Sialic Acid Metabolism and Systemic Pasteurellosis

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Received 24 June 2004/Returned for modification 13 August 2004/Accepted 27 October 2004

Pasteurella multocida subsp. multocida is a commensal and opportunistic pathogen of food animals, wildlife, and pets and a zoonotic cause of human infection arising from contacts with these animals. Here, an investigation of multiple serotype A strains demonstrated the occurrence of membrane sialyltransferase. Although P. multocida lacks the genes for the two earliest steps in de novo sialic acid synthesis, adding sialic acid to the growth medium resulted in uptake, activation, and subsequent transfer of sialic acid to a membrane acceptor resembling lipooligosaccharide. Two candidate-activating enzymes with homology to Escherichia coli cytidine 5'-monophospho-N-acetylneuraminic acid synthetase were overproduced as histidine-tagged polypeptides. The synthetase encoded by pm0187 was at least 37 times more active than the pm1710 gene product, suggesting pm0187 encodes the primary sialic acid cytidylyltransferase in P. multocida. A sialate aldolase (pm1715) mutant unable to initiate dissimilation of internalized sialic acid was not attenuated in the CD-1 mouse model of systemic pasteurellosis, indicating that the nutritional function of sialate catabolism is not required for systemic disease. In contrast, the attenuation of a sialate uptake-deficient mutant supports the essential role in pathogenesis of a sialylation mechanism that is dependent on an environmental (host) supply of sialic acid. The combined results provide the first direct evidence of sialylation by a precursor scavenging mechanism in pasteurellae and of a potential tripartite ATP-independent periplasmic sialate transporter in any species.

Pasteurella multocida subsp. multocida (hereafter referred to as P. multocida) is an economically important opportunistic pathogen of livestock (hemorrhagic septicemia, shipping fever, and atrophic rhinitis), poultry (fowl cholera), wildlife (avian cholera), and laboratory rabbits (snuffles) (1). As a commensal of cats and dogs, P. multocida also is a significant cause of zoonotic abscesses arising mainly from bites or scratches, but it has been increasingly associated with pulmonary disease, sepsis, and meningitis in patients with underlying medical conditions that may compromise their immune status (4, 10, 13, 15, 16, 20, 21, 29, 30, 35, 38, 48). Although the so-called dermonectic toxin synthesized by some P. multocida strains produces atrophic rhinitis, the toxin is not essential for respiratory or systemic disease. The P. multocida virulence factors responsible for life-threatening invasive infections are poorly defined despite complete genomic DNA sequencing (36), microarray gene-expression profiling (7, 8), in vivo expression technology (25), and signature tagged mutagenesis (19, 23). Known or suspected virulence factors include adhesins, systems for iron acquisition, lipooligosaccharide (LOS), capsules, and neuraminidases (1). However, with the exception of capsule (11), the function in pathogenesis or relative importance of most other virulence factors is either unknown or uncertain. Evidence is provided here that the metabolism of sialic acids should be included as a potentially essential disease factor in systemic pasteurellosis.

We are interested in the metabolism of the sialic acids, a structurally diverse group of nine-carbon keto sugars (3, 9), and have hypothesized that the interplay between its metabolism and the host-microbe interaction is a unifying theme for understanding diseases caused by a wide range of invasive pathogens or commensals (51–53). For example, Escherichia coli K1 and certain meningococci synthesize sialic acid by a de novo pathway for assembly of sialic acid into outer membrane capsules or lipooligosaccharides (LOS) that are known to inhibit host innate immunity (44). Other pathogens, such as Neisseria gonorrhoeae and Haemophilus spp., do not synthesize sialic acid but scavenge host-derived (environmental) sialic acids for cell surface decoration (modification) involving endogenous sialyltransferases. All known sialyltransferases use the activated nucleotide sugar cytidine 5'-monophospho (CMP)-sialic acid as the obligate donor substrate for transfer of sialyl units to appropriate acceptor substrate molecules. In addition to de novo synthesis or scavenging of sialic acid, some pathogens may also catabolize environmental sialic acid for nutrition (carbon, nitrogen, and energy) or as a source of amino sugars for cell wall biosynthesis (40). How organisms that both decorate their surfaces with sialic acid and use it for nutrition regulate the metabolic decision between surface modification and degradation has not been investigated until recently (27, 41, 42, 54, 55).

Here, for the first time, sialyltransferase is demonstrated in P. multocida, and the role of sialometabolism in the host-pathogen interaction is investigated in an animal model of systemic pasteurellosis. In addition, the first evidence is pro-
provided for a novel sialate uptake system involving a putative tripartite ATP-independent periplasmic (TRAP) transporter of the type that functions in carboxylic acid transport in a wide range of bacterial species (18, 28). The combined results indicate that sialometabolism may be a common feature of the entire genus and begin to delineate the importance of sialylation to systemic Pasteurella. (A preliminary account of a portion of this study was presented by E. Vimr and C. Lichtensteiger at the 101st General Meeting of the American Society for Microbiology in Orlando, Fla., 2001 [abstr. B-446].)

FIG. 1. Genetic organization of sialocatabolic systems in selected gram-negative bacteria. On the basis of the known functions encoded by the nan genes in E. coli (A) (nanR, transcriptional regulator; nanA, sialate lyase; nanT, sialate transporter; nanE, ManNAc 6-phosphate epimerase; nanK, ManNAc kinase, and yhcH, function unknown [27, 40, 51]), homologous genes in H. influenzae (B) and P. multocida (C) were assigned equivalent functions as indicated by the color-coded ORFs (large arrows). Note that the function of pm1710 is unknown, but is presumed to encode a cytidylyltransferase, whereas H10141 and H10140 encode glucosamine deaminase and N-acetylglucosamine deacetylase, respectively. Bent arrows indicate known or predicted promoters. The functions of H10148 and pm1707 also are unknown. Open triangles indicate the insertion of the kanamycin-resistant transposon described in reference 19. Arrows underneath triangles indicate transcriptional direction of the insertionally inactivated genes. Note that an insertion in the same transcriptional frame as the inactivated gene is nonpolar (14).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Pm70 is a spontaneous capsule-negative, nalidixic acid-resistant turkey isolate kindly provided by V. Kapur (36). TF5 is a nalidixic acid-resistant type A bovine isolate with defined systemic virulence in the CD-1 mouse model (19). TF5E (PM1B-E11) and TF5P (PM3-C9) are TF5-derived strains bearing kanamycin-resistant disruptions of pm1711 and pm1709, respectively, isolated from CD-1 mice after signature-tagged mutagenesis of TF5 (19). The transcriptional orientations of the kanamycin cassettes in these mutants are given in Fig. 1. X-73 is a type A avian isolate kindly provided by M. Wolkett (National Wildlife Health Center, Madison, Wis.). Cincy is a type A strain isolated from a clinical case of human pneumonia kindly provided by M. Wolcott (National Wildlife Health Center, Madison, Wis.). Cincy was passaged once before cryopreservation. The strain involving pulmonary contusions; it was given to us by J. Solomkin (University of Wisconsin). Cincy is a type A strain isolated from a clinical case of human pneumonia kindly provided by M. Wolcott (National Wildlife Health Center, Madison, Wis.). Cincy was passaged once before cryopreservation. The P. multocida strains were grown in vitro in the presence of kanamycin to select for cointegrate maintenance.

CML-sialic acid synthetase overproduction. The genes pm1710 and pm0187 encoding putative activating enzymes were cloned into pCR CT TOPO TA cloning kit (version B) after PCR amplification of the complete open reading frames (ORFs) with N- and C-terminal primers to generate pSX1000 and pSX1001, respectively, by previously described methods (41). All PCR primers were synthesized by IDT (Corvald, Iowa). Polypeptides were overproduced by IPTG (isopropyl-p-D-thiogalactopyranoside) induction as C-terminal His, fusions (42).

Biochemical assays. N-Acetylneuraminic acid (Neu5Ac; the most common sialic acid) was measured by colorimetric thiobarbituric acid assay (53). Sialyltransferase was detected by incubating membrane samples derived by sonic disruption in 20 mM Tris (pH 8.0), 5 mM magnesium acetate, and 2 mM dithiothreitol (TMD) buffer with CMP-[4-14C]Neu5Ac (50 mCi/mmol), followed by descending paper chromatography in solvent system I (7:3, ethanol-ammonium acetate [pH 7.5]) and liquid scintillation spectrometry of radioactivity remaining at the origin essentially as described previously (54, 55). Quantitative estimation of sialyltransferase activity is described in detail below. Protein was estimated by dye binding (Pierce Chemical Co., Chicago, Ill.) with bovine serum albumin as standard.

Sialidase was measured as relative fluorescence units with the fluorogenic substrate 2′-(4-methylumbelliferyl)-Neu5Ac (MuNeu5Ac) as previously described (50). Sialic acid uptake was measured by depletion assay using unlabelled or [4-14C]Neu5Ac (55 mCi/mmol) as previously described (53, 54). CMP-sialic acid synthetase was detected autoradiographically after incubation of soluble cell extracts with 10 mM CTP and 18 μM radiolabeled Neu5Ac, followed by chromatography in solvent system I or by thin-layer chromatography as previously described (45). Quantitative estimation of sialyltransferase activity was accomplished by excising regions of the chromatograms containing CMP-Neu5Ac product, followed by liquid scintillation spectrometry. Sialate aldolase was detected autoradiographically after incubation of extracts with radiolabeled Neu5Ac and chromatography in solvent system II (n-propanol-M sodium acetate [pH 5.0]-water, 7:1:2), with [1-14C]ManNAc (50 mCi/mmol) as the standard (41). All radiochemicals were purchased from American Radiochemical Company (St. Louis, Mo.). Type V sialidase from Clostridium perfringens and all other chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.).

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TABLE 1. Putative P. multocida ORFs involved in sialic acid metabolism

<table>
<thead>
<tr>
<th>Homolog</th>
<th>P. multocida ORF*</th>
<th>% Identity (no. of residues)</th>
<th>Function†</th>
<th>Proposed gene designation in P. multocida</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeuA</td>
<td>0187</td>
<td>38 (220)</td>
<td>CMP-sialate synthetase</td>
<td>neuA</td>
</tr>
<tr>
<td>NeuB</td>
<td>1710</td>
<td>30 (187)</td>
<td>Cytidylyltransferase‡</td>
<td>None</td>
</tr>
<tr>
<td>NeuC</td>
<td>None</td>
<td>0</td>
<td>Sialate synthase</td>
<td>None</td>
</tr>
<tr>
<td>NanA</td>
<td>1715</td>
<td>37 (290)</td>
<td>UDP-GlcNAc 2-epimerase</td>
<td>nanA</td>
</tr>
<tr>
<td>NanE</td>
<td>1711</td>
<td>54 (212)</td>
<td>Sialate aldolase</td>
<td>nanE</td>
</tr>
<tr>
<td>NanK</td>
<td>1712</td>
<td>41 (277)</td>
<td>ManNAc-6-P 2-epimerase</td>
<td>nanK</td>
</tr>
<tr>
<td>NanT</td>
<td>0835</td>
<td>35 (191)</td>
<td>ManNAc kinase</td>
<td>None</td>
</tr>
<tr>
<td>NagA</td>
<td>0874</td>
<td>54 (377)</td>
<td>GlcNAc-6-P deacetylase</td>
<td>nagA</td>
</tr>
<tr>
<td>NagB</td>
<td>0875</td>
<td>72 (266)</td>
<td>GlcN-6-P deaminase</td>
<td>nagB</td>
</tr>
<tr>
<td>YecH</td>
<td>1713</td>
<td>45 (129)</td>
<td>Unknown</td>
<td>None</td>
</tr>
<tr>
<td>ST0160b</td>
<td>0188</td>
<td>37 (387)</td>
<td>α2,6-Sialyltransferase</td>
<td>neuS1</td>
</tr>
<tr>
<td>NanH †</td>
<td>0663</td>
<td>&lt;25</td>
<td>Sialidase-1</td>
<td>nanHp</td>
</tr>
<tr>
<td>NanH †</td>
<td>1000</td>
<td>&lt;25</td>
<td>Sialidase-2</td>
<td>nanHp</td>
</tr>
<tr>
<td>YiaO</td>
<td>1709</td>
<td>30 (305)</td>
<td>Sialate uptake</td>
<td>nanP</td>
</tr>
<tr>
<td>HI0147f</td>
<td>1708</td>
<td>82 (629)</td>
<td>Sialate (up)take</td>
<td>nanU</td>
</tr>
<tr>
<td>HI0148f</td>
<td>1707</td>
<td>&lt;25</td>
<td>Unknown</td>
<td>None</td>
</tr>
<tr>
<td>HI1699f</td>
<td>0508</td>
<td>40 (124)</td>
<td>α2,3-Sialyltransferase</td>
<td>neuS2</td>
</tr>
</tbody>
</table>

* Unless indicated otherwise, all homologs refer to E. coli K-12 or K1 gene products.
† May et al. (36).
‡ P. phosphatase.
§ Photobacterium damsela (57).
¶ Salmonella enterica Typhimurium strain LT2 (24).
‖ H. influenzae strain Rd (17).
* pm1710 also encodes a region that is 80% identical over 198 amino acid residues and 57% identical over 195 residues with the putative lysophospholipases of Actinobacillus pleuropneumoniae.

Sialyltransferase assay. Quantitative comparisons of sialyltransferase activities in mammalian and avian P. multocida were carried out by a modification of the procedure described above. Single colonies from the indicated strains were inoculated into 25 ml of BHI and grown to stationary phase. Cultures of encapsulated strains were treated for 1 h at room temperature with 100 μl of hyaluronidase to facilitate cell collection by centrifugation. All succeeding preparative steps were carried out at 0 to 4°C. Cell pellets were resuspended in 0.5 ml of TMD buffer and disrupted by sonication (Branson Cell Disrupter 185 with microtip): four cycles of 20 s each on an ice bath output setting of 4 with 45 s of cooling between each cycle. Intact cells were removed by centrifugation at 4,000 × g for 4 min. Supernatants were diluted with TMD to a final volume of 3 ml, and membrane-bound activities were determined by analysis of variance (P < 0.0001), followed by comparisons of means by the Tukey method (α = 0.05). Animal experiments. Outbred (5- to 7-week-old) CD-1 female mice were purchased from Charles River Laboratories (Wilmington, Mass.) and housed at the University of Illinois animal care office after institutional review according to National Institutes of Health guidelines. CI differing from 1.0 was analyzed by a one sample, two-way t test using log transformation.

In vivo detection of sialyl acceptor. Wild-type P. multocida strain Pm70 or the Pm70A mutant were grown in 2 ml of BHI and exposed at an A600 of 0.4 for 1 h to 5 μg of Neu5Ac/ml containing a tracer amount of radiolabeled Neu5Ac. Bacteria were collected by centrifugation; residual liquid was removed by careful wicking the tube walls around the pellets. Wild-type and mutant bacteria removed 40 and 70%, respectively, of the label from the culture medium during the 1-h incubation period. Bacterial pellets were resuspended in 9 μl of 10 mM Tris (pH 8.0) containing 5 μg of lysozyme/ml, followed by the addition of 1 μl of 0.5 mM EDTA. Cells were ruptured by three freeze-thaw cycles, and 0.6 μl of 1 M MgCl2 was added prior to treatment with DNase I as previously described (49).

Membrane and soluble samples were prepared by centrifugation. Half of each soluble fraction was analyzed by descending paper chromatography in solvent system II by autoradiography, as previously described (42). The membrane fraction was resuspended in 9 μl of water and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 4 to 20% gradient gel. The gel was stained with Coomassie blue, fluorescent, and subjected to autoradiography for 3 days prior to film development. Tricine gels (16.5%) were purchased from Bio-Rad.

RESULTS

Predicted functions of P. multocida strain Pm70 ORFs with presumed roles in sialometabolism. When a BLAST (2) comparison of known E. coli K1 sialometabolic gene products and the P. multocida strain Pm70 genomic DNA sequence (36) was carried out, orthologs of NeuB (sialate synthase) and NeuC (UDP-GlcNAc 2-epimerase) were not detected (Table 1). The absence of neuB- and neuC-like genes indicated that P. multocida lacks the two earliest steps in the pathway for de novo sialic acid synthesis (53). In contrast, P. multocida potentially encodes two CMP-Neu5Ac synthetases (pm0187 and pm1710), an ortholog of the α2,6-sialyltransferase (encoded by pm0188)
from the marine organism *Photobacterium damsela* (57), and a potential α2,3-sialyltransferase that is homologous to one of the *Haemophilus influenzae* LOS sialyltransferases (8). The absence of genes for de novo sialate synthesis but the presence of ORFs potentially coding for activating enzyme(s) and sialyltransferases suggested that *P. multocida* may use a sialylation pathway similar to that described previously for *H. influenzae* (53). This precursor scavenging pathway involves the uptake of environmental sialic acid, followed by its activation by CMP-sialic acid synthetase (NeuA) and subsequent transfer of sialic acid to an appropriate membrane acceptor by sialyltransferase (Fig. 2). As shown in Table 1, *P. multocida* also potentially encodes a complete pathway for sialic acid catabolism, including two membrane sialidases with distinct substrate specificities for hydrolyzing host sialoglycoconjugates (37). The combined in silico analysis of Pm70 ORFs suggests that, with the exception of de novo Neu5Ac biosynthesis, *P. multocida* is genetically equipped to carry out a full range of sialometabolic functions including uptake, and either activation of internalized sialic acid to an appropriate membrane acceptor by sialyltransferase (Fig. 2). As shown in Table 1, *P. multocida* also potentially encodes a complete pathway for sialic acid catabolism, including two membrane sialidases with distinct substrate specificities for hydrolyzing host sialoglycoconjugates (37). The combined in silico analysis of Pm70 ORFs suggests that, with the exception of de novo Neu5Ac biosynthesis, *P. multocida* is genetically equipped to carry out a full range of sialometabolic functions including uptake, and either activation of internalized sialic acid for cell surface modification or cleavage to N-acetylmannosamine (ManNAc) and pyruvate for nutrition or source of amino sugars for cell wall biosynthesis (Fig. 2). Since *P. multocida* is an obligate commensal of mammals, birds, and reptiles, including dragons (38), all of which synthesize sialic acid, sialometabolism is likely to be a central aspect of this bacterium’s unusually broad range of host interactions, including the potential to cause disease.

**Detection of sialyltransferase.** If *P. multocida* uses a sialylation mechanism similar to that of *H. influenzae* (53), the isolated membrane fraction should transfer sialic acid from exogenous CMP-Neu5Ac substrate to endogenous membrane acceptor(s). To test for this activity, we incubated the membrane fractions from sonically disrupted bacteria with radiolabeled CMP-Neu5Ac and measured the incorporation of labeled sialic acid into membranes by descending paper chromatography in solvent system I. Table 2 indicates that all of the *P. multocida* strains tested (including two type D strains, TF5E, and X-73 [results not shown]) had detectable transferase activity. The highest specific activity was consistently observed with mammalian isolates, a finding comparable in magnitude to the activities of *E. coli* and meningococcal polysialyltransferases (46). A control experiment with radiolabeled Neu5Ac instead of CMP-Neu5Ac as the substrate did not result in detectable sialyltransferase activity, which is consistent with the known substrate dependency of sialyltransferases for activated sialic acid.

To determine whether the variability in sialyltransferase specific activities resulted from endogenous sialidases (Fig. 2),
hydrolysis of the fluorogenic sialoside MuNeu5Ac was measured at basic and acidic pH. As expected from the known acidic pH optima of *P. multocida* sialidases (37), activity against the sialoside was greater at pH 6.8 than at pH 8.0 (Table 2). However, there were no appreciable differences between sialidase activities of mammalian or the avian isolates tested. The greater activity of the aldolase-negative derivative of Pm70 (Pm70A) may reflect induction by trace sialic acid in the medium, as previously observed for the inducible nan system in *E. coli* (41, 42, 49).

To directly investigate the susceptibility of the sialylated acceptors to endogenous sialidases, radiolabeled Pm70 membranes were diluted 50-fold into assay buffer, pelleted by centrifugation, and resuspended to the same volume as for the initial radiolabeling. Samples assayed over a 3-h period showed no loss of radioactivity, indicating no effect of endogenous sialidase on sialic acid release. In contrast, 0.5 U of exogenous *C. perfringens* sialidase released over half of the radioactivity in 1 h, indicating sialyl residues were added by sialyltransferase in their expected terminal α-glycoketosidic linkages to endogenous acceptor(s). Furthermore, proteinase K digestion did not release label, and Pm70 lacks the type A hyaluronate capsule, strongly suggesting that the endogenous acceptor is neither protein nor capsular polysaccharide. We concluded that sialyltransferase is a consistent phenotype of at least *P. multocida* subsp. *multocida*, which is responsible for most animal disease (1).

Table 2. Sialyltransferase and sialidase specific activities in *P. multocida*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Sialyltransferase (pmol/mg of protein)*</th>
<th>Sialidase (RFU/mg of protein)² at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH 8.0</td>
<td>pH 6.8</td>
</tr>
<tr>
<td>Cincy</td>
<td>Human</td>
<td>413 ± 51*</td>
<td>700</td>
</tr>
<tr>
<td>Pm70</td>
<td>Avian</td>
<td>133 ± 29</td>
<td>1,200</td>
</tr>
<tr>
<td>Pm70A</td>
<td>Avian</td>
<td>159 ± 37</td>
<td>2,800 ND</td>
</tr>
<tr>
<td>TF5</td>
<td>Bovine</td>
<td>397 ± 50*</td>
<td>500</td>
</tr>
</tbody>
</table>

*Activity higher than avian strains (see Materials and Methods for a description of the statistical method). ND, not done; RFU, relative fluorescence units.

Identification of CMP-Neu5Ac synthetase. The absence of orthologs of *neuB* and *neuC* but the presence of sialyltransferase implies *P. multocida* scavenges environmental sialic acid and activates it intracellularly using one or both of the putative CMP-Neu5Ac synthetase candidates indicated in Table 1. One of these candidate synthetases (encoded by pm0187) is located adjacent to the putative α2,6-sialyltransferase encoded by pm0188, whereas the other (pm1710) is predicted to be the first gene of the *P. multocida* nan-like operon (Fig. 1). PCR analysis of 32 *P. multocida* type A or D isolates from our swine collection (33) resulted in the successful amplification of the expected DNA products from pm1710 or pm0187 in all strains tested (data not shown). The ubiquity of these candidate *neuA* orthologs in *P. multocida* is consistent with the sialyltransferase-positive phenotype documented in Table 2.

*Campylobacter* spp. and *Legionella pneumophila* have been shown to express multiple orthologs of *neuA* or *neuB* (32, 34), suggesting that both pm0187 and pm1710 could encode functional sialate activating enzymes despite the greater similarity of the pm1710 gene product to phospholipidase than cytidylyltransferase (Table 2). To determine whether either of these ORFs codes for an active CMP-Neu5Ac synthetase, the respective genes were cloned into a vector designed to express gene products in frame with C-terminal His₆ tags. As shown in Fig. 3A, pm0187 complemented an *E. coli* K12/K1 hybrid strain with a mutation in *neuA*. This gene normally encodes CMP-sialic acid synthetase, which catalyzes a necessary step in polysialic acid synthesis (K1 antigen) synthesis (55). To detect complementation of the *neuA* defect in EV5, bacteria expressing basal amounts of pm0187 or pm1710 are cross-streaked against K1-specific bacteriophage painted (arrows) down the center of the plate. Strains expressing the K1 capsule are sensitive to the lytic action of the bacteriophage, whereas those not expressing the capsule are resistant and grow beyond the phage streak. (A) Complementation by pSX1001 (pm0187); (B and C) lack of complementation by two independent EV5 transformants harboring the two independent pSX1000 constructs (pm1710). (D) EV5 transformed with pUC18.

![FIG. 3. Complementation of EV5 (*neuA*). EV5 is an *E. coli* K12/K1 hybrid strain with a mutation in *neuA*. This gene normally encodes CMP-sialic acid synthetase, which catalyzes a necessary step in polysialic acid biosynthesis, whereas pm1710 did not (Fig. 3B), suggesting that only pm0187 encodes a functional synthetase. Similarly, while an extract of wild-type Pm70 had both detectable Neu5Ac aldolase and CMP-Neu5Ac synthetase activity (Fig. 4A, lane 3), only the His₆-tagged pm0187 product produced CMP-Neu5Ac from CTP and radiolabeled Neu5Ac (compare lanes 2 to 4 to lanes 5 to 7 in Fig. 4B). The results of complementation and direct biochemical analyses indicate that the pm0187 is a functional NeuA ortholog.

To further investigate the potential cytidylyltransferase activity of pm1710, we compared its relative activity to that of the pm0187-encoded NeuA ortholog. As shown in Fig. 5, NeuA had, as expected, high synthetase activity that saturated between 56 and 113 μg of protein. In contrast, no activity of the extract containing the overproduced pm1710 gene product was detected until 159 μg of protein was tested, resulting in at least a 37-fold-lower specific activity than NeuA. These results suggest that if pm1710 encodes a cytidylyltransferase, its substrate is unlikely to be Neu5Ac. Further work is necessary to deter-
mine the function of the pm1710 gene product, but the present results indicate that NeuA encoded by pm0187 is the primary if not the sole Neu5Ac synthetase in *P. multocida*. The results in Table 1 suggest that pm1710 may encode a phospholipase or esterase, similar to the C-terminal domain of *E. coli* NeuA (31).

**Scavenging environmental sialic acid.** Although *P. multocida* lacks candidate genes for the two earliest steps in sialic acid synthesis (Table 1), the results described above demonstrate that it has the genetic information for Neu5Ac activation and sialyl transfer. In addition, an extract of wild-type *P. multocida* has a sialate aldolase activity that could initiate the catabolism of environmental sialic acid, as indicated by the product migrating with the same relative migration (*R*<sub>f</sub>) value (0.83) as authentic ManNAc (Fig. 4A, lane 3). Therefore, if *P. multocida* uses a precursor scavenging mechanism for cell surface sialylation, there must be an uptake system for sialic acid that provides the substrate for both intracellular catabolic and activating enzymes (Fig. 2). The results presented in Table 1 suggested that pm0835 could encode an ortholog of NanT, whereas pm1708, together with pm1709, was a likely candidate for a TRAP sialate transporter.

Sialate uptake was demonstrated directly by a chromogenic depletion assay (53), indicating that wild-type strain TF5 quantitatively removed Neu5Ac during growth in supplemented HTM (Table 3). In contrast, growth of TF5P in supplemented HTM did not result in Neu5Ac depletion (Table 3), strongly suggesting that the polypeptide encoded by pm1709 is required for sialate uptake. This conclusion was confirmed by uptake of radiolabeled Neu5Ac during growth of the wild type but not TF5P in HTM containing 10 μM unlabeled and a tracer amount of labeled Neu5Ac. Wild-type TF5 took up nearly 50% of the label by 1 h, whereas the input counts per minute (cpm) remaining in the culture medium of TF5P remained constant when sampled at 1-, 2-, or 3-h intervals (99.2% ± 1.3% [standard deviation]). Interestingly, with wild-type bacteria the number of extracellular cpm actually increased after the initial 1-h labeling period, resulting in ca. 20% more radioactivity detected in the medium by 3 h compared to that present after the first hour. We interpret this increase in cpm as the excretion of fermentative end products, because Neu5Ac is clearly metabolized after uptake as shown by the nearly 70% growth stimulation of TF5 relative to TF5P (Table 3). Note that the uptake results imply *P. multocida* uses only one system for sialic acid transport, at least under the conditions of laboratory growth used for these experiments, suggesting that the putative NanT ortholog encoded by pm0835 is not involved in sialate uptake (Fig. 2).

**Isolation and characterization of *P. multocida* sialate aldolase mutants.** To confirm the presence of a functional sialate dissimilatory system in *P. multocida*, we constructed a gene disruption of the strain Pm70 *nanA* ortholog (pm1715) by homologous recombination with a pGPKan derivative harboring an internal (500 bp) fragment of the gene; the resulting mutant was designated Pm70A. ORF pm1715 is predicted to be the last gene of the *P. multocida* nan-like dissimilatory operon (Fig. 1C); thus, the gene disruption would have no effect other than loss of the proposed aldolase activity encoded by pm1715. An identical gene disruption was constructed in the bovine *P. multocida* isolate TF5 (18), which was designated...
TF5A. The loss of aldolase activity was detected by direct biochemical assay with radiolabeled Neu5Ac (Fig. 6, lane 6). Extracts prepared from either Pm70A or TF5A lacked detectable sialate aldolase activity (Fig. 6, lanes 1 and 5, respectively), whereas extracts from either wild-type parent (Fig. 6, lanes 2 and 3), as expected, produced a radiolabeled product with the same mobility as authentic ManNAc (Fig. 4, lane 7). An extract of TF5E bearing a nonpolar kanamycin resistance cassette (14) transcribed in the same direction as pm1711 expressed wild-type aldolase activity (Fig. 4, lane 4). Although we did not directly confirm that pm1711 encodes ManNAc-6-P epimerase, its homology to E. coli nanE (Table 1) and its proximity to sialyltransferase(s), we expected to identify the in vivo acceptor recognized by P. multocida sialyltransferase(s), we expected that a growing culture of Pm70A would efficiently transport and activate exogenous radiolabeled Neu5Ac, because there would be no competition for label from the catabolic system, as originally observed with E. coli nanA mutants (55). However, most of the label accumulated by Pm70A remained as free Neu5Ac (Fig. 7, lane 1), making it difficult to identify the endogenous membrane acceptor (Fig. 8A and B, lanes 5 and 7). In contrast, wild-type strain Pm70 produced a readily detectable CMP-Neu5Ac signal (Fig. 7, lane 2) that was correlated with a membrane-associated product with electrophoretic mobility near the dye front and apparent mass of <14 kDa (Fig. 8A and B, lanes 4 and 6). The radiolabeled product remaining in the wells is likely to be peptidoglycan, as sug-

FIG. 5. Relative Neu5Ac activation by pm0187 or pm1710 gene products. Aliquots containing 14, 28, 56, or 113 μg of protein from an induced soluble extract of strain BL21(DE3) Star harboring pSX1001 (pm0187) were incubated with CTP and radiolabeled Neu5Ac for 30 min at 37°C. The nucleotide sugar produced by the reaction (inset) was quantified and expressed as picomoles of CMP-Neu5Ac under the defined assay conditions (○). Aliquots containing 40, 59 and 159, or 318 μg of protein were assayed in the same manner (○) with an extract of the induced strain harboring pSX1000 (pm1710). Values in parentheses indicate the specific activities of each enzyme preparation determined from the linear portion of the curves, indicating at least 37 times greater activity in the extract containing the pm0187 gene product. Extracts contained similar amounts of overproduced polypeptides as judged after SDS-PAGE and staining with Coomassie blue.

TF5 Wild type 1.77 ± 0.03 7.3 ± 8.5e
TF5P nanP 1.05 ± 0.03c 940 ± 37.5

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Growtha (A600)</th>
<th>Residual Neu5Aconc (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF5</td>
<td>Wild type</td>
<td>1.77 ± 0.03</td>
<td>7.3 ± 8.5e</td>
</tr>
<tr>
<td>TF5P</td>
<td>nanP</td>
<td>1.05 ± 0.03c</td>
<td>940 ± 37.5</td>
</tr>
</tbody>
</table>

a Cells from overnight cultures grown in HTM without aeration were diluted to an A600 of 0.02 in fresh HTM and allowed to grow aerobically for 6 h in the presence of approximately 1 μg of Neu5Ac/ml. The data shown are for three independent experiments. Note that growth of the wild type and the mutant strain was identical in unsupplemented HTM.

b At the end of the 6-h growth period, cells were collected by centrifugation, and the Neu5Ac remaining in the spent culture medium was quantified by colorimetric assay.

c Mutant not stimulated by sialic acid (Student t test; P = 0.0004).

d Wild-type transported exogenous sialic acid (Student t test; P = 0.0003)
gested by past results with *E. coli* in which ManNAc dissimi-
lation resulted in rapid labeling of the insoluble cell wall frac-
tion (55). This suggestion is consistent with the low detectable
amount of ManNAc in wild type (Fig. 7, lane 2) and with the
absence of any detectable peptidoglycan in Pm70A (Fig. 8B,
lanes 5 and 7), which cannot produce the ManNAc precursor.

Note that, as expected, all of the soluble radiolabel that was in
low-molecular-weight products passed through the gel in front
of the dye marker, exiting into the anode buffer (Fig. 7B, lanes
2 and 3). When membranes that were used for the sialyltrans-
ferases assays presented in Table 2 were solubilized and frac-
tionated by Tricine SDS-PAGE, only the broad band migrating
at <14 kDa was detected after autoradiography (data not
shown), a result which is consistent with the in vivo analysis
shown in Fig. 8. We conclude that the sole macromolecular
sialyl acceptor is a relatively low molecular weight product
resembling LOS in its electrophoretic behavior. This conclu-
sion is consistent with the biochemical results presented above,
and previous results in the *H. influenzae* system (see reference
53 and references therein). Both the products of the in vivo
and in vitro sialylation reactions will now be open to structural
characterization.

**Requirement for sialometabolism during systemic pasteu-
rellosis.** *P. multocida* strain Pm70 lacks the type A hyaluronate
capsule that is essential for systemic virulence. Therefore, all
animal experiments were carried out with encapsulated TF5 or
its mutant derivatives. As summarized in Table 4, strain TF5
was previously found to be highly virulent in the CD-1 mouse
model of systemic pasteurellosis (LD<sub>50</sub> of <10 CFU), whereas TF5E and TF5P had >10,000 times
higher LD<sub>50</sub> values in this model (19). Because the data pre-
sented above strongly suggest that the pm1709 gene product is
required for sialate transport, we concluded that sialometabo-
listism is likely to be a necessary feature of systemic pasteurel-
losis. To correlate the CI with LD<sub>50</sub> as an alternative measure-
ment of virulence, we determined the CI for TF5E, indicating
that a 500-fold decrease in relative fitness corresponds to a

**TABLE 4. Nutritional function of sialometabolism is not required
in the CD-1 mouse model of systemic pasteurellosis**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;</th>
<th>CF vs. wild type (no. of animals) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF5</td>
<td>Wild type</td>
<td>&lt;10</td>
<td>1.0</td>
</tr>
<tr>
<td>TF5E</td>
<td>nanE</td>
<td>3 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.002 ± 0.002 (3)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>TF5A</td>
<td>nanA</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.958 ± 0.236 (6)</td>
</tr>
<tr>
<td>TF5P</td>
<td>nanP</td>
<td>6 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> LD<sub>50</sub> values are taken from Fuller et al. (19).
<sup>b</sup> ND, not done.
<sup>c</sup> CI of the indicated strain versus the wild type, with the number of animals
used for the determination shown in parentheses. Note that a CI of 1.0 indicates
no advantage to either strain in the mixed challenge infection.
<sup>d</sup> One-sample t test (CI ≠ 1.0); P = 0.0001
>10,000-fold increase in LD50 (Table 4). Because both the pm1711 and the pm1709 gene products are required for sialate catabolism, but only that of pm1709 is required for LOS sialylation, we sought to distinguish between the possible nutritional requirement for Neu5Ac in vivo and its alternative use for surface modification.

To distinguish between these possibilities, we carried out mixed infections with wild type and the TF5A aldolase-deficient mutant. As shown in Table 4, despite the relatively weak activation of Neu5Ac by Pm70A grown in HTM (Fig. 7), TF5A was as fit as the wild type for systemic propagation in the CD-1 mouse infection model. This result formally excludes a primary nutritional function of sialic acid catabolism in vivo, strengthening our hypothesis, as we previously described for the related H. influenzae system (53), that LOS sialylation is dependent on an external (environmental) source of sialic acid. Attenuation of TF5P is consistent with this hypothesis, since failure to transport exogenous sialic acid would result in the expression of unsialylated LOS. Independent analysis of H. influenzae sialylation has shown that very little sialic acid must be incorporated in vivo to provide a fitness advantage during otitis media (6). We suggest the same is likely to be true for P. multocida during systemic infection.

Although our combined results point to an essential role of sialylation in vivo, the attenuation of TF5E is difficult to reconcile with this hypothesis and suggests that, whereas Neu5Ac catabolism per se is not required for pathogenesis, as shown by the relatively wild-type fitness of the NanA mutant (Table 4), the ManNAc 6-phosphate expected to accumulate in a nanE mutant (41, 42) may be toxic during in vivo infection. Alternatively, a second mutation in TF5E, unrelated to the defect in pm1711, may account for the observed attenuation of this mutant. The single-infection control experiment described in Materials and Methods rules out instability of the pm1715 mutation as an explanation. Further biochemical analysis of LOS structure and genetic complementation will be necessary to determine the exact function of LOS sialylation in pasteurellosis and to understand the possible function of the pm1711 gene product in pathogenesis.

**DISCUSSION**

Since the discovery that H. influenzae sialylates its LOS by a precursor scavenging mechanism (52, 53), analogous pathways have been described in H. ducreyi (43) and H. somnus (26), suggesting that all haemophili may depend on an environmental source of sialic acid for LOS modification. Thus, bacteria that lack orthologs of neuB and neuC, which are needed for the two earliest steps in sialic acid biosynthesis (Fig. 2) (41, 42), but contain one or more copies of neuA and at least one sialyltransferase gene are likely to use precursor scavenging as a mechanism for surface sialylation. H. influenzae was subsequently shown to sialylate its LOS during infection in the chinchilla model of otitis media, and it was also shown that this surface modification was crucial to virulence (6). Although the exact function of sialylation during animal infection remains unclear, two recent studies have shown that sialic acid is required for normal H. influenzae biofilm formation, which may be a necessary process for colonization of mucosal surfaces (22, 47). In contrast, we have provided evidence that P. multocida sialylation, or at least sialate transport, may be necessary for systemic pasteurellosis, probably by protecting the sialylated bacteria from innate host defense mechanisms because mice do not normally carry P. multocida and would, therefore, not be expected to mount an antibody-dependent immune response under our conditions of experimental infection (1).

Perhaps in addition to a role of sialylation during systemic disease, the ability of obligate commensals and symbionts to modify their repertoire of surface carbohydrates arose as a mechanism for avoiding detection and clearance by the host’s immune system. This idea would imply that sialylation may be required for persistence and that, if so, a therapeutic approach blocking surface modification could have broad clinical value by causing certain microbes to become sensitive to host clearance mechanisms. Evidence that surface carbohydrate modification plays a role in symbiosis comes from Bacteroides spp., in which the synthesis of eight structurally distinct capsular polysaccharides varies randomly in response to a site-specific recombinase for reversible inversion of the different capsule biosynthetic operon promoters (12). In contrast, our results point to the variation at the level of LOS sialylation being dependent on a supply of host sialic acid.

An elevated serum sialoglycoconjugate concentration has been shown to be a general marker of inflammation, suggesting that endogenous or microbial sialidases could increase the extracellular free sialic acid concentration in response to any ongoing inflammatory process (51). Thus, by increasing their cell surface sialylation in response to elevated environmental sialic acid, some microbes may be protected under conditions that normally activate innate defense mechanisms that would ordinarily lead to detection and clearance of unsialylated species (51). The P. multocida lifestyle appears to be unusually committed to sialometabolism, including two sialidases, two potential sialyltransferases, at least one activating enzyme, and a complete system for sialic acid dissimilation, including sialic acid transport (Fig. 2). The present results suggest that sialic acid is essential for at least systemic propagation. This robust sialometabolic system might also account in part for the wide P. multocida host range that includes diverse mammalian, avian, and reptilian species.

A functional precursor scavenging system minimally requires mechanisms for sialic acid uptake, activation, and some way to regulate the metabolic decision between sialate catabolism and surface modification. To date, all known sialy acceptors in microbes that have been found to use a precursor scavenging mechanism are lipopolysaccharides in the outer membranes of gram-negative nasopharyngeal commensals. In the case of P. multocida, we have identified an activating enzyme (encoded by pm1087) and shown that a relatively low molecular weight membrane component resembling LOS is the endogenous acceptor. We have presented evidence here that at least the first gene (pm1709) of the potential operon defined by pm1007-1009 is required for sialate uptake. This uptake system is homologous to the TRAP transporters for carboxylic acids in diverse bacterial species, including H. influenzae (Fig. 1B). In contrast, the E. coli sialate transporter encoded by nanT (Fig. 1A) is a member of the major facilitator superfamily (MFS), indicating the existence of at least two distinct mechanisms of bacterial sialate uptake. Our results
Mizan, S., A. Henk, A. Stallings, M. Maier, and M. D. Lee.


Liu, G., J. Chunsheng, and C. Jin.

Kelly, D. J., and G. H. Thomas.

Editor: J. T. Barbieri