass window was adjusted to include the total molecular ion isotope cluster. The selected ions were fragmented in a collision cell using air as the collision gas and analyzed in the orthogonal TOF instrument operating at an acceleration potential of 20 kV. Spectra were externally calibrated using MS/MS fragment ions of a glufibrinogen peptide standard (*m*/*z* 187.0719 and 1285.4995), providing a mass accuracy of ±50 ppm, whereas internal calibration gave masses accurate to ±5 ppm.

Computer Programs. The isotope pattern calculation program Isotope version 1.6 was used for simulating isotope patterns to determine the [^{13}C]NeuAc incorporation efficiency (L. Arnold, University of Waikato, Waikato, New Zealand). The *m* over *z* program (version 8.6 for Windows NT, ProteoMetrics) was applied to MALDI-MS files to quantify the ratio between O-deacylated LOS glycoforms and to determine the overall sialylation level.

RESULTS AND DISCUSSION

It has been recently shown that *N*-acylmannosamine derivatives can be metabolically converted into their corresponding sialic acids and subsequently incorporated into mammalian cell surface glycoconjugates (*16*, *18*, *19*). In this study, we examined the pathogenic bacterium *H. ducreyi* as a model prokaryote system for metabolic oligosaccharide engineering. *H. ducreyi* was chosen for this study since we have previously shown that this human pathogen produces a high concentration of sialylated glycolipids (or LOS) on its surface (*12*), making it a potentially ideal bacterial system for direct characterization of the resulting sialylated products. Furthermore, the ability to modulate sialic acid structures may provide new avenues for vaccine development or immunotherapy.

In the first part of this study, several *N*-acylmannosamine derivatives that have been previously shown to be metabolized by mammalian cells (*16*, *18*) were added to the medium of *H. ducreyi* wild-type strain 35000. These derivatives possessed varying *N*-acyl substitutions, such as *N*-pentanoyl-

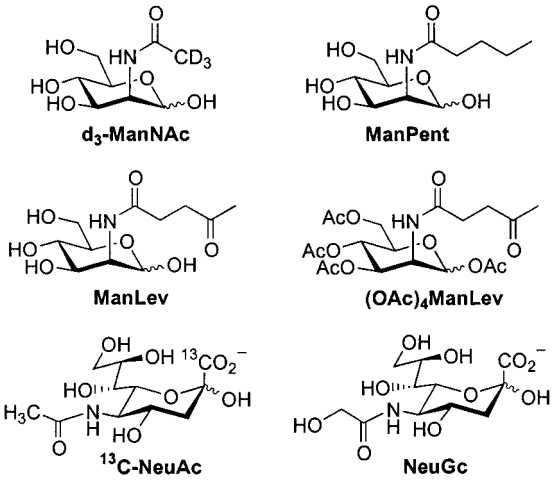


FIGURE 2: Various *N*-acylmannosamine derivatives and *N*-acylneuraminic acid substrates were added to the *H. ducreyi* growth medium. Incorporation studies were performed by adding modified mannosamine derivatives with *N*-acyl groups containing deuterium labels (d_3-ManNAc), alkyl chains (ManPent), and functionalized alkyl chains bearing a ketone group (ManLev and its tetraacetylated analogue, $(OAc)_4$-ManLev. Sialic acid substrates were isotopically labeled [^{13}C]NeuAc and NeuGc.

Table 1: Incorporation Efficiencies of Unnatural Sialic Acids Produced by Metabolism of *N*-Acylmannosamine Precursors

N-acyl Man precursor	concn (mM)	incorporation efficiency (%)[a]
d_3-ManNAc	10	0
$(OAc)_4$-d_3-ManNAc	0.1	0
ManLev	10	<3
$(OAc)_4$-ManLev	0.1	<3
ManPent	10	<3
$(OAc)_4$-ManPent	0.1	<3

[a] Incorporation efficiency is defined as the ratio of the relative peak abundance of modified sialylated LOS to that of total sialylated LOS glycoforms.

mannosamine (ManPent) and *N*-levulinoylmannosamine (ManLev) (Figure 2). After incubation for 2 days, the LOS were isolated from these organisms using standard micro phenol extraction protocols (*30*) and O-deacylated with hydrazine to form water-soluble O-LOS, a form more amenable to mass spectrometric analysis (*9*). Determination of the molecular masses of these O-LOS species by MALDI-MS showed masses that were identical to those found under normal growth conditions without these added sugars; no evidence was found for the presence of any unnatural sialic acid residues (Table 1). Electrospray ionization mass spectrometry (ESI-MS) on a quadrupole orthogonal time-of-flight instrument of these same LOS mixtures also failed to reveal the presence of new LOS species with altered mass values, despite the higher mass accuracy and sensitivity that this latter technique can provide (data not shown).

Given the failure to observe any modified sialic acid-containing LOS glycoforms when ManPent and ManLev were added to the growth medium, we investigated whether *H. ducreyi* was capable of transporting and metabolizing *N*-acetylmannosamine itself. Data from the literature are not conclusive as to whether *Haemophilus* or other bacterial species are capable of transporting ManNAc presursors (*25*). To test this hypothesis, deuterium-labeled d_3-*N*-acetylmannosamine (d_3-ManNAc) was added to the growth medium and the resulting LOS were isolated and analyzed by MALDI-MS and by ESI-MS as before. Any O-LOS glycoforms containing sialic acid originating from d_3-ManNAc would be expected to show a molecular mass shifted 3 Da higher, i.e., $M_{calc} = 3250.10$ versus the natural sialylated O-LOS at $M_{calc} = 3247.10$. If d_3-ManNAc was only partially utilized, the distribution of the natural isotope pattern for the sialic acid would be partially perturbed by d_3-sialic acid and its percent contribution could be calculated using available software. However, LOS isolated from bacteria grown in the presence of d_3-ManNAc also revealed no mass shift and no change in the isotope distribution. To confirm this observation, a tandem mass spectrum (ESI-MS/MS) was obtained by selecting the mass region that would contain both the normal acid and d_3-labeled sialic acid LOS species (if the latter were present), and examining the low-mass region for characteristic fragment ions. Although several ions were observed originating from fragments containing non-labeled sialic acid (e.g., at *m*/*z* 657.3 and 292.1), no ions were seen for the analogous deuterium-labeled species (data not shown).

The failure to observe any metabolic products of exogenous d_3-ManNAc at a wide range of concentrations could be due to the lack of a specific transport system (i.e., permease). To address this possibility, the peracetylated derivative, tetra-*O*-acetyl-d_3-*N*-acylmannosamine [$(OAc)_4$-d_3-ManNAc], was added to the growth medium. This latter hydrophobic derivative would be expected to cross the bacterial membrane via passive transport and then undergo intracellular conversion and O-deacetylation of the sugar to d_3-ManNAc. Esterases cleaving acetylated sugars have been described for mammalian cells (*35*), and there are several putative genes in the *H. ducreyi* genome that encode proteins that are homologous to known esterases and/or lipases (*36*). As summarized in Table 1, none of the mannosamine derivatives, including $(OAc)_4$-d_3-ManNAc, were metabolized by *H. ducreyi* to yield sialic acid-containing LOS, at least at the detection limits of the mass spectrometry analysis (>3% base glycoform peak). The failure of these mannosamine derivatives to show evidence of metabolism would tend to argue against the requirement for an active transport mechanism such as a permease, and rather suggest that *N*-acetylmannosamine is not part of the sialic acid biosynthetic pathway.

Recently, an alternative mechanism for the formation of sialylated LOS based primarily on genetic analysis was suggested for a related organism, *H. influenzae* (*25*, *37*). In this scheme, sialic acid is directly imported from the medium, presumably by a permease, and then either catabolized by a NeuAc aldolase or used to form the activated sugar nucleoside CMP-NeuAc (see Figure 1). To test this possibility, *H. ducreyi* was grown in liquid medium supplemented with *N*-acetyl[1-^{13}C]neuraminic acid at various concentrations. Control experiments were also performed in which no sugar (blank) or native *N*-acetylneuraminic acid ([^{12}C]NeuAc) was added to the growth medium.

LOS from *H. ducreyi* grown with [^{13}C]NeuAc and the two controls were isolated, converted to their O-deacylated forms, and analyzed by MALDI-MS. Under linear conditions, the molecular ions for individual O-LOS species typically appeared as an unresolved isotope cluster whose centroids correspond to the average mass. The deprotonated molecular

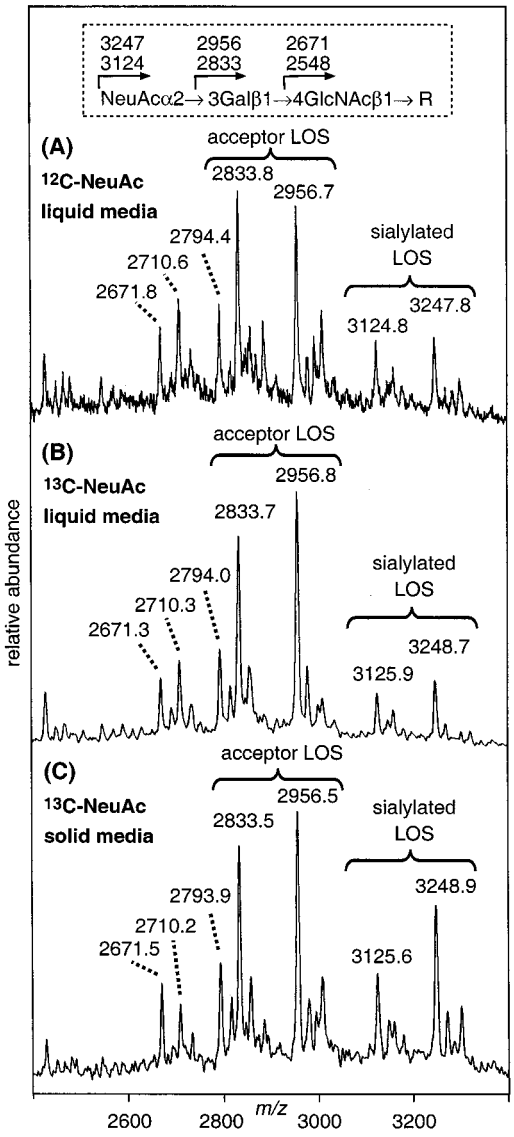


FIGURE 3: Negative-ion MALDI-TOF mass spectrum showing $[M - H]^-$ ions for O-deacylated LOS isolated from *H. ducreyi*. Spectra were recorded in the linear mode yielding average masses. Sialylation of the acceptor LOS was observed under the following growth conditions: (A) liquid medium with addition of 1 mM unlabeled NeuAc, (B) liquid medium with addition of 1 mM [^{13}C]-NeuAc, and (C) solid medium with addition of 1 mM [^{13}C]NeuAc.

ions for O-LOS containing either natural or isotopically labeled [^{13}C]NeuAc are expected to differ by 1 mass unit. For example, unlabeled O-LOS yield ions at *m*/*z* 3247/3124 while those containing [^{13}C]NeuAc are 1 Da higher at *m*/*z* 3248/3125, respectively. MALDI mass spectra of O-LOS isolated from bacteria grown in liquid medium in the presence of 1 mM NeuAc (control) or [^{13}C]NeuAc were recorded and are shown in panels A and B of Figure 3. Deprotonated molecular ion peaks $[M - H]^-$ at *m*/*z* 2956.7 and 2833.8 corresponded to the major wild-type glycoforms terminating in *N*-acetyllactosamine, Gal-GlcNAc-Gal-Hep-Glc-Hep$_3$-(PEA)$_{0,1}$-KdoP(PEA)$_1$-lipid A, substituted with one or two PEA groups (Figure 3A). The ions at *m*/*z* 2794.4 and 2671.8 appear to arise from the additional loss of the terminal galactose residue. Sialylated glycoform species containing native NeuAc were observed at *m*/*z* 3247.8 and 3124.8 (Figure 3A). Figure 3B shows molecular ion peaks $[M - H]^-$ at *m*/*z* 3248.7 and 3125.9, instead indicating LOS glycoforms terminating in ^{13}C-labeled sialyl-*N*-acetyllactosamine. To optimize sialic acid incorporation, different experimental conditions were tested, including growth on solid medium containing added modified sialic acid substrates. Figure 3C shows a MALDI-MS spectrum of O-LOS extracted from *H. ducreyi* grown on solid medium (addition of 1 mM [^{13}C]NeuAc). Two molecular ion peaks ($[M - H]^-$) were observed at *m*/*z* 2956.5 and 2833.5 and were assigned as *N*-acetyllactosamine terminating glycoforms (LacNAc LOS). At even higher masses, the two molecular ions at *m*/*z* 3248.9 and 3125.6 suggested the incorporation of [^{13}C]-NeuAc, since in both cases a mass shift of 292 Da was observed, and not 291 Da (mass shift for unlabeled NeuAc).

Although the MALDI-MS data were an indication of the successful metabolic incorporation of [^{13}C]NeuAc into several LOS glycoforms, the mass resolution and accuracy of these spectra were not sufficient to determine a precise incorporation efficiency of [^{13}C]NeuAc relative to unlabeled sialic acid, the latter of which is present in the solid medium at low concentrations (~0.5 μM). To obtain spectra at a higher resolving power, Fourier transform ion cyclotron resonance (FT-ICR) mass spectra of O-deacylated LOS were recorded in the negative-ion mode under conditions where the molecular ions were isotopically resolved. The LOS isolated from *H. ducreyi* grown in liquid medium (addition of 1 mM [^{12}C]NeuAc) exhibited a triply charged molecular ion peak ($[M - 3H]^{3-}$) at *m*/*z* 1081.3563 that corresponded to a sialylated LOS glycoform (containing two PEA molecules) with the exact mass M_{obs} of 3247.0924 and a mass accuracy of 1.66 ppm (Figure 4A). The observed isotope distribution was identical to an isotope pattern calculated for a LOS species containing unlabeled NeuAc. Mass spectra of O-deacylated LOS isolated from bacteria grown in medium that contained isotopically labeled [^{13}C]NeuAc are presented in Figure 4B (liquid medium) and Figure 4C (solid medium). LOS extracted from *H. ducreyi* grown in liquid medium (addition of 1 mM [^{13}C]NeuAc) revealed a molecular ion isotope distribution pattern of the sialylated LOS glycoform corresponding to the presence of 64% [^{13}C]NeuAc and 36% endogenous [^{12}C]NeuAc (Figure 4B and Table 2). The incorporation efficiency (64%) was defined as the ratio of the relative abundance of the molecular ions for LOS species containing ^{13}C-labeled sialic acid to that of total sialic acid-containing LOS glycoforms (total, [^{13}C]NeuAc + [^{12}C]-NeuAc). When *H. ducreyi* was grown under conditions of low to moderate levels of exogenous [^{13}C]NeuAc, incomplete incorporation of the labeled sialic acid into the various sialylated LOS glycoforms was explained by competition with unlabeled sialic acid present in the liquid medium. Components of liquid and solid media such as fetal bovine serum or IsoVitaleX are known to contain sialic acid, but unlike those for many bacteria, conditions for growing *H. ducreyi* in defined medium without such contaminants have not been determined. Using a highly sensitive fluorometric assay, the solid medium was found to contain the least sialic acid, at a concentration of approximately 0.5 μM. Therefore, solid medium supplemented with various sialic acid derivatives appeared to be the preferred experimental approach. Indeed, under these conditions, high incorporation efficiencies were revealed as determined by FT-ICR mass spectrometry. For example, as shown in Figure 4C, LOS obtained from *H. ducreyi* grown on solid medium (addition of 1 mM

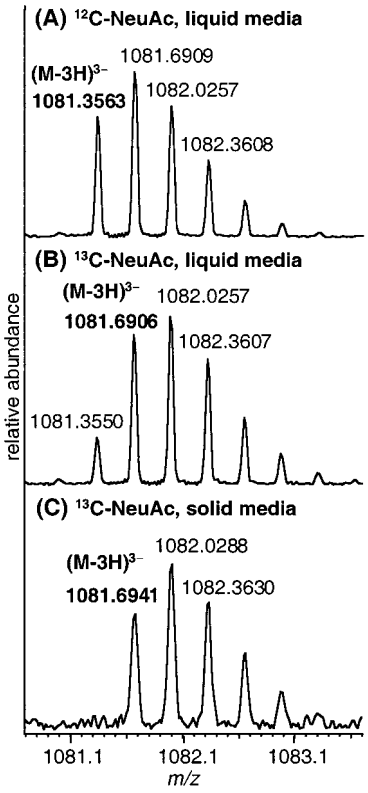


FIGURE 4: High-resolution mass measurement of O-deacylated LOS (FT-ICR mass spectrometer, negative-ion mode). Incorporation of natural NeuAc and [^{13}C]NeuAc into *H. ducreyi* LOS was observed under varying experimental conditions. (A) LOS isolated from bacteria grown in liquid medium (1 mM NeuAc) exhibited a molecular ion ([M − 3H]$^{3-}$) at *m*/*z* 1081.3563 (M_{obs} = 3247.0924). (B) LOS isolated from bacteria grown in solid medium (1 mM [^{13}C]-NeuAc) exhibited a molecular ion ([M − 3H]$^{3-}$) at *m*/*z* 1081.6906 (M_{obs} = 3248.0953). (C) LOS isolated from bacteria grown in solid medium (1 mM [^{13}C]NeuAc) exhibited a molecular ion ([M − 3H]$^{3-}$) at *m*/*z* 1081.6941 (M_{obs} = 3248.1058).

Table 2: Exact Masses of Sialylated O-Deacylated LOS and Incorporation Efficiencies of Unnatural Sialic Acids

precursor	expected M_r (error, ppm)	incorporation efficiency (%)[a]
[^{12}C]NeuAc (broth, 1 mM)	3247.0924 (−1.66)	−
[^{13}C]NeuAc (broth, 1 mM)	3248.0953 (−1.82)	64
[^{12}C]NeuAc (plate, 1 mM)	3247.0918 (−1.08)	−
[^{13}C]NeuAc (plate, 1 mM)	3248.1058 (1.42)	100
NeuGc[b] (plate, 1 mM)	3263.0977 (1.53)	38[b]

[a] Incorporation efficiency is defined as the ratio of the relative peak abundance of modified LOS (containing [^{13}C]NeuAc or NeuGc) to that of total sialylated LOS glycoforms. [b] Commercial NeuGc contained 10% NeuAc. Masses were determined as triply charged ions in the negative-ion mode, [M − 3H]$^{3-}$, (FT-ICR).

[^{13}C]NeuAc) exhibited a triply charged molecular ion peak ([M − 3H]$^{3-}$) at *m*/*z* 1081.6941, which corresponds to a molecular mass M_{obs} of 3248.1058. No isotope peak at *m*/*z* 1081.35 was detected, and the observed isotope distribution clearly showed an incorporation efficiency approaching 100% for [^{13}C]NeuAc. All FT-ICR data obtained from different experimental conditions are summarized in Table 2. The mass accuracy of the observed molecular masses was in a range of ±2−3 ppm compared to calculated masses.

H. ducreyi grown on solid medium showed an increase in the incorporation efficiency and the overall level of sialylation relative to the *N*-acetyllactosamine acceptor LOS (LacNAc LOS) compared to these organisms grown in liquid medium. For example, at 1 mM [^{13}C]NeuAc, MALDI-MS measurements indicated that the level of sialylation of the acceptor LOS is substantially higher for bacteria grown on solid medium (62%, Figure 3C) than for bacteria grown on liquid medium (25%, Figure 3B). A similar observation was previously described for *H. ducreyi* glycosyl knockout mutants (*13*). More importantly, the incorporation efficiency of [^{13}C]NeuAc was found to approach 100% when bacteria were grown on solid medium supplemented with [^{13}C]NeuAc (Figure 4C and Table 2). Given the advantages of solid medium, all subsequent experiments were performed on solid medium under the described optimized experimental conditions using single-plate incubations. Despite the small amount of LOS that could be obtained from single-culture plates, the mass spectrometric methods that were employed were more than adequate for their characterization.

The successful incorporation of exogenous labeled sialic acid confirms that a pathway exists for the exogenous uptake and utilization of this sugar. Given this result, we examined whether other sialic acids could access this same uptake and subsequent incorporation pathway. Tullius et al. (*38*) recently described the enzymatic synthesis of CMP-NeuGc with non-native *N*-glycolylneuraminic acid (NeuGc) under in vitro conditions with *H. ducreyi* CMP-*N*-acetylneuraminic acid synthetase. To test whether *H. ducreyi* would incorporate NeuGc into its LOS, 1 mM NeuGc was added to the solid-phase growth medium. After incubation for 2 days, LOS were isolated, converted to their O-deacylated LOS forms, and analyzed by MALDI-MS and FT-ICR. Under negative-ion conditions, MALDI-MS spectra of the O-LOS glycoforms yielded abundant deprotonated molecular ions at *m*/*z* 3140.7 and 3263.6 (average masses, corresponding to glycoforms containing one and two PEA molecules, respectively). The mass shift of 16 Da relative to the normal sialylated forms (Δm = 3263−3247 Da) corresponds to an additional hydroxyl group in NeuGc compared to NeuAc. FT-ICR analysis of this same sample yielded an exact mass M_{obs} of the O-LOS of 3263.0977 [*m*/*z* 1086.6914 for the triply charged ion ([M − 3H]$^{3-}$)] within 1.53 ppm of the predicted mass (Table 2). The ratio of NeuGc-LOS to total sialylated LOS was determined by FT-ICR, measuring the intensity of the second, most abundant isotope peak of the considered molecular ion species. *N*-Glycolylneuraminic acid was incorporated into the LOS revealing 38% of *N*-glycolyl sialylated LOS and 62% of *N*-acetyl sialylated LOS (i.e., 38% incorporation efficiency for NeuGc). The added substrate NeuGc competed with the native substrate NeuAc, which was estimated to be present in this commercial NeuGc preparation at approximately 10% (w/w).

Up to this point, LOS that contained sialic acids such as [^{13}C]NeuAc and NeuGc had been identified only by their mass and/or isotope distribution. To obtain more detailed structural proof and to determine the precise location of these sialic acids, tandem mass spectra were acquired on their doubly charged partially sodiated molecular ions on a quadrupole time-of-flight instrument. For these experiments, LOS were O-deacylated and then directly analyzed in the positive-ionization mode. The exact mass was measured by ESI-MS, and sialic acid specific B_1 and B_3 fragment ions were obtained by ESI MS/MS. The abundant sialyllactosamine fragment ions (B_3 ions), in particular, clearly

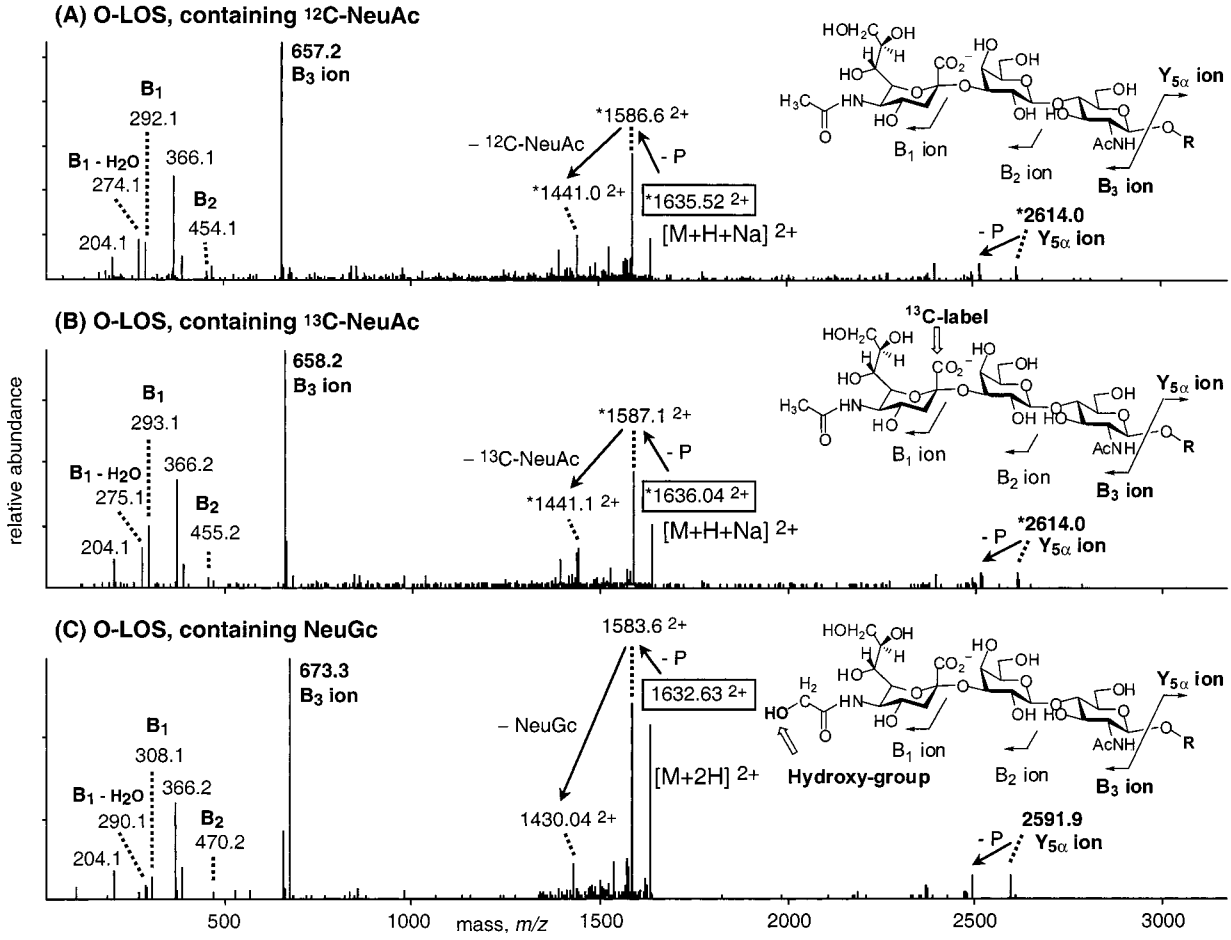


FIGURE 5: Positive-ion ESI-MS/MS spectra of O-deacylated LOS. (A) MS/MS spectrum of [^{12}C]NeuAc containing O-LOS; selected precursor ion ([M + H + Na]$^{2+}$) at *m/z* 1635.52 (M_{obs} = 3247.04). (B) MS/MS spectrum of [^{13}C]NeuAc containing O-LOS; selected precursor ion ([M + H + Na]$^{2+}$) at *m/z* 1636.04 (M_{obs} = 3248.08). (C) MS/MS spectrum of NeuGc containing O-LOS; selected precursor ion ([M + 2H]$^{2+}$) at *m/z* 1632.63 (M_{obs} = 3263.26). Fragment ions marked with asterisks are sodiated. The ion at *m/z* 366.2 was assigned as an internal *N*-acetyllactosamine ion (i.e., Galβ1−4GlcNAc). The ion nomenclature is that proposed by Domon and Costello (*42*).

identified the incorporated sialic acid residues. ESI MS/MS spectra of some sialylated O-LOS are shown in Figure 5. The investigated O-LOS contained either [^{12}C]NeuAc (Figure 5A), [^{13}C]NeuAc (Figure 5B), or NeuGc (Figure 5C), and doubly charged molecular ions were selected as precursor ions and subsequently fragmented. Loss of a phosphate group and subsequent loss of the sialic acid from the precursor ion was observed. Although the fragmentation pattern was not extensive, characteristic nonreducing terminal fragment ions, such as B_1−B_3 ions, were revealed. The major fragmentation site yielded abundant sialyllactosamine fragment ions (B_3 ions, base peak) and the corresponding weaker $Y_{5\alpha}$ ions. The abundant B_3 ions were observed at *m/z* 657.2 for **[^{12}C]-NeuAc**-Gal-GlcNAc (Figure 5A), *m/z* 658.2 for **[^{13}C]NeuAc**-Gal-GlcNAc (Figure 5B), and *m/z* 673.3 for **NeuGc**-Gal-GlcNAc (Figure 5C). Similar to observed B_3 fragment ions, B_1 ions at *m/z* 292.1, 293.1, and 308.1 and the corresponding dehydrated B_1 ions at *m/z* 274.1, 275.1, and 290.1 (B_1 − H_2O ions) can also be used to confirm the incorporation of a modified sialic acid residue. B_2 fragment ions were detected as weak signals at *m/z* 454.1, 455.2, and 470.2.

To improve upon the ionization efficiencies in the positive-ionization mode and to provide a more extensive set of fragment ions, O-deacylated LOS were treated with aqueous HF to remove phosphate and PEA prior to tandem MS analysis. For example, Figure 6 shows an ESI-MS/MS spectrum of an HF-treated sialylated O-LOS glycoform isolated from bacteria grown on solid medium with 1 mM [^{13}C]NeuAc. As sodiated molecular ions have been shown to fragment more efficiently than their protonated analogues, a doubly charged ion [M + H + Na]$^{2+}$ at *m/z* 1393.03 (M_r = 2762.06) was selected as the precursor ion. Although this precursor ion still contained components of the original Lipid A moiety, the majority of the fragment ions were found to be derived from the oligosaccharide moiety. A series of sequence ions was generated that resulted from cleavage of various glycosidic bonds, such as Y ions, e.g., *m/z* 1247.0^{2+}, 2128.1, 1966.0, and 1773.9, and B ion fragments, e.g., *m/z* 293.1, 455.2, and 658.3 (Figure 6, inset). It was recently observed for partially sodiated precursor ions that the positive charge on singly charged B ion fragments was due to protonation, whereas Y ions were sodiated (B. Schilling et al., unpublished data). A preferred fragmentation site next to the *N*-acetylglucosamine residue (GlcNAc) yielded a characteristic trisaccharide B_3 ion at *m/z* 658.3 (sialyllactosamine fragment ion) and the corresponding $Y_{5\alpha}$ ion at *m/z* 2128.1. The observed B_3 ion at *m/z* 658.3 (B_3 − H_2O ion at *m/z* 640.3) and the B_1 ion at *m/z* 293.1 (B_1 − H_2O ion at *m/z* 275.1) were especially significant as they contained [^{13}C]NeuAc. Fragmentation of the glycosidic bond between the Lipid A and the oligosaccharide moiety yielded a series of sodiated oligosaccharide fragments at *m/z* 1992.7, 1700.7,

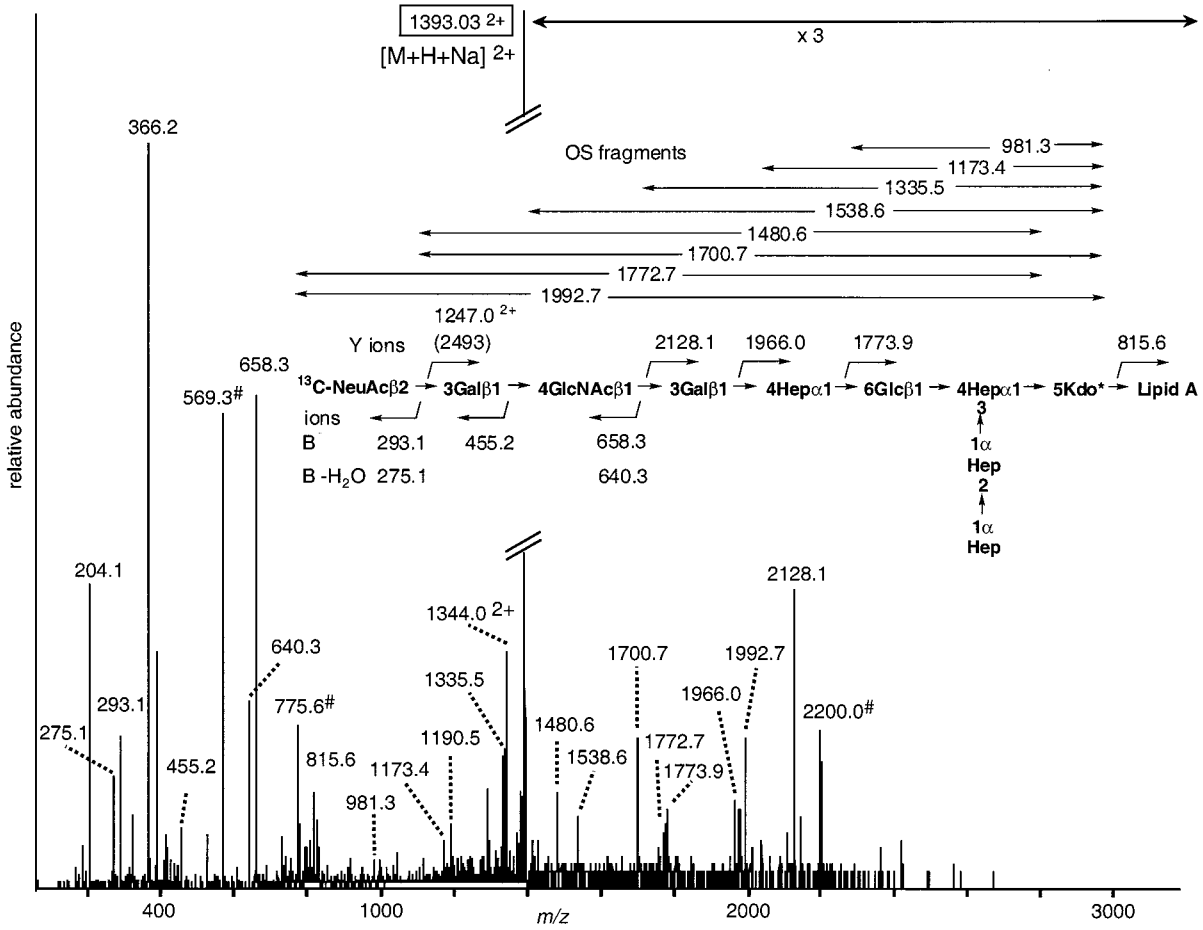


FIGURE 6: Positive-ion ESI-MS/MS spectra of HF-treated O-deacylated LOS with 100% incorporated [^{13}C]NeuAc. The sialylated glycoform was isolated from bacteria grown in solid medium containing 1 mM [^{13}C]NeuAc. Partially sodiated precursor ion ([M + H + Na]$^{2+}$) at *m/z* 1393.03 (M = 2762.06). Observed B-type ions are protonated, whereas Y-type ions and oligosaccharide fragment ions are sodiated. Ions at *m/z* 569.3 and 775.6 have been observed in the MS/MS spectra of structurally related O-LOS glycoforms and likely originate from the conserved core (*m/z* 569.3, sodiated Glc-Hep$_2$) and Lipid A region (*m/z* 775.6, protonated and dehydrated Lipid A). The ion at *m/z* 2200.0 is unassigned.

1538.6, 1335.5, 1173.4, and 981.3. Each of these oligosaccharide fragment ions resulted from a subsequent loss of a carbohydrate residue from its nonreducing terminus confirming the sequence [^{13}C]NeuAc→Gal→GlcNAc→Gal→Hep→Glc→Hep$_3$Kdo.

Having established a procedure for incorporating modified sialic acid substrates into *H. ducreyi* LOS, we carried out competition experiments using different sialic acid substrates. *H. ducreyi* was grown on plates in the presence of various concentrations of [^{12}C]NeuAc and [^{13}C]NeuAc at a given ratio (Table 3). ESI-MS/MS spectra (QSTAR) of the corresponding sialylated O-deacylated LOS glycoforms in the positive-ionization mode allowed us to analyze the isotopic distribution of the abundant sialyllactosamine B_3 ions (Figure 7). The observed B_3 ion isotope patterns were compared and then matched with simulated isotope distributions calculated for [^{13}C]NeuAc:[^{12}C]NeuAc ratios. The incorporation efficiencies were determined by finding the best match between the observed and simulated isotope distribution and deviated less than 4% from the calculated percentages corresponding to the actual ^{13}C:^{12}C sialic acid concentration in the medium (Table 3).

This so-called "B_3 ion method" was used to monitor a titration experiment in which different concentrations of [^{13}C]-NeuAc where added to solid medium, ranging from 0.0001 to 1 mM. The results are summarized in Table 4 and show that 100% incorporation of an isotopically labeled sialic acid can be obtained by adding substrate concentrations of ≥0.1 mM to solid medium. At lower concentrations, incorporation efficiencies decreased due to competition with endogenous sialic acid (concentration of endogenous [^{12}C]NeuAc of 0.52 μM). We were especially interested in changes of the overall sialylation of the *N*-acetyllactosamine acceptor of LOS and its dependence on the concentration of total available sialic acid (sum of added and contaminating sialic acid from the medium). As depicted in Figure 8, the acceptor LOS retained

Table 3: Efficiency of [^{13}C]NeuAc Incorporation into LOS in the Presence of Varying Concentrations of [^{12}C]NeuAc

[^{13}C]NeuAc (mM)	[^{12}C]NeuAc (mM)	incorporation efficiency (%)[a]	calcd %
1.0	0.0	100	100
1.0	0.1	89	91
1.0	0.5	65	67
1.0	1.0	49	50
0.5	1.0	37	33
0.1	1.0	10	9
0.0	1.0	–	0

[a] Incorporation efficiency is defined as the ratio of the relative peak abundance of [^{13}C]NeuAc-LOS to that of total [^{12}C]- and [^{13}C]NeuAc-LOS glycoforms.

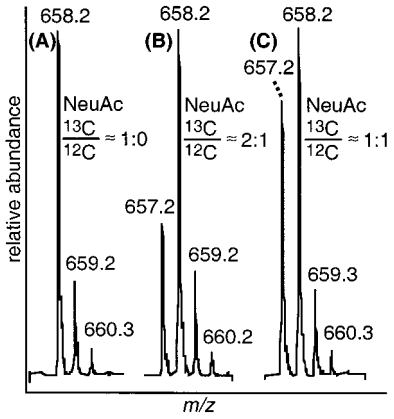


FIGURE 7: Molecular ion isotope pattern of the observed B_3 fragment ion obtained from ESI-MS/MS of sialylated O-deacylated LOS. LOS were isolated from bacteria grown in solid medium containing various concentrations of [^{12}C]NeuAc and [^{13}C]NeuAc. Incorporation efficiencies for [^{13}C]NeuAc are indicated: (A) medium with 1 mM [^{13}C]NeuAc (observed, 100% [^{13}C]NeuAc; calcd, 100%), (B) medium with 1 mM [^{13}C]NeuAc and 0.5 mM [^{12}C]NeuAc (observed, 65% [^{13}C]NeuAc; calcd, 67%), and (C) medium with 1 mM [^{13}C]NeuAc and 1 mM [^{12}C]NeuAc (observed, 49% [^{13}C]NeuAc; calcd, 50%).

Table 4: Sialylation of LOS and Efficiency of Incorporation of [^{13}C]NeuAc into LOS Depending on the Sialic Acid Concentration in the Medium

medium	[^{13}C]NeuAc (mM)	sialylation %[a]	incorporation efficiency (%)[b]
liquid	0	19	−
liquid	1	25	64
solid	0	15	−
solid	1	62	100
solid	0.5	60	100
solid	0.1	61	100
solid	0.05	58	96
solid	0.01	64	89
solid	0.005	62	82
solid	0.001	48	51
solid	0.0005	26	35
solid	0.0001	19	0

[a] Sialylation of the LacNAc glycoform. [b] Incorporation efficiency is defined as the ratio of the relative peak abundance of [^{13}C]NeuAc-LOS to that of total [^{12}C]- and [^{13}C]NeuAc-LOS glycoforms. The concentration of endogenous sialic acid in medium was 0.5 μM.

a level of sialylation of ~61% at sialic acid concentrations ranging from 0.005 to 1 mM of added [^{13}C]NeuAc substrate. At lower sialic acid concentrations, the overall sialylation level dropped drastically and seemed to reach half the observed maximum level (~30%) at a total sialic acid concentration of approximately 1.3 μM. In the case of no added sialic acid where the level of contaminant sialic acid was determined to be ~0.5 μM (blank, plate cultures), the sialylation level was as low as 15%.

CONCLUSIONS

In this study, we have shown that *H. ducreyi* is capable of incorporating sialic acids such as NeuAc, [^{13}C]NeuAc, and NeuGc into their LOS when these sugars are supplied to the growth medium. This is the first report to our knowledge of biosynthetic sialic acid engineering in a microbial organism. In contrast, a series of *N*-acylmannosamine derivatives, including d_3-ManNAc, ManPent, and ManLev, showed no evidence of metabolism to sialosides.

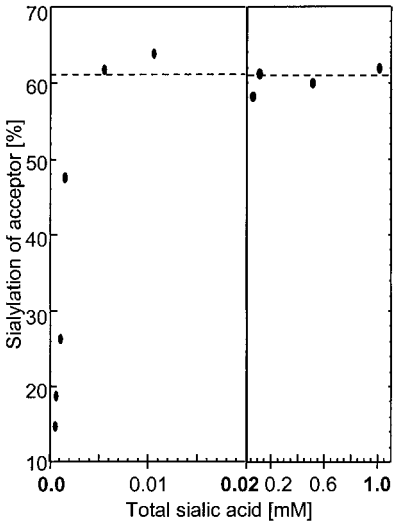


FIGURE 8: Concentration dependence of sialylation of LOS on total exogenous sialic acid. Sialylation of acceptor LOS glycoform depends on the total amount of sialic acid present in the medium which is defined as the amount of added [^{13}C]NeuAc and contaminating unlabeled NeuAc present in the medium (~0.5 μM).

Likewise, a corresponding series of the more hydrophobic tetra-*O*-acetyl derivatives of these same *N*-acylmannosamines failed to show evidence of metabolism. Together, these data are consistent with a biosynthetic pathway that does not include the metabolism of mannosamine precursors, but rather requires the direct uptake and biosynthetic incorporation of sialic acid and similar substrates. Indeed, our studies clearly show that exogenous sialic acids were efficiently transported into the cell and subsequently metabolized. Moreover, these results point to the presence of a permease capable of efficient uptake of NeuAc and NeuGc.

[^{13}C]NeuAc was used to examine details of the incorporation of exogenous sialic acid over a broad concentration range (from 0.1 μM to 1 mM). The use of a ^{13}C-labeled substrate was necessitated by the low level of contaminating NeuAc in the growth medium (~0.5 μM in solid medium) and for the purpose of distinguishing any endogenous sialic acid that might be formed from other sources. A combination of mass spectrometry techniques, including MALDI-MS and ESI-MS, provided sufficient sensitivity and resolution to accurately quantify the extent of substrate incorporation in the intact LOS glycoforms. These experiments demonstrated that small changes in low-level sialic acid concentrations can have a significant impact on the overall level of sialylation of LOS. Within a total sialic acid concentration range of 1−6 μM (solid medium), sialylation of the LacNAc-containing LOS glycoforms changed from ~20% to the highest observed level of ~60%. This dramatic change in LOS sialylation in response to relatively small changes in exogenous sialic acid concentration suggests that, in the human host, serum or tissue levels of sialic acid are likely to play an important role in regulating this modification. It is interesting to note that a similar mechanism has been reported for several species of trypanosomes (*39*). In the procyclic form of *Trypanosoma brucei*, for example, a trans-sialidase activity is responsible for tranferring sialic acid from the mammalian glycoproteins to the parasite's surface glycoproteins (*40*), presumably as a means of warding off attack from glycosidases and proteases, or blunting the immune response. How the formation of sialylated LOS species in vivo effects

mechanisms of bacterial infectivity and pathogenesis remains to be determined.

Now that the pathway leading to the formation of sialylated LOS glycoforms has been clarified, we have embarked on a separate study attempting to introduce substantially modified *N*-acylneuraminic acids containing functional groups such as ketones, or photoactive azide or dibenzoyl groups, into the LOS. The activation of sialic acids containing photoactive groups could generate cross-links to proteins or other macromolecules involved in the cell adhesion or invasion processes, and could therefore allow us to examine the molecular interactions of LOS under conditions that preserve the multivalent and native features of these complex processes. Alternatively, LOS that have been modified by the incorporation of unnatural sialic acids may have altered immunological properties that could be useful in vaccine development. Preliminary data using a series of *N*-alkyl extended neuraminic acids seem to suggest that these approaches are possible (S. Goon et al., unpublished data).

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