Biosynthesis of CMP-*N*,*N*′-Diacetyllegionaminic Acid from UDP-*N*,*N*′-Diacetylbacillosamine in *Legionella pneumophila*[†]

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ABSTRACT: Legionaminic acid is a nine-carbon α-keto acid that is similar in structure to other members of the sialic acid family that includes neuraminic acid and pseudaminic acid. It is found as a component of the lipopolysaccharide in several bacterial species and is perhaps best known for its presence in the O-antigen of the causative agent of Legionnaires' disease, *Legionella pneumophila*. In this work, the enzymes responsible for the biosynthesis and activation of *N*,*N*′-diacetyllegionaminic acid are identified for the first time. A cluster of three *L. pneumophila* genes bearing homology to known sialic acid biosynthetic genes (*neuA,B,C*) were cloned and overexpressed in *Escherichia coli*. The NeuC homologue was found to be a hydrolyzing UDP-*N*,*N*′-diacetylbacillosamine 2-epimerase that converts UDP-*N*,*N*′-diacetylbacillosamine into 2,4-diacetamido-2,4,6-trideoxymannose and UDP. Stereochemical and isotopic labeling studies showed that the enzyme utilizes a mechanism involving an initial *anti* elimination of UDP to form a glycal intermediate and a subsequent *syn* addition of water to generate product. This is similar to the hydrolyzing UDP-*N*-acetylglucosamine 2-epimerase (NeuC) of sialic acid biosynthesis, but the *L. pneumophila* enzyme would not accept UDP-GlcNAc as an alternate substrate. The NeuB homologue was found to be a *N*,*N*′-diacetyllegionaminic acid synthase that condenses 2,4-diacetamido-2,4,6-trideoxymannose with phosphoenolpyruvate (PEP), although the in vitro activity of the recombinant enzyme (isolated as a MalE fusion protein) was very low. The synthase activity was dependent on the presence of a divalent metal ion, and the reaction proceeded via a C−O bond cleavage process, similar to the reactions catalyzed by the sialic acid and pseudaminic acid synthases. Finally, the NeuA homologue was shown to possess the CMP-*N*,*N*′-diacetyllegionaminic acid synthetase activity that generates the activated form of legionaminic acid used in lipopolysaccharide biosynthesis. Together, the three enzymes constitute a pathway that converts a UDP-linked bacillosamine derivative into a CMP-linked legionaminic acid derivative.

Legionaminic acid (5,7-diamino-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid or Leg)[1] is a nine-carbon α-keto acid that is related in structure to sialic acid (*N*-acetylneuraminic acid or NeuAc) (Figure 1). Its name refers to its presence, and role as an antigen, in the lipopolysaccharide (LPS) of *Legionella pneumophila*, which is a facultative intracellular parasite that is the cause of Legionnaires' disease (*1*–*3*). This disease is an often fatal pneumonia that was first recognized in a large outbreak at an American Legion convention in Philadelphia in 1976. The O-polysaccharide in the LPS of *L. pneumophila* is composed of a repeating homopolymer of α-(2→4)-linked 5-*N*-acetimidoyl-7-*N*-acetyllegionaminic acid [Leg5Am7Ac (Figure 1)] (*4*). In strain Philadelphia 1, the hydroxyl at C-8 is acetylated such that the polymer bears both positive and negative charge but no free hydroxyl groups (*5*). Legionaminic acid derivatives have also been found in the O-antigens of *Pseudomonas fluorescens* (*6*), *Vibrio alginolyticus* (*7*), *Vibrio salmonicida* (*8*), and *Acinetobacter baumannii* (*9*). More recently, Leg5Am7Ac has been detected in *Campylobacter coli* where it is found as a posttranslational

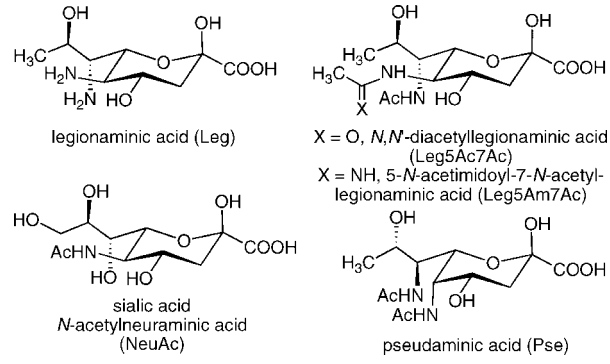

FIGURE 1: Structures of legionaminic acid, sialic acid, and pseudaminic acid.

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[1] Abbreviations: Leg, legionaminic acid or 5,7-diamino-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid; LPS, lipopolysaccharide; Leg5Am7Ac, 5-*N*-acetimidoyl-7-*N*-acetyllegionaminic acid; Bac, bacillosamine, 4-amino-4-deoxyquinovosamine, or 2,4-diamino-2,4,6-trideoxyglucose; Bac2Ac4Ac, *N*,*N*′-diacetylbacillosamine; Leg5Ac7Ac, *N*,*N*′-diacetyllegionaminic acid; PEP, phosphoenolpyruvate.

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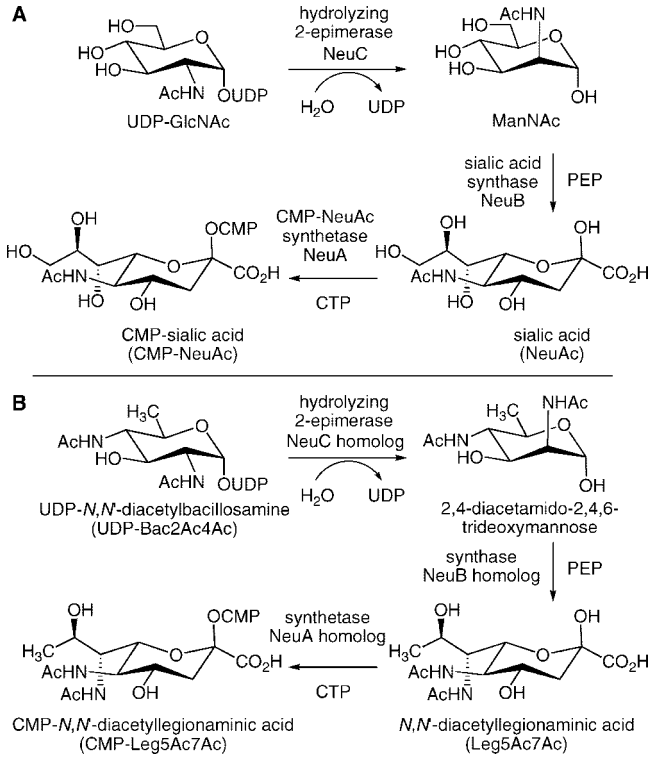

FIGURE 2: Biosynthetic pathways of (A) CMP-sialic acid and (B) CMP-*N*,*N*-diacetyllegionaminic acid.

modification on the flagellar proteins (*10*). *Campylobacter* sp. are the primary cause of bacterial diarrhea worldwide and require flagella for motility and pathogenicity. The flagellin proteins are heavily glycosylated with derivatives of both legionaminic acid and pseudaminic acid [an isomer of *N*,*N*′-diacetyllegionaminic acid (Figure 1)], and these modifications are required for the assembly of functional flagella. In early studies, the configuration of legionaminic acid was unclear, but once synthetic samples of the material became available, it was established that it bore the D-*glycero*-D-galacto configuration, similar to that of sialic acid (*11*). It should be noted that epimers of legionaminic acid are also known, such as 8-epilegionaminic acid (L-*glycero*-D-*galacto* configuration) that is found in the LPS of *Pseudomonas aeruginosa* (*11, 12*).

The biosyntheses of both sialic acid and pseudaminic acid start with UDP-linked hexoses derived from UDP-GlcNAc, and it is reasonable to expect that a similar strategy is employed in the biosynthesis of legionaminic acid (*13, 14*). The biosynthesis of sialic acid in bacteria involves two enzymes and is shown in Figure 2A. The first enzyme is a hydrolyzing UDP-*N*-acetylglucosamine 2-epimerase, NeuC, that catalyzes both an inversion of configuration at C-2 and a hydrolysis of the glycosidic-UDP linkage to convert UDP-GlcNAc into free ManNAc and UDP (*15, 16*). The second enzyme is a sialic acid synthase, NeuB, that condenses the three-carbon unit derived from phosphoenolpyruvate (PEP) with ManNAc to give sialic acid (*17–20*). A subsequent enzyme, CMP-sialic acid synthetase or NeuA, then activates the sialic acid as a CMP derivative that is used as a donor in biosynthesis (*21, 22*). Since the stereochemistry of sialic acid and legionaminic acid is the same, it is reasonable to suspect that a similar biosynthetic strategy is employed in the latter case (Figure 2B). For legionaminic acid biosynthesis, the hydrolyzing 2-epimerase would act on UDP-*N*,*N*′-

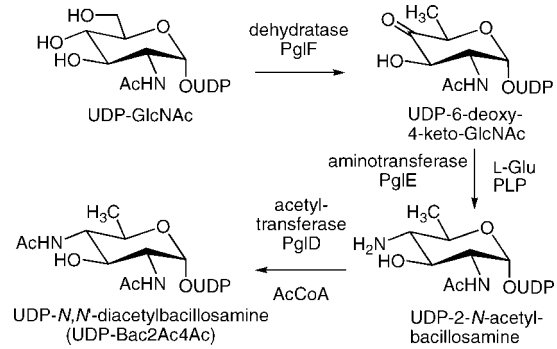

FIGURE 3: Biosynthesis of UDP-*N*,*N*′-diacetylbacillosamine (UDP-Bac2Ac4Ac). In this work, PglF and PglE were used in the preparation of UDP-2-*N*-acetylbacillosamine and the final acylation was done nonenzymatically.

diacetylbacillosamine (UDP-Bac2Ac4Ac) which is a 6-deoxy-4-acetamido derivative of UDP-GlcNAc (or UDP-4-acetamido-4-deoxy-*N*-acetylquinovosamine). The product, 2,4-diacetamido-2,4,6-trideoxymannose, would then be condensed with phosphoenolpyruvate by legionaminic acid synthase to give *N*,*N*′-diacetyllegionaminic acid. Finally, the α-keto acid would be activated as CMP-Leg5Ac7Ac by the corresponding synthetase. It is expected that modification of the C-5 acetamido group into an acetamidino group will occur at the level of the CMP-nucleotide, which is thought to be the case in acetamidino-containing derivatives of pseudaminic acid (*23*). The required starting material, UDP-Bac2Ac4Ac, is found in *Campylobacter jejuni* where it is used in the N-linked protein glycosylation system (*24–26*). This bacterium decorates at least 30 of its periplasmic and cell-surface proteins with a heptasaccharide, where the first residue is an asparagine-linked Bac2Ac4Ac. Recently, the biosynthesis of UDP-Bac2Ac4Ac in *C. jejuni* has been shown to require three enzymes (Figure 3) (*24, 25*). The first is a UDP-GlcNAc dehydratase, PglF, that generates a 4-keto-6-deoxy derivative of UDP-GlcNAc. The second and third are an aminotransferase, PglE, and an acetyltransferase, PglD, respectively, that install the acetamido group at C-4. Since homologues of PglD-F are all found in the *L. pneumophila* genome (*27*), it is reasonable to suspect that this organism could produce the UDP-Bac2Ac4Ac required for legionaminic acid biosynthesis. Previous work on *L. pneumophila* also identified a 30 kb locus that contained many of the genes required for LPS biosynthesis and included homologues of NeuA-C (*28*). Complementation studies showed that these NeuA and NeuB homologues could restore the biosynthesis of capsular polysialic acid in the corresponding *Escherichia coli* K1 mutants. This suggested that the *L. pneumophila* genes encode enzymes that could synthesize either legionaminic acid (from UDP-Bac2Ac4Ac) or sialic acid (from UDP-GlcNAc).

In this work, we demonstrate that the NeuC homologue of *L. pneumophila* is a hydrolyzing UDP-Bac2Ac4Ac 2-epimerase that converts UDP-Bac2Ac4Ac into 2,4-diacetamido-2,4,6-trideoxymannose and UDP. The reaction proceeds with an inversion of stereochemistry at C-2 and retention of stereochemistry at C-1. Evidence of the cleavage of the C-1−O bond and the C-2−H bond supports an elimination/hydration mechanism similar to that of NeuC in sialic acid bisoynthesis. The NeuB homologue was found to possess low levels of *N*,*N*′-diacetyllegionaminic acid

synthase activity and condenses 2,4-diacetamido-2,4,6-trideoxymannose with PEP to give give *N*,*N*′-diacetyllegionaminic acid. This reaction proceeds via a C−O bond cleavage mechanism similar to that of the NeuB of sialic acid biosynthesis. The *L. pneumophila* NeuC and NeuB did not show measurable activity with UDP-GlcNAc and ManNAc, respectively, indicating that they were specific for legionaminic acid biosynthesis. Finally, mass spectral evidence is presented showing that the *L. pneumophila* NeuA can convert *N*,*N*′-diacetyllegionaminic acid and CTP into CMP-*N*,*N*′-diacetyllegionaminic acid.

EXPERIMENTAL PROCEDURES

Materials and General Methods. UDP-*N*-acetylglucosamine, lactate dehydrogenase, and pyruvate kinase were purchased from Sigma-Aldrich. ^{18}O-enriched H_2O (95%) was purchased from Cambridge Isotope Laboratories. ^{1}H and ^{31}P NMR spectra were recorded on Bruker AV300, AV400, and AV600 NMR spectrometers. Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard (*29*). ESI-MS was performed on a Bruker Esquire LC mass spectrometer.

Cloning of L. pneumophila neuA (lpg0751), neuB (0752), and neuC (0753). The *neuA* (lpg0751), *neuB* (lpg0752), and *neuC* (lpg0753) genes were obtained from genomic DNA of *Legionella pneumophila* subsp. *pneumophila* ATCC 33152D by PCR amplification with Phusion DNA polymerase (New England Biolabs Inc.), according to the manufacturer's instructions. The primers used incorporated either NdeI or SalI cloning sites (underlined) and a six-His tag (italics) in either the 5P or 3P PCR primer. The following primer pairs were used: neuA5PHis, 5′-CTAGCTAGCTAGCATATG*CATCACCATCACCATCAC*AGAATATTGGCAGTAATCCCGGC-3′ (forward) and 5′-CTAGCTAGCTAGGTCGACTTATTATACTAGAGCCTCTTGGTTTAATTCC-3′ (reverse); neuA3PHis, 5′-CTAGCTAGCTAGCATATGAGAATATTGGCAGTAATCCCGGC-3′ (forward) and 5′-CTAGCTAGCTAGGTCGACTTATTA*GTGATGGTGATGGTGATG*TACTAGAGCCTCTTGGTTTAATTCC-3′ (reverse); neuB5PHis, 5′-CTAGCTAGCTAGCATATG*CATCACCATCACCATCAC*ACTTGTTTTATTATTGCTGAAGCAGG-3′ (forward) and 5′-CTAGCTAGCTAGGTCGACTTATTAATATGTTCCCATAACAAAGTTAGTACCCGC-3′ (reverse); neuB3PHis, 5′-CTAGCTAGCTAGCATATGACTTGTTTTATTATTGCTGAAGCAGG-3′ (forward) and 5′-CTAGCTAGCTAGGTCGACTTATTA*GTGATGGTGATGGTGATG*ATATGTTCCCATAACAAAGTTAGTACCCGC-3′ (reverse); neuC5PHis, 5′-CTAGCTAGCTAGCATATG*CATCACCATCACCATCAC*ATCAGAAAAATAATTTATGTTACAGGTACTCG-3′ (forward) and 5′-CTAGCTAGCTAGGTCGACTTATTAGTATGCATTGCATTTATTCAATATTTGTGAG-3′ (reverse); and neuC3PHis, 5′-CTAGCTAGCTAGCATATGATCAGAAAAATAATTTATGTTACAGGTACTCG-3′ (forward) and 5′-CTAGCTAGCTAGGTCGACTTATTA*GTGATGGTGATGGTGATG*GTATGCATTGCATTTATTCAATATTTGTGAG-3′ (reverse). The PCR products were gel purified and cloning sites generated by double digestion with NdeI and SalI restriction enzymes according to the manufacturer's suggested protocol (New England Biolabs Inc.). The genes were cloned into NdeI- and SalI-digested plasmid pCWori$^+$ and the constructs maintained in *E. coli* AD202.

Purification of L. pneumophila NeuA and NeuC. For the expression and purification of either NeuA or NeuC, the appropriate recombinant plasmid was transformed into *E. coli* BL21(DE3) competent cells which were incubated in 10 mL of Luria-Bertani (LB) medium containing 50 mg/L ampicillin at 37 °C and 225 rpm for 10 h. This culture was then poured into 500 mL of LB medium containing 50 mg/L ampicillin and shaken at 37 °C and 225 rpm until an OD_{600} of 0.6–1.0 was reached. Cultures were induced with 1 mM isopropyl β-D-galactopyranoside (IPTG), and after incubation for 5 h at 37 °C, the cells were harvested by centrifugation and stored in pellet form at −80 °C. The pellets were resuspended in 10 mL of phosphate buffer (20 mM, pH 8.0) containing 2 mM dithiothreitol (DTT), 1 mg/L aprotinin, and 1 mg/L pepstatin A at 4 °C. The cells were then lysed by being passed through a French pressure cell at 20000 psi. The lysate was centrifuged at 6000*g* for 1 h, passed through a 0.45 and 0.22 μm filters, and loaded onto a column containing 10 mL of Chelating Sepharose Fast Flow resin (Pharmacia Biotech), which had previously been charged with 100 mM $NiSO_4$ and washed with sodium phosphate buffer [20 mM (pH 8.0), containing 0.5 M NaCl and 5 mM imidazole]. The purification process was monitored using a Flow Thru UV monitor spectrometer at 280 nm. Nonspecifically bound proteins were washed away by applying buffer containing first 5 mM, and then 125 mM, imidazole. Finally, bound enzyme was eluted using 500 mM imidazole buffer. For NeuC, the fractions containing the desired enzyme were combined and concentrated using Amicon Ultra Centricons (Millipore) before being flash-frozen with liquid N_2 in the presence of 10% glycerol. For NeuA, the corresponding fractions were combined and dialyzed against 20 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl before being concentrated and flash-frozen with liquid N_2 in the presence of 10% glycerol.

Subcloning of neuB for a MalE−NeuB Fusion Protein. The NeuB5PHis clone, verified by sequence analysis, was double digested with NdeI and SalI restriction enzymes. The liberated insert was gel purified and subcloned into pCWOri$^+$ containing the *E. coli malE* gene with a downstream thrombin cleavage recognition sequence that was cloned as a BamHI to NdeI fragment. The constructs were maintained in *E. coli* AD202, and cells bearing positive clones were identified by colony PCR using specific *malE* and *neuB* primers and by restriction mapping. The resulting *malE*−*neuB* plasmid was transformed into *E. coli* BL21(DE3) competent cells, which were then grown at 37 °C in 2YT medium supplemented with 50 mg/L ampicillin. Overexpression of fusion protein was induced by the addition of IPTG to a final concentration of 1 mM at an A_{600} of 0.5, and growth continued for a further 6 h. Cells were harvested by centrifugation at 10000*g* for 15 min, resuspended in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mg/L aprotinin and 1 mg/L pepstatin A, and lysed by being passed through a French pressure cell at 20000 psi. The cell lysate was clarified by centrifugation at 27000*g* for 30 min and cell debris discarded. Total membrane and soluble protein fractions were obtained from clarified cell extracts by ultracentrifugation at 100000*g* for 60 min. Following adjustment to 200 mM NaCl and 1 mM EDTA, the soluble protein fraction was

passed through a 20 mL amylose resin (New England Biolabs Inc.) column previously equilibrated with 200 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 1 mM EDTA. The column was washed with 3 column volumes of equilibration buffer, and bound protein was eluted with equilibration buffer containing 10 mM maltose. Fractions containing the protein of interest, as judged by SDS−PAGE, were pooled and dialyzed against 200 mM NaCl and 20 mM Tris-HCl (pH 7.5). Glycerol (10%) was added to the solution, and aliquots were flash-frozen in liquid nitrogen and stored at −80 °C.

Purification of PglF and PglE as His$_6$-Tagged Proteins. The plasmids pNRC40.1 and pNRC41.3 were used in the overexpression of *pglF* (Cj1120c) and *pglE* (Cj1121c) to give His-tagged proteins, as described previously (*25*). In each case, the appropriate plasmid was transformed into *E. coli* BL21(DE3) competent cells which were incubated in 10 mL of Luria-Bertani (LB) medium containing 50 mg/L ampicillin at 37 °C and 225 rpm for 10 h. The culture was then added to 500 mL of LB medium containing 50 mg/L ampicillin and shaken at 37 °C and 225 rpm until an OD_{600} of 0.6 was reached. The culture was allowed to continue to grow for 5 h after 70 mg/L IPTG had been added. Cells were harvested by centrifugation, and the pellet was stored at −80 °C. The pellet was resuspended in 10 mL of phosphate buffer (10 mM, pH 7.0) containing 2 mM dithiothreitol (DTT), 1 mg/L aprotinin, and 1 mg/L pepstatin A. The cells were lysed by being passed through a French Pressure cell at 20000 psi. The lysate was centrifuged at 10000*g* for 1 h and passed through 0.45 and 0.22 μm filters. A column containing 10 mL of Chelating Sepharose Fast Flow resin (Pharmacia Biotech) was charged with 20 mL of 100 mM $NiSO_4$ and washed with 20 mL of distilled H_2O and 30 mL of sodium phosphate buffer [10 mM (pH 7.0), containing 0.5 M NaCl and 5 mM imidazole]. The lysate was loaded onto the column and eluted with the same buffer containing increasing amounts of imidazole in a stepwise fashion (5, 125, and 500 mM). Fractions containing the protein of interest, as judged by SDS−PAGE, were pooled and dialyzed against 20 mM phosphate buffer (pH 7.0) before being flash-frozen with liquid N_2 in the presence of 10% glycerol.

Chemoenzymatic Synthesis of UDP-Bac2Ac4Ac. UDP-Bac2Ac4Ac was prepared under conditions slightly modified from those described by Olivier et al. (*24*). A purified sample of PglF (5 mg) was added to 50 mL of phosphate buffer (10 mM, pH 7.0) containing 500 mg of UDP-*N*-acetylglucosamine disodium salt and 200 μM NAD^+. The solution was incubated for 6 h at 37 °C, and the reaction progress was followed by negative ESI mass spectroscopy. Almost all of the starting material was converted to a UDP-4-keto sugar. To synthesize the UDP-4-amino sugar, the following components were added to the mixture: 5 mg of purified PglE, L-glutamate (final concentration of 15 mM), and pyridoxal 5′-phosphate (PLP) (final concentration of 100 μM). The solution was incubated for 4 h at 37 °C, and the reaction progress was monitored by negative ESI mass spectroscopy. It was determined that >95% of the UDP-4-keto sugar (*m*/*z* 587, $[M - H]^-$) was converted to UDP-Bac2Ac (*m*/*z* 589, $[M - H]^-$) during this time. Enzymes were then removed by centrifugal ultrafiltration, and the resultant filtrate was loaded onto a 220 mL column of DEAE-cellulose (DE-52, Whatman Inc.) and eluted with a linear gradient from 0 to 0.5 M triethylammonium bicarbonate buffer. The A_{254} of the eluant was monitored, and UV-active fractions were analyzed by negative ESI mass spectroscopy. Those containing UDP-Bac2Ac were lyophilized to dryness. The lyophilized sugar (295 mg) was stirred with 1.2 mL of acetic anhydride in 25 mL of methanol at room temperature for 24 h. Negative ESI-MS showed that the starting material was completely converted to UDP-Bac2Ac4Ac (*m*/*z* 631, $[M - H]^-$) during this time. After removal of the solvent under reduced pressure, the product was loaded onto a DE-52 anion exchange column and subjected to linear gradient elution as described above. After lyophilization, the product was dissolved in 10 mL of H_2O and lyophilized again. This procedure was repeated twice more to yield 151 mg (28%) of the UDP-Bac2Ac4Ac as its triethylammonium salt. The ^1H NMR and ^{31}P NMR spectral characteristics of the product were identical to those described in the literature (*24*).

NeuC Homologue Activity Assay by NMR Spectroscopy and Mass Spectrometry. A glycerol stock solution of the NeuC homologue (70 μg) was subjected to buffer exchange with a 25 mM phosphate buffer (pH 7.5, final volume of 150 μL) using centrifugal ultrafiltration. This was added to a solution of UDP-Bac2Ac4Ac (3.0 mg) dissolved in 850 μL of H_2O (final volume of 1.0 mL), and ^{31}P NMR and positive ion ESI mass spectra were acquired at timed intervals. Once the reaction was complete, the enzyme was removed by centrifugal ultrafiltration and the resultant filtrate was passed through a column (15 mL) of AG-1X8 resin (100–200 mesh, formate form) and eluted with water to remove the UDP. The flowthrough was lyophilized to dryness and redissolved in D_2O for spectral analysis. The ^1H NMR spectrum of the product was identical to that previously reported for the mixture of anomers of 2,4-diacetamido-2,4,6-trideoxymannose (*30*). Material prepared in this fashion was also analyzed by a two-dimensional heteronuclear NMR experiment (HMQC) both with and without the $^1H-^{13}C$ coupling constant retained in the ^1H dimension, to establish the identity of the H1 signals for each of the anomers (vide infra).

Kinetic Studies of the NeuC Homologue Using a Continuous Coupled Assay. Enzyme kinetics were measured using a continuous coupled assay for UDP formation (*31*). Each cuvette contained 50 mM NaH_2PO_4 buffer (pH 7.5), 10 mM $MgCl_2$, 2 mM PEP, 0.2 mM NADH, 20 units of lactate dehydrogenase, 18 units of pyruvate kinase, and UDP-Bac2Ac4Ac (varying from 25 to 1000 μM) in a total volume of 800 μL. The concentrations of stock UDP-sugar solutions were determined by measuring A_{260} ($\epsilon = 9890\ M^{-1}\ cm^{-1}$). Enzymatic reactions were initiated by the addition of 20 μL of a 0.05 mg/mL enzyme solution (final concentration of 2.0 nM). Rates were measured by monitoring the decrease in A_{340} at 37 °C. Kinetic parameters were determined by fitting initial velocities to the Michaelis–Menten equation using GraFit 4.0. No detectable background release of UDP was observed in the absence of added NeuC homologue.

NeuC Homologue Reaction Stereochemistry and Deuterium Incorporation. A glycerol stock solution of the NeuC homologue (70 μg) was subjected to buffer exchange with a 25 mM phosphate/D_2O buffer (pD 7.4, final volume of 200 μL) using centrifugal ultrafiltration. This was added to a solution of UDP-Bac2Ac4Ac (3 mg) dissolved in 800 μL

of D_2O (final volume of 1 mL), and ^1H NMR spectra were immediately acquired at timed intervals during incubation at 25 °C.

Metal Dependency of the NeuC Homologue. Two aliquots of a solution containing UDP-Bac2Ac4Ac (3 mg per aliquot) in 25 mM phosphate buffer (pH 7.5) were prepared. One aliquot received $MgCl_2$ and the other EDTA tetrasodium salt, each at a final concentration of 10 mM in a total volume of 990 μL. The NeuC homologue (70 μg) was added to each sample, and the mixtures were incubated for 2 h at room temperature. The progress of the reactions was monitored by ^{31}P NMR spectroscopy with integration of the diphosphate signals.

^{18}O Incorporation Experiment. A solution of 25 mM phosphate buffer (pH 7.5, 1.60 mL) was prepared from 50% $H_2{}^{16}O$ and 50% $H_2{}^{18}O$ (95% isotopic enrichment) and divided into two aliquots. To one aliquot was added UDP-Bac2Ac4Ac (2.0 mg), and to the other was added 2,4-diacetamido-2,4,6-trideoxymannose (1.0 mg). The NeuC homologue (70 μg) was added to each sample, and the mixtures were incubated at room temperature. Isotope incorporation was monitored by both positive (sugar detection) and negative (UDP detection) ESI-MS as a function of time. The extent of incorporation into 2,4,6-trideoxydiacetamidomannose was deduced from the ratio of peaks at *m/z* 269 (^{16}O, $[M + Na]^+$) and *m/z* 271 (^{18}O, $[M + 2 + Na]^+$).

Determination of the Activity of the NeuB Homologue. To monitor the NeuB reaction run to completion, a solution containing 2,4-diacetamido-2,4,6-trideoxymannose (12 mM) and PEP (20 mM) in Tris-DCl buffer prepared using D_2O (700 μL, 10 mM, pD 7.4) was placed in a NMR tube. Initial ^1H and proton-decoupled ^{31}P NMR spectra were recorded. The solution was removed from the tube and mixed with 5 mg of the NeuB homologue and 1 mM $MgCl_2$ in the same deuterated buffer (total volume of 1 mL). After incubation of the reaction mixture for 20 h at 25 °C, Chelex-100 resin (~20 mg, previously rinsed with D_2O) was added, and the solution was incubated for an additional 1 h at room temperature. The resulting mixture was analyzed directly by ^1H and ^{31}P NMR spectroscopy.

To determine the activity of the NeuB homologue under initial velocity conditions, a solution containing 2,4-diacetamido-2,4,6-trideoxymannose (6.4 mM), PEP (20 mM), $MgCl_2$ (1 mM), and the NeuB homologue (6.5 mg) in Tris-DCl buffer prepared using D_2O (10 mM, pD 7.4, total volume of 1.0 mL) was placed into a NMR tube and immediately monitored by ^1H NMR spectroscopy. Spectra were recorded every 5 min for a period of 1 h while the samples were incubated at 25 °C. The conversion rate was calculated by comparing the integrals of the signals due to the acetamido methyl protons of both 2,4-diacetamido-2,4,6-trideoxymannose anomers (2.03, 2.04, 2.08, and 2.12 ppm) to those of the product *N*,*N*′-diacetyllegionaminic acid (1.99 and 2.01 ppm). The rate of the reaction was determined by using the data that were accumulated during the first 15% of the reaction.

Isolation and Characterization of N,N′-Diacetyllegionaminic Acid. The NeuB homologue was removed from the enzymatic activity test reaction mixtures by centrifugal ultrafiltration, and the resulting filtrate was loaded onto a 15 mL column of Dowex AG-1X8 resin (formate form, 100–200 mesh, Bio-Rad) pre-equilibrated with water. A stepwise gradient from 0 to 1.0 M formic acid in water with 0.1 M increments (50 mL per increment) was used to elute the product. *N*,*N*′-Diacetyllegionaminic acid eluted from the column in the 0.2 and 0.4 M fractions which were concentrated in vacuo and then lyophilized. *N*,*N*′-Diacetyllegionaminic acid was characterized using ^1H NMR and negative ESI-MS, and the spectroscopic data were identical to those reported for the synthetically produced material (*11, 30*).

C−O versus P−O Bond Cleavage. [2-^{18}O]PEP disodium salt was prepared as described previously and found to incorporate 54% of the isotopic label as analyzed by ^{31}P NMR and mass spectral analysis (*32*). A solution containing 2,4-diacetamido-2,4,6-trideoxymannose (12 mM) and [2-^{18}O]PEP (20 mM) in Tris-DCl buffer (700 μL prepared using D_2O, 10 mM, pH 7.4) was placed in a NMR tube. Chelex-100 resin (~20 mg) was added, and an initial proton-decoupled ^{31}P NMR spectrum was recorded using a previously reported procedure (*32*). The Chelex resin was removed by decanting the solution, and the solution was mixed with 5 mg of the NeuB homologue and 1.0 mM $MgCl_2$ in 250 μL of the same Tris-DCl buffer. The reaction mixture was incubated for 20 h at 25 °C, and a solution of EDTA tetrasodium salt in D_2O (final concentration of 10 mM) was added to the mixture. Another proton-decoupled ^{31}P NMR spectrum was acquired with the same parameters: ^{31}P NMR δ 0.16 (s, P_i-^{16}O), 0.14 (s, P_i-^{18}O), −3.01 (s, P-^{16}O, PEP), −3.03 (s, P-^{18}O, PEP).

Activity Test for NeuA. A glycerol stock solution of the NeuA homologue (containing 1.25 mg of enzyme) was subjected to buffer exchange with a 20 mM Tris-HCl buffer (pH 7.5). The enzyme was added to a solution containing 500 μM *N*,*N*′-diacetyllegionaminic acid, 1.5 mM cytidine 5′-triphosphate disodium salt, and 1.0 mM $MgCl_2$ in the same buffer (final volume of 1.0 mL). The reaction mixture was incubated at 25 °C, and the progress was monitored using negative ion ESI mass spectrometry. After 2 h, all of the starting material at *m/z* 333 ($[M - H]^-$) was converted to CMP-*N*,*N*′-diacetyllegionaminic acid product at *m/z* 660 ($[M + Na - 2H]^-$).

RESULTS

Identification and Activity of a Hydrolyzing UDP-N,N′-Diacetylbacillosamine 2-Epimerase. Past studies of LPS biosynthesis in *L. pneumophila* implicated the *neuC* homologue (lpg0753, Orf 23, *mnaA*, or *siaA*) as the gene encoding an enzyme required in the biosynthesis of legionaminic acid (*28*). The gene product is a protein that shares homology with NeuC of sialic acid biosynthesis in *Neisseria meningitidis* and *E. coli* K1 (30 and 28% identical, respectively), and it is clustered with other genes resembling those of sialic acid biosynthesis (*neuA* and *neuB*). Later work on the bacterial biosynthesis of sialic acid in *N. meningitidis* and *E. coli* K1 demonstrated that NeuC is a hydrolyzing UDP-*N*-acetylglucosamine 2-epimerase (Figure 2A) (*15, 16*). This suggests that the *L. pneumophila* NeuC is a hydrolyzing UDP-*N*,*N*′-diacetylbacillosamine 2-epimerase that converts UDP-Bac2Ac4Ac into 2,4-diacetamido-2,4,6-trideoxymannose and UDP (Figure 2B). *L. pneumophila neuC* (lpg0753) was therefore cloned and overexpressed in *E. coli* to give a protein bearing an N-terminal hexahistidine tag in excellent

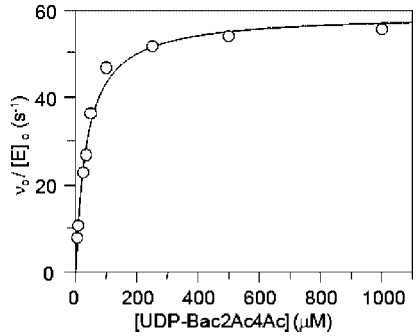

FIGURE 4: Plot of $v_o/[E]_o$ vs substrate concentration for the reaction catalyzed by the hydrolyzing UDP-*N*,*N*′-diacetylbacillosamine 2-epimerase. The kinetic parameters obtained by fitting the data to Michealis−Menten kinetics are as follows: $k_{cat} = 59.1 \pm 1.6\ s^{-1}$, $K_M = 36.5\ \mu M$, and $k_{cat}/K_M = 1.6 \times 10^6\ M^{-1}\ s^{-1}$.

yield (52 mg/L). The protein was purified by metal affinity chromatography and was found to be >95% pure as analyzed by SDS−PAGE (see the Supporting Information).

A barrier to studying the activity of the *L. pneumophila* NeuC is the difficulty in obtaining a sample of UDP-Bac2Ac4Ac. Fortunately, recent interest in the N-linked glycosylation system of *C. jejuni* has led researchers to uncover the biosynthetic pathway of this compound and has made the corresponding enzymes available (*24, 25*). A sample of UDP-GlcNAc was therefore treated with the dehydratase PglF (Cj1120c) and the aminotransferase PglE (Cj1121c) in the presence of glutamate and PLP to give UDP-2-*N*-acetylbacillosamine (Figure 3). This compound was purified and then chemically N-acetylated with acetic anhydride in methanol to give UDP-Bac2Ac4Ac.

To test the activity of the *L. pneumophila* NeuC, a sample of UDP-Bac2Ac4Ac was incubated with the enzyme and the reaction was monitored by ^{31}P NMR spectroscopy. The signals of the sugar nucleotide at −11.01 and −12.82 ppm were converted into the signals of free UDP at −7.48 and −10.26 ppm, respectively, indicating that a hydrolysis reaction was taking place. Similarly, mass spectral analysis confirmed that UDP was produced along with a compound whose mass corresponded to 2,4-diacetamido-2,4,6-trideoxymannose. Following the removal of UDP by ion exchange chromatography, the hexose product was analyzed by ^1H NMR spectroscopy and was found to be a 1:1 mixture of anomers of 2,4-diacetamido-2,4,6-trideoxymannose. The small $J_{H1,H2}$ values observed in both of the anomers (0.91 and 1.47 Hz) indicated that the acetamido group at C-2 was in an axial position, and therefore, an inversion of stereochemistry had occurred at C-2. The ^1H NMR data that were obtained were in complete agreement with those reported in the literature for the synthetic compound (*30*). This analysis confirms that the *L. pneumophila* NeuC homologue is a hydrolyzing UDP-Bac2Ac4Ac 2-epimerase.

The kinetic constants for this reaction were measured using a continuous spectrophotometric assay for UDP formation that employs lactate dehydrogenase and pyruvate kinase (*31*). The reaction was found to obey Michaelis–Menten kinetics with the following kinetic constants: $k_{cat} = 59.1 \pm 1.6\ s^{-1}$, $K_M = 36.5\ \mu M$, and $k_{cat}/K_M = 1.6 \times 10^6\ M^{-1}\ s^{-1}$ (Figure 4). The reasonably large value of the specificity constant suggests that UDP-Bac2Ac4Ac is the physiologically correct substrate for this enzyme. When UDP-GlcNAc was incubated

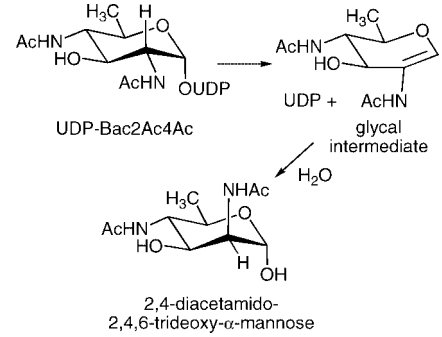

FIGURE 5: Proposed mechanism of the reaction catalyzed by the hydrolyzing UDP-*N*,*N*′-diacetylbacillosamine 2-epimerase.

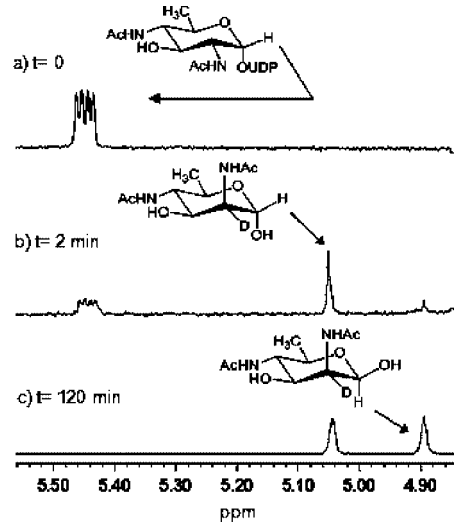

FIGURE 6: Enzymatic formation of α-[2-^2H]-2,4-diacetamido-2,4,6-trideoxymannose in D_2O as followed by ^1H NMR spectroscopy. (a) Before addition of enzyme. (b) Spectrum taken within 2 min of addition of enzyme that shows the α-anomer is first formed. (c) Spectrum taken after 2 h showing an equilibrated mixture of anomers.

with the enzyme, no product formation was observed. This indicates that the epimerase is specifically involved in the biosynthesis of *N*,*N*′-diacetyllegionaminic acid and not in the biosynthesis of sialic acid.

Mechanistic Studies of the Hydrolyzing UDP-Bac2Ac4Ac 2-Epimerase. A proposed mechanism for the reaction catalyzed by the hydrolyzing UDP-Bac2Ac4Ac 2-epimerase is shown in Figure 5. This mechanism is analogous to that of the previously studied hydrolyzing UDP-GlcNAc 2-epimerase (*15, 33*). An initial *anti* elimination of UDP generates a glycal intermediate and UDP, and a subsequent *syn* hydration of the glycal gives the α-anomer of 2,4-diacetamido-2,4,6-trideoxymannose as the first formed product (nonenzymatic mutarotation will readily generate a mixture of the two anomers). This elimination mechanism invokes a cleavage of both the C-2−H bond and the C-1−O bond during the formation of the glycal intermediate. A key mechanistic experiment in studying such an epimerization involves running the reaction in a dilute buffer prepared from D_2O and monitoring the initial stages of the reaction by ^1H NMR spectroscopy (Figure 6). This experiment identifies which anomer is formed first and also probes for a C-2−H bond cleavage process by analyzing incorporation of deuterium into the C-2 position during catalysis. A dilute buffer is employed to slow any buffer-catalyzed mutarotation

process that could prevent a determination of which anomer forms first. A sample of UDP-Bac2Ac4Ac in deuterated buffer shows an anomeric signal at 5.45 ppm that appears as a doublet of doublets due to coupling with both the β-phosphorus of the UDP group and the proton at C-2 (Figure 6a). To this sample is added a relatively large amount of the epimerase, and the spectrum is immediately re-recorded to minimize the possibility of mutarotation (Figure 6b). A new singlet appears at 5.06 ppm that corresponds to the first formed anomer of 2,4-diacetamido-2,4,6-trideoxy-mannose. As the reaction proceeds, subsequent spectra show a singlet at 4.90 ppm due to the other anomer that is formed by a nonenzymatic mutarotation process (Figure 6c). To establish the identity of the first formed anomer, it was not possible to use the $J_{H1,H2}$ values of the undeuterated product since they are very similar for sugars of the *manno* configuration. Instead, the method of Bock and Pedersen was employed that relies on measuring the $J_{C1,H1}$ values of the two anomers (*34*). The α-anomers of mannose derivatives are known to have $J_{C1,H1}$ values that are typically 10 Hz larger than those of the β-anomers. A two-dimensional hetero-nuclear NMR experiment (HMQC) was first used to establish that the first formed proton signal correlated to a carbon signal at 92.93 ppm and the second formed proton signal correlated to a carbon signal at 92.86 ppm. The $J_{C1,H1}$ values could not be determined directly using a ^{1}H-coupled ^{13}C NMR experiment due to the small quantities of product available. Instead, a HMQC experiment with a ^{1}H−^{13}C coupling constant retained in the ^{1}H dimension was conducted with an equilibrated sample of isolated product and showed that the α-anomeric ^{13}C signal at 92.93 ppm had a $J_{C1,H1}$ value of 173 Hz and the β-anomeric ^{13}C signal at 92.86 ppm had a $J_{C1,H1}$ value of 163 Hz. This finding establishes that the α-anomer is the first formed product and that the reaction proceeds with a net retention of stereochemistry at C-1.

This experiment also supports the proposal of a C-2−H bond cleavage process in the epimerization reaction. The H1 signal of each product anomer appears as a singlet since the proton at C-2 has been replaced with deuterium and the $J_{H1,D2}$ values are negligible. As expected, the signals due to the C-2 proton are also absent in these spectra (not shown). The solvent isotope incorporation is further confirmed by mass spectral analysis of 2,4-diacetamido-2,4,6-trideoxymannose that is isolated from such an incubation and found to be one mass unit larger than a sample prepared in H_2O.

The anticipated glycal mechanism predicts that the hydrolysis of the glycosidic bond proceeds via a C-1−O bond cleavage process with water ultimately being added to the anomeric carbon in the second step of the reaction (Figure 5). An alternative mechanism would involve a P−O bond cleavage process in which water attacks the β-phosphorus directly and displaces the sugar. To distinguish between these possibilities, the reaction was run in a 50:50 mixture of $H_2{}^{18}O$ and $H_2{}^{16}O$ and the products were immediately analyzed by mass spectrometry. The mass of the UDP generated in the reaction corresponded to material lacking any ^{18}O isotope, whereas that of the 2,4-diacetamido-2,4,6-trideoxymannose showed a 1:1 ratio of $M + Na^+$ and $M + Na^+ + 2$, indicating that an ^{18}O label had been incorporated into the sugar. To confirm that the label had been incorporated during the enzymatic reaction and not after hydrolysis, a control sample of unlabeled 2,4-diacetamido-2,4,6-trideoxymannose was incubated under identical conditions. A slow wash-in of ^{18}O could be observed over an extended incubation period; however, this rate was orders of magnitude too low to account for the incorporation observed during catalysis.

The observations that the hydrolyzing UDP-Bac2Ac4Ac 2-epimerase reaction proceeds with both cleavage of the C-2−H bond and the C-1−O bond provide support for the formation of a glycal intermediate in the reaction mechanism. The finding that the reaction proceeds with an inversion of stereochemistry at C-2 and a retention of stereochemistry at C-1 indicates that the mechanism involves an *anti* elimination of UDP from UDP-Bac2Ac4Ac followed by a *syn* hydration of the glycal double bond (Figure 5). These findings are in complete agreement with the accepted mechanism for the reaction catalyzed by the hydrolyzing UDP-GlcNAc 2-epimerase of sialic acid bisoynthesis (*15, 33*).

Identification and Activity of a N,N′-Diacetyllegionaminic Acid Synthase. Past work also identified a *neuB* homologue (lpg0752, Orf 24, *siaC*) in *L. pneumophila* that is clustered with the *neuC* and *neuA* homologues and was thought to encode a putative *N*,*N*′-diacetyllegionaminic acid synthase (*28*). The *L. pneumophila* NeuB is 61% identical in sequence with the *N. meningitidis* NeuB that is known to possess sialic acid synthase activity. This suggests that the *L. pneumophila* NeuB will catalyze the condensation of 2,4-diacetamido-2,4,6-trideoxymannose and phosphoenolpyruvate to give *N*,*N*′-diacetyllegionaminic acid (Figure 2B). *L. pneumophila neuB* was therefore cloned and overexpressed in *E. coli* as both an N-terminal and C-terminal His-tagged protein. In both cases, expression levels were high, but the vast majority of recombinant protein was insoluble and was located in the cell pellet. This is likely due to formation of inclusion bodies consisting of misfolded protein, as opposed to membrane association, since an examination of hydrophobicity plots does not indicate that a membrane insertion sequence is present in this protein. Very small amounts of soluble protein could be isolated from the cell lysate using metal affinity chromatography (4.4 μg/L), and this protein was found to possess low levels of synthase activity. However, the difficulties in obtaining reasonable quantities of protein precluded its use in further studies. Growth at lower temperatures resulted in a reduction of the total amount of recombinant protein that was obtained and did not increase the levels of soluble protein. The *L. pneumophila neuB* was also expressed as a MalE−NeuB fusion protein, and very high yields (54 mg/L) of soluble protein could be readily purified using an amylose column (see the Supporting Information). Subsequent studies showed that the fusion protein possessed very low, but measurable, levels of *N*,*N*′-diacetyllegionaminic acid synthase activity. In an attempt to obtain a more active synthase, the MalE protein was removed by thrombin treatment. Analysis of the cleavage reaction by SDS−PAGE indicated that the cleavage was complete and that the resulting NeuB had not been digested into smaller protein fragments. NeuB prepared in this fashion was soluble and could be purified by metal affinity chromatography (demonstrating that the N-terminal His tag was still present); however, no activity could be detected either before or after the purification procedure.

To identify NeuB as an *N*,*N*′-diacetyllegionaminic acid synthase, a sample of 2,4-diacetamido-2,4,6-trideoxymannose

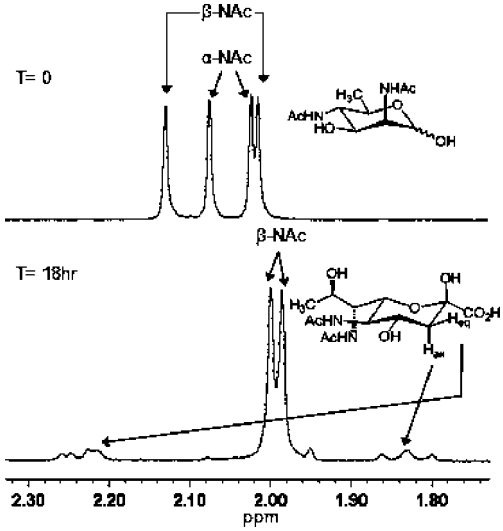

FIGURE 7: ^{1}H NMR spectra monitoring the conversion of 2,4-diacetamido-2,4,6-trideoxymannose into *N*,*N*-diacetyllegionaminic acid by the NeuB homologue. The top panel shows the spectrum before the addition of enzyme. The bottom panel shows the spectrum after incubation for 18 h.

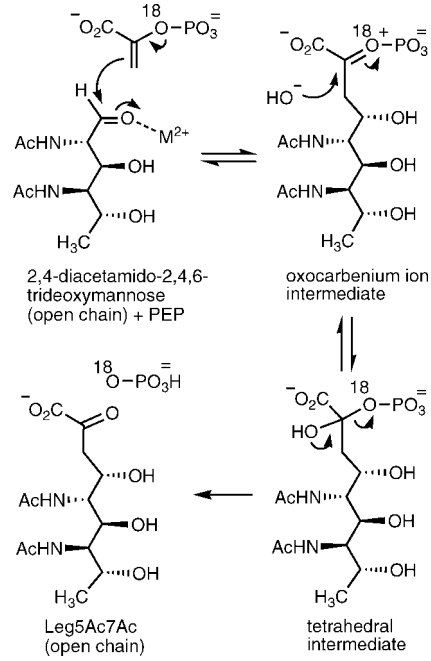

FIGURE 8: Proposed mechanism of the reaction catalyzed by *N*,*N*-diacetyllegionaminic acid synthase. ^{18}O isotopic labels are included to highlight the fate of the bridging phosphate oxygen of PEP during catalysis.

and phosphoenolpyruvate was incubated with the MalE−NeuB fusion protein in Tris-DCl buffer prepared using D_2O and containing $MgCl_2$. The resulting reaction was monitored by ^{31}P NMR spectroscopy, and the signal of PEP at −3.01 ppm was observed to convert into the signal of free phosphate at 0.14 ppm, indicating that a hydrolysis reaction was taking place. This hydrolysis did not take place in the absence of 2,4-diacetamido-2,4,6-trideoxymannose, indicating that it was not due to a low level of a phosphatase contaminant. The reaction was also monitored using ^{1}H NMR spectroscopy, and the characteristic signals of a sialic acid analogue were clearly observed in the upfield region of the spectrum (Figure 7). Before the addition of the synthase, the 50:50 mixture of 2,4-diacetamido-2,4,6-trideoxymannose anomers showed four singlets due to the acetamido methyl protons (2.03, 2.04, 2.08, and 2.12 ppm in Figure 7; $T = 0$). After the reaction was completed, the more stable β-anomer of *N*,*N*′-diacetyllegionaminic acid was the predominant form in solution as indicated by the two acetamido methyl signals (1.99 and 2.01 ppm in Figure 7; $T = 18$ h). Strong support for the formation of a nonulosonic acid is evident by the appearance of the C-3 methylene protons at 1.83 and 2.23 ppm (axial and equatorial, respectively). The signal for the axial proton is a doublet of doublets that appears as a triplet due to two large proton–proton coupling constants that are similar in magnitude ($J_{3ax,3eq} = 12.9$ Hz, and $J_{3ax,4} = 12.4$ Hz). The large coupling constant with the proton on C-4 indicates that the newly formed hydroxyl group occupies an equatorial position as expected for the correct stereochemistry of legionaminic acid. This indicates that C-3 of phosphoenolpyruvate attacked the *si* face of the 2,4-diacetamido-2,4,6-trideoxymannose aldehyde to generate an (*S*) configuration at C-4. By monitoring the initial rate of product formation using ^{1}H NMR spectroscopy, we were able to estimate the specific activity of the MalE−NeuB fusion protein to be 4.4×10^{-3} μmol min^{-1} mg^{-1} at room temperature. As this is a very low activity that does not likely reflect that expected for in vivo conditions with an unmodified protein, no further attempts were made to kinetically characterize the reaction. A similar determination could not be made with the N-terminally His-tagged enzyme due to the requirement for extended incubation times with the quantity of soluble protein that was available. However, a qualitative assessment suggested that it was only 50-fold more active than the fusion enzyme. When the activity of the MalE−NeuB fusion protein was examined with 1.0 mM EDTA in place of $MnCl_2$, no product formation was observed. This is consistent with the notion that the synthase utilizes a divalent cation during catalysis, which has clearly been demonstrated in the case of sialic acid synthase (*17, 20*). Similarly, when *N*-acetylmannosamine was employed in place of 2,4-diacetamido-2,4,6-trideoxymannose, no product formation was observed (a rate that was 20-fold lower would still be detectable in this assay). This supports the notion that the enzyme is specifically involved in legionaminic acid biosynthesis and not in sialic acid biosynthesis.

To fully characterize the product of the reaction as *N*,*N*′-diacetyllegionaminic acid, the reaction mixture was subjected to ion exchange chromatography and the nonulosonic acid was purified to homogeneity. The resulting compound had ^{1}H NMR (see the Supporting Information) and mass spectral properties identical to those of the synthetic product as reported in the literature (*11, 30*). This unambiguously demonstrates that the *L. pneumophila* MalE−NeuB fusion protein possesses a low, but measurable, level of *N*,*N*′-diacetyllegionaminic synthase activity.

Mechanistic Studies of N,N′-Diacetyllegionaminic Synthase. The mechanism of the reaction catalyzed by *N*,*N*′-diacetyllegionaminic synthase is expected to be similar to that catalyzed by sialic acid synthase and pseudaminic acid synthase (Figure 8) (*17, 18, 32*). An initial attack of C-3 of phosphoenolpyruvate on the aldehyde of 2,4-diacetamido-2,4,6-trideoxymannose (open chain form) generates an oxocarbenium ion intermediate. This attack is facilitated by the divalent cation that serves as an electrophilic catalyst and

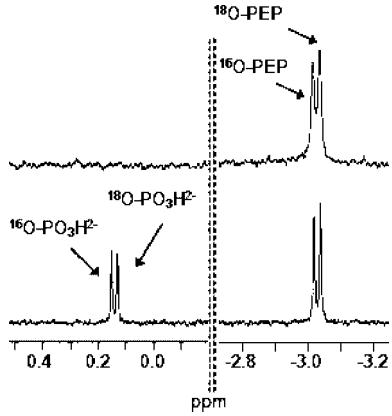

FIGURE 9: ^{31}P NMR spectra monitoring the conversion of partially labeled [2-^{18}O]PEP into ^{18}O-labeled phosphate by *N*,*N*-diacetyllegionaminic acid synthase. The top panel shows the spectrum before the addition of enzyme. The bottom panel shows the spectrum taken after incubation for 20 h.

polarizes the carbonyl of the aldehyde. Water then adds to the oxocarbenium ion intermediate to give a tetrahedral intermediate that subsequently collapses to generate phosphate and the product α-keto acid (open chain form). A key feature of this mechanism is that the release of phosphate ultimately involves a C−O bond cleavage process. This distinguishes it from potential mechanisms in which water attacks the phosphorus of phosphoenolpyruvate directly to release an enolate via a P−O bond cleavage process. By using ^{18}O-labeled PEP, it is possible to demonstrate that a C−O bond cleavage process has occurred since the label will reside in the phosphate that is produced and not in the carbonyl of the α-keto acid (see the labeled atoms in Figure 8). A sample of PEP was therefore synthesized that contained a 54% level of incorporation of an ^{18}O label in the C−O−P bridging position. A ^{31}P NMR spectrum of this material shows two signals at −3.03 and −3.01 ppm due to the labeled and unlabeled compounds, respectively, since the isotopic substitution causes a small upfield shift in the chemical shift of the ^{18}O-labeled compound (Figure 9, top panel) (*35*). When this material was utilized in the synthase reaction and the progress was monitored by ^{31}P NMR spectroscopy, two signals at 0.14 and 0.16 ppm appeared that correspond to ^{18}O-labeled and unlabeled phosphate, respectively, in the same ratio as that of the starting material (Figure 9, bottom panel). This demonstrates that the reaction proceeds through a C−O bond cleavage mechanism in a fashion similar to that of the reactions catalyzed by sialic acid synthase and pseudaminic acid synthase.

Identification and Activity of a CMP-N,N′-Diacetyllegionaminic Acid Synthetase. To demonstrate that the third gene of the cluster encodes a CMP-*N*,*N*′-diacetyllegionaminic acid synthetase (Figure 2B), the *neuA* homologue (lpg0751, Orf25, *siaB*) of *L. pneumophila* was cloned and overexpressed as a histidine-tagged protein in *E. coli*. Large quantities of the protein (51 mg/L) could readily be purified by metal affinity chromatography and were found to be >95% pure as analyzed by SDS−PAGE (see the Supporting Information). Because of the relatively small amounts of *N*,*N*′-diacetyllegionaminic acid available from the action of the NeuB synthase, a single experiment was used to investigate the activity of this enzyme. A sample of *N*,*N*′-diacetyllegionaminic acid was incubated with CTP, $MgCl_2$, and the NeuA homologue, and the resulting reaction was monitored by mass spectrometry. A signal corresponding to the expected mass of CMP-*N*,*N*′-diacetyllegionaminic acid readily replaced that of the starting nonulosonic acid, indicating that the NeuA homologue possessed CMP-*N*,*N*′-diacetyllegionaminic acid synthetase activity.

DISCUSSION

This work demonstrates that the cluster of the three *neuA*, *neuB*, and *neuC* homologues in *L. pneumophila* encodes enzymes involved in the biosynthesis and activation of *N*,*N*′-diacetyllegionaminic acid. The NeuC homologue was found to be an active hydrolyzing UDP-Bac2Ac4Ac 2-epimerase with kinetic constants larger than that of the hydrolyzing UDP-GlcNAc 2-epimerase of sialic acid biosynthesis (*15*). It was specific for UDP-Bac2Ac4Ac as a substrate and showed no activity with UDP-GlcNAc, indicating it is exclusively involved in legionaminic acid biosynthesis. The stereochemical and mechanistic studies performed with this enzyme support a mechanism that involves an initial *anti* elimination of UDP to give a glycal intermediate, followed by a *syn* addition of water to generate product (Figure 5).

The NeuB homologue was found to possess very low, but measurable, levels of *N*,*N*′-diacetyllegionaminic acid synthase activity. It is difficult to explain why the activity was so low, but the need for a fusion protein to obtain soluble enzyme may indicate that the synthase is normally active as a complex with another protein in vivo. No such problems in obtaining active protein were encountered in previous studies on the NeuB of sialic acid biosynthesis in *N. meningitidis*, indicating that it is not due to an inherent property of such synthases. Nevertheless, studies of the NeuC of sialic acid biosynthesis in *E. coli* were hampered by extremely low levels of product formation (2-acetamidoglucal and UDP were formed instead) (*16*). It was suggested that the in vitro assay conditions did not accurately reflect those required for in vivo activity, possibly lacking key protein–protein interactions. One other potential explanation for the low activity in this study could be that the true substrate is actually 2,4,6-trideoxy-2-acetamidino-4-acetamidomannose with the amidine group installed into the free sugar prior to its condensation with PEP. We feel that this scenario is unlikely given the strong evidence in support of such a modification occurring at the level of a CMP-sugar nucleotide in the pseudaminic acid biosynthetic pathway (*23*), and the existence of unmodified *N*,*N*′-diacetyllegionaminic acid in other bacterial species (*7, 9*). This low level of activity was not observed when ManNAc was used in place of 2,4-diacetamido-2,4,6-trideoxymannose, indicating that the NeuB homologue is not involved in the biosynthesis of sialic acid. This is somewhat contradictory to the report that the *L. pneumophilla* neuB homologue gene complements capsule negative *E. coli* K1 mutants that were deficient in sialic acid biosynthesis (*28*). A possible explanation could be that the levels of synthase activity are much higher in vivo when the protein is in its natural environment, and even a low background rate of sialic synthase activity that was not measurable in vitro could result in complementation. Despite the low level of *N*,*N*′-diacetyllegionaminic acid activity, it was possible to show that the stereochemistry of the product was identical to that of synthetic material, indicating that

the PEP added to the *si* face of the open chain aldehyde. Furthermore, the activity was dependent on the presence of a divalent metal ion, and the reaction proceeded via a C−O bond cleavage process, similar to the reactions catalyzed by the sialic acid and pseudaminic acid synthases (Figure 8) (*17, 18, 20*).

Finally, the NeuA homologue readily converted *N*,*N*′-diacetyllegionaminic acid and CTP into CMP-*N*,*N*′-diacetyllegionaminic acid as expected for a CMP-Leg5Ac7Ac synthetase. Together with the three enzymes that convert UDP-GlcNAc into UDP-Bac2Ac4Ac, these three enzymes complete a pathway for the biosynthesis and activation of legionaminic acid in *L. pneumophila*. Their three genes, *lpg0751*−*lpg0753*, form a tight group with the *neuA* and *neuB* genes overlapping by 4 bp and the *neuB* and *neuC* genes being separated by only 6 bp. The remainder of the LPS locus (*28*) also has candidate genes for some of the other steps in the overall LPS biosynthetic pathway. The two adjacent genes, *lpg0754* and *lpg0755*, are strong candidates for the second and third genes involved in Bac2Ac4Ac biosynthesis, i.e., the aminotransferase and the acetyltransferase. *lpg0754* is annotated as an acetyltransferase, and this gene overlaps the *neuC*, by 10 bp. Though it belongs to an acetyltransferase family different from that of PglD, so do many other *N*-acetyltransferases with amino sugar substrates. Additionally, it is not the *lag-1* gene responsible for the 8-O-acetylation of the LPS (*28*). *lpg0755* is the closest homologue of PglE (Cj1121c) in the *Legionella* genome and, therefore, a strong candidate for the Bac aminotransferase. With regard to the third enzyme required for Bac synthesis, the dehydratase PglF (Cj1120c), there are dehydratase genes in the LPS locus, currently annotated as rhamnose biosynthesis genes, but they exhibit a level of sequence identity to Cj1120c lower than that of the gene *lpg0966*, which lies outside the LPS locus. On the basis of the Leg pathway in *C. coli* (*10*), after CMP-Leg synthesis, the next enzyme in the pathway to the LPS polymer would carry out the conversion of the 5-acetyl substituent to acetamidino. There is a homologue of the relevant *Campylobacter* enzyme Cj1324 (*10*) nearby, *lpg0748*. The activities of the Neu homologues reported here therefore confirm the core part of the pathway postulated for Leg synthesis from UDP-Bac2Ac4Ac (*36*), and the assignments given above suggest the genes for other key steps in the overall process are also adjacent in the locus.

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SUPPORTING INFORMATION AVAILABLE

SDS−PAGE gels for the purification of the NeuA−C homologues (Figures S1−S3) and ^{1}H NMR spectra for the substrates of each enzyme (Figures S4−S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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