INFECTION AND IMMUNITY, Sept. 2000, p. 5261–5268 Vol. 68, No. 9
0019-9567/00/$04.00+0
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Lipooligosaccharide P^k (Galα1-4Galβ1-4Glc) Epitope of *Moraxella catarrhalis* Is a Factor in Resistance to Bactericidal Activity Mediated by Normal Human Serum

ANTHONY ZALESKI,[1] N. KAROLINE SCHEFFLER,[2] PETER DENSEN,[3] FRANK K. N. LEE,[1] ANTHONY A. CAMPAGNARI,[4] BRADFORD W. GIBSON,[2] AND MICHAEL A. APICELLA[1]*

Departments of Microbiology[1] and Internal Medicine,[3] The University of Iowa, Iowa City, Iowa; College of Medicine and Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California[2]; and Department of Microbiology, State University of New York at Buffalo, Buffalo, New York[4]

Received 5 April 2000/Returned for modification 5 June 2000/Accepted 13 June 2000

***Moraxella catarrhalis* is a respiratory pathogen responsible for acute bacterial otitis media in children and exacerbation of chronic bronchitis in adults. *M. catarrhalis* strains are frequently resistant to the bactericidal activity of normal human serum. In order to determine if the lipooligosaccharide (LOS) of *M. catarrhalis* has a role in serum resistance, the UDP-glucose-4-epimerase (*galE*) gene was identified, cloned, and sequenced and a deletion/insertion mutation was introduced into *M. catarrhalis* strain 2951. GalE enzymatic activity, measured in whole-cell lysates, was ablated in *M. catarrhalis* 2951 *galE*. Mass spectrometric analysis of LOS isolated with hot phenol-water confirmed that strain 2951 produced a type A LOS. These studies showed that the LOS from 2951 *galE* had lost two hexose residues due to the *galE* mutation and that the resultant LOS structure lacked the (Galα1-4Galβ1-4Glc) P^k epitope found on *M. catarrhalis* 2951. Wild-type *M. catarrhalis* 2951 is resistant to complement-mediated serum bactericidal activity. In contrast, a greater than 2-$\log_{10}$-unit reduction in CFU occurred after incubation of 2951 *galE* in either 50 or 25% pooled human serum (PNHS), and CFU in 10% PNHS decreased by about 1 $\log_{10}$ unit. These studies suggest that the P^k epitope of the LOS may be an important factor in the resistance of *M. catarrhalis* to the complement-mediated bactericidal effect of normal human serum.**

Moraxella catarrhalis is a human respiratory pathogen that is currently the third leading cause of otitis media along with *Streptococcus pneumoniae* and *Haemophilus influenzae* (10). Studies from various centers in the United States, Europe, and Asia used tympanocentesis to demonstrate that 15 to 20% of the middle-ear infections occurring in young children were caused by *M. catarrhalis* (10, 15, 16, 18, 46). *M. catarrhalis* has also been implicated as an important cause of respiratory disease in adults with predisposing conditions (41). Studies from several centers have reported clusters of nosocomial outbreaks of *M. catarrhalis*, most of which occurred in pulmonary care units (43, 45).

Although multiple studies have described specific bacterial components considered potential virulence factors, the steps involved in the pathogenesis of *M. catarrhalis* colonization and infection remain elusive (28, 41). One feature of this organism which has stimulated the interest of a number of investigators is its resistance to killing by normal human serum. Recent studies have focused on components of the bacterial outer membrane, as these structures would most likely be available for interaction with the host immune response. One prominent bacterial surface component, implicated as a potential virulence factor, is the lipooligosaccharide (LOS).

The LOS is similar to those of other airway pathogens such as *H. influenzae*, *Neisseria meningitidis*, and *Bordetella pertussis* in lacking O antigens typical of the enteric gram-negative bacilli. There are three *M. catarrhalis* serotypes (A, B, and C) based on chemically defined differences in the LOS antigen structures (12, 13, 38, 52). The LOSs of all three serotypes consist of a multiantenneray carbohydrate structure, but in all three serotypes, one of the oligosaccharide chains terminates in Galα1-4Galβ1-4Glc. Mandrell and Apicella showed that *M. catarrhalis* LOS reacted with monoclonal antibody (MAb) Gal 1-3 specific for the P^k (Galα1-4Galβ1-4Glc) epitope (36). The role of the *Moraxella* LOS in human infection has not been clearly defined. Most *Moraxella* strains have been shown to be highly resistant to complement-mediated killing in normal human serum (41, 53). In this paper, we present studies that investigate the role that the terminal Galα1-4Galβ1-4Glc structure of *Moraxella* LOS plays in resistance to complement-mediated killing by normal human serum. To perform these investigations, we created a mutation in the UDP-glucose 4-epimerase gene, resulting in a truncated LOS structure lacking terminal galactose residues. This change resulted in the loss of the P^k epitope from the LOS. These studies indicate that the P^k epitope may be a factor responsible for the resistance of *M. catarrhalis* to the complement-mediated bactericidal effect of normal human serum.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacteria and plasmids used in this study are described in Table 1. All *M. catarrhalis* clinical isolates were kindly provided by Timothy Murphy (Veterans Administration Medical Center, Buffalo, N.Y.) and Howard Faden (Children's Hospital, Buffalo, N.Y.). *Neisseria gonorrhoeae* strain 1291 and the 1291a-e pyocin mutant were described elsewhere (11, 26).

Development of MAb 4G5. MAb 4G5 was isolated from a previously described fusion (34). The antibody was defined as an immunoglobulin G2a using mouse MonoAb-ID (Zymed Laboratories). MAb 9E9, which is specific for the high-molecular-mass (HMW) protein of *M. catarrhalis*, was a gift from Timothy Murphy (Veterans Administration Medical Center, Buffalo, N.Y.) (30).

Bacterial growth. *Escherichia coli* was grown at 37°C in Luria-Bertani medium with or without agar (1.5%) and supplemented with antibiotics as needed. Wild-

* Corresponding author. Mailing address: Department of Microbiology, The University of Iowa, 51 Newton Rd., Iowa City, IA 52242. Phone: (319) 335-7807. Fax: (319) 335-9006. E-mail: michael-apicella@uiowa.edu.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype, relevant phenotype, or selection marker	Source or reference
Strains		
E. coli XL1-Blue MRF′	Δ(*mcrA*) *183* Δ(*mcrCB-hsdSMR-mrr*) *173 endA1 sup E44 thi-1 recA1 gyrA96 relA1 lac* [*F′ proAB lacI*^{q} *ZΔM15* Tn*10* (Tet^r)]	Stratagene
M. catarrhalis	Wild-type serotype A	ATCC 25238
M. catarrhalis 2951	Wild-type clinical isolate, serotype A	This study
M. catarrhalis 2951 *galE*	UDP-glucose 4-epimerase deficient	This study
Plasmids		
pMCSau2	Ampicillin	This study
pMCApoA	Ampicillin	This study
pMCPvu1a	Ampicillin	This study
pMCgalE	Ampicillin	This study
pMCgalEBBR	Ampicillin, spectinomycin	This study
pUC18	Ampicillin	Pharmacia Biotech
pUC19	Ampicillin	Pharmacia Biotech
pTAV1	Ampicillin	6
pMC2951galETA	Ampicillin	This study
pABR3	Ampicillin, spectinomycin	2
pD2galE	Kanamycin	31

type *M. catarrhalis* was grown either on gonococcal agar (GCA) supplemented with 1% IsoVitaleX (BBL Laboratories, Cockeysville, Md.) or brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) supplemented with 2.5% heat-inactivated fetal calf serum (FCS) at 37°C in 5% CO_2 with 85% relative humidity. Spectinomycin-resistant *M. catarrhalis* was grown on supplemented BHI agar with 15 μg of spectinomycin/ml or in supplemented BHI broth containing 5.0 μg of spectinomycin/ml. Selection was carried out without CO_2.

Recombinant DNA and transformation methods. All recombinant DNA techniques were performed as outlined previously (47). Transformations of *Moraxella* were performed as previously described by Catlin (8) and modified by Stephens et al. (48).

Cloning and mutagenesis of the UDP-glucose 4-epimerase gene (*galE*). The cloning of *M. catarrhalis* strain 25238 *galE* was accomplished in three steps (Fig. 1A to D). A ^{32}P-labeled probe made from bp 1 to 931 of the *N. meningitidis galE* (31) was used to probe a Southern blot of a *Sau*3AI partial digest of *M. catarrhalis* genomic DNA. This probe hybridized to a 2.8-kb DNA genomic fragment which was ligated into pUC18 and used to transform DH5α. Transformants were screened with the same probe. Plasmids isolated from colonies to which this probe hybridized contained an *M. catarrhalis* DNA fragment that contained the 3′ 425 bp of the putative *M. catarrhalis galE* (pMCSau2) (Fig. 1A).

The *M. catarrhalis* genomic DNA fragment from pMCSau2 was used to probe an *Apo*I digest of *M. catarrhalis* chromosomal DNA. A 1.9-kb fragment was identified and cloned into pUC19. Plasmids were isolated from colonies which hybridized to this probe. Sequence analysis revealed another 414 bp of *M. catarrhalis galE* 5′ to that found in pMCSau2. This plasmid was designated pMCApoA (Fig. 1B). DNA sequencing reactions were performed by using dye terminator cycle sequencing chemistry with AmpliTaq DNA polymerase and FS enzyme (PE Applied Biosystems, Foster City, Calif.). The reactions were run on and analyzed with an Applied Biosystems model 373A stretch fluorescence automated sequencer at the University of Iowa DNA Facility.

A third Southern blot was produced using a randomly primed ^{32}P probe constructed from a 482-bp *Apo*I/*Pvu*II fragment of the *M. catarrhalis* DNA in pMCApoA. This probed hybridized to a 4,000-bp fragment. A sublibrary was constructed in pUC18 with this DNA fragment. Clones containing portions of the *M. catarrhalis* putative *galE* open reading frame (ORF) were identified with the same randomly primed probe. The plasmid within these clones contained 710 bp of the 5′ end of *M. catarrhalis galE* in addition to the region upstream of this gene. This plasmid was designated pMCPvu1a (Fig. 1C).

Based on information gained from the above transformations and sequence analysis, a plasmid was constructed by ligating the *Bam*HI/*Sma*I 1,506-bp fragment from pMCApoA and the *Bam*HI/*Ecl*136II fragment (6,676 bp) from pMCPvu1a. This plasmid was designated pMCgalE. The *M. catarrhalis* DNA in the plasmid was sequenced (6,471 bp) and was demonstrated to contain the entire *M. catarrhalis galE* (Fig. 1D). *galE* from *M. catarrhalis* strain 2591 was amplified by PCR from genomic DNA using the forward primer 5′TATGACA AACACAGGGACAAC′3 and the reverse primer 5′ATCAATGCCCACAACC AG. The resulting 1,140-bp product was cloned into the pTAV1 cloning vector.

UDP-glucose 4-epimerase enzyme assays. *M. catarrhalis* strains were grown in BHI broth supplemented with 2% FCS and spectinomycin as required. To prepare extracts for enzymatic assay, 100-ml cultures were inoculated with 0.01 volume of fresh overnight culture grown in supplemented BHI broth and incubated at 37°C with shaking. Separate cultures were grown to either exponential phase or stationary phase and washed twice in 1× phosphate-buffered saline. The washed pellets were resuspended in 5 ml of assay buffer (125 mM potassium bicinate [pH 8.5], 1 mM phenylmethylsulfonyl fluoride) and lysed in a French press using 16,000 lb/in^2. The bacterial debris was sedimented by centrifugation at 15,800 × *g* for 30 min at 4°C. The supernatants were transferred to prechilled microcentrifuge tubes and kept on ice. The total protein content of the crude cell extracts was determined using the Bio-Rad protein assay reagent by following the microassay protocol with bovine serum albumin as the standard. Extracts containing equal amounts of total protein were added to the two-step UDP-glucose 4-epimerase assay mixture as described below.

The two-step UDP-glucose 4-epimerase assay described here is a modification of a procedure described elsewhere (57, 58). We modified the assay slightly to optimize it for *Moraxella* extracts. The first step of the two-step assay was carried out in a 500-μl reaction volume (125 mM bicinate [pH 8.5], 0.44 mM UDP-galactose) at 37°C for 15 min. The reaction mixture was then placed in a boiling water bath for 90 s, chilled on ice for 5 min, and then centrifuged at 15,800 × *g*

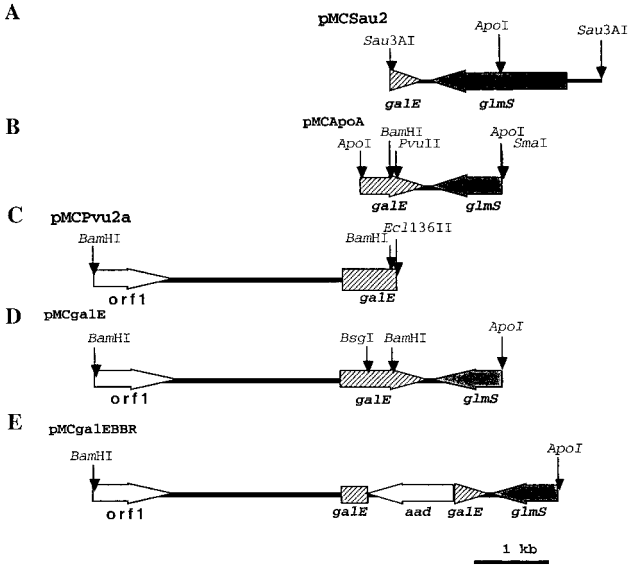

FIG. 1. *M. catarrhalis* DNA inserts from the five plasmids used to create the mutant and wild-type constructs. (A) *M. catarrhalis* DNA fragment from pMCSau2 cloned into the *Bam*HI site of pUC18. (B) *M. catarrhalis* DNA fragment from pMCApoA cloned into the *Eco*RI site in pUC19. (C) *M. catarrhalis* DNA fragment from pMCPvu2a cloned into the *Sma*I site in pUC18. (D) *M. catarrhalis* DNA fragment constructed from those shown in panels B and C cloned into pUC18. (E) *M. catarrhalis* DNA fragment from pMCgalE with the *aad* gene inserted into the *Bsg*I and *Bam*HI gap of *galE* in pUC18. Multiple restriction enzyme sites have been omitted for clarity.

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for 10 min at 4°C. A 400-μl aliquot of the supernatant was added to the mixture from the second step of the assay in a 600-μl total volume (0.125 mM bicinate, [pH 8.5], 1.25 mM NAD^+, 0.02 U of UDP-glucose dehydrogenase). The reaction was observed in a quartz cuvette, and the increase in absorbance was measured every 15 s at 340 nm. All extracts including appropriate controls were assayed in triplicate.

Determination of UDP-glucose 4-epimerase activity levels. The net absorbance was determined after adjusting for endogenous UDP-galactose and UDP-glucose and UDP-glucose contamination of exogenous UDP-galactose preparations. The initial velocities (V_i) of the second reaction (UDP-glucose to UDP-glucuronic acid) were determined over the first 30 s. V_i, which is indicative of the starting concentration of UDP-glucose, was converted to nanomoles of NADH generated per minute per nanogram of total protein using the Beer-Lambert law and $\varepsilon_{NADH} = 6.2 \times 10^3\ M^{-1}\ cm^{-1}$.

SDS-PAGE and Western blotting of isolated LOS. LOS was isolated from 6 liters of supplemented BHI broth for strain 2951 and 6 liters of supplemented BHI broth cultures with 5 μg of spectinomycin/ml for the 2951 *galE* mutant by a modified Westphal hot phenol-water preparation (31). Whole-bacterial-cell proteinase K lysates were made from bacteria grown on supplemented BHI agar. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Lesse et al. (32). Western blotting was performed by the method of Towbin et al. (51).

Mass spectrometric analysis. LOS structures from *M. catarrhalis* 2951 and 2951 *galE* were analyzed by mass spectrometry (MS). In each case, approximately 0.5 mg of LOS was treated with mild hydrazine for 30 min at 37°C (21) for conversion into the corresponding water-soluble O-deacylated LOSs, which are more amenable to mass spectrometric analysis (19).

O-deacylated samples were taken up in water and desalted by drop dialysis using a 0.025-μm-pore-size nitrocellulose membrane (Millipore, Bedford, Mass.). The dialyzed sample was mixed in a 1:1 ratio with 320 mM 2,5-dihydroxybenzoic acid solution in 4:1 (vol/vol) acetone-water containing 175 mM 1-hydroxyisoquinoline (40), desalted with cation-exchange resin beads (DOWEX, 50X; NH_4^+) (42), and then air dried on a stainless steel target.

Samples were then analyzed by matrix-assisted laser desorption ionization (MALDI)-MS using a PE Biosystems (Framingham, Mass.) Voyager DE time-of-flight mass spectrometer operated with a nitrogen laser (337 nm) in the negative-ion mode under delayed-extraction conditions (55). The delay time was 175 ns, and the grid voltage was 93.5% of full acceleration voltage (20 to 30 kV). Spectra were acquired, averaged, and mass calibrated with an external calibrator consisting of an equimolar mixture of angiotensin II, bradykinin, luteinizing hormone-releasing factor, bombesin, α-MSH (CZE mixture; Bio-Rad), and adrenocorticotropin 1-24 (Sigma).

Electrospray mass spectra were obtained using a quadrupole ion trap mass analyzer fitted with an electrospray ionization source (Finnigan LCQ; Finnigan MAT, San Jose, Calif.). For sample delivery, direct infusion with a syringe pump at a flow rate of 0.5 to 2 μl/min was used. The mobile phase was 70% acetonitrile in water. Ions were produced with a spray voltage of 2.9 keV with the heated capillary set at 200°C. Spectra were collected in the negative-ion mode by averaging 20 individual scans, consisting of three "microscans." Collision-induced dissociation was carried out in the mass analyzer on an ion selected from the mass spectrum by using He as the collision gas in the ion trap.

Bactericidal assay. Bacteria were grown to early log phase, $A_{600} = 0.2$, in supplemented BHI broth. A 0.5-ml aliquot of each strain was centrifuged for 1 min at 2,000 × *g* in a Beckman microcentrifuge at room temperature. The pellet was resuspended in 1.0 ml of phosphate-buffered salt solution (PBSS) consisting of 10 mM K_2HPO_4, 10 mM KH_2PO_4, 136 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.3 mM $MgCl_2 \cdot 6H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, and 0.01% bovine serum albumin, pH 7.0.

The bactericidal assay, modified from that reported by Andreoni et al. (4), was carried out in a 96-well plate in a 200-μl final volume. Pooled normal human serum (PNHS; a 20-donor pool of serum from human volunteers who had no previous history of neisserial infections) was diluted to 10, 25, or 50% in PBSS. A control containing PNHS heat-inactivated for 30 min at 56°C was included in each experiment. Ten microliters (10^6 cells) of the resuspended bacteria was diluted into 190 μl of PBSS, and serial 1/10 dilutions were made in PBSS. Twenty microliters of each dilution was spread on GCA and grown overnight at 37°C in 5% CO_2. The colonies in these reaction mixtures were counted and used as the initial CFU. Ten microliters of the bacterial stock was incubated in the diluted serum for 30 min with shaking at 200 rpm in a 37°C incubator (Inova 4080; New Brunswick Scientific, Edison, N.J.). Serial 1/10 dilutions of the reaction mixtures were diluted into PBSS and were spread on GCA plates. These were grown overnight at 37°C in 5% CO_2, and emerging colonies were counted the next day. The resulting CFU value was the 30-min value.

Killing was assessed by comparing the number of CFU from the 30-min serum incubation with the number of initial CFU. Results were expressed as the $\log_{10}$ change in CFU at 30 min compared to the initial CFU.

Statistical analysis and DNA sequence construction and analysis. Statistical analysis of the data from bactericidal and UDP-glucose 4-epimerase activity assays was carried out using the paired *t* test and analysis of variance functions found in Statview, version 4.0 (Abacus Concepts, Inc., Berkeley, Calif.).

DNA sequence construction and analysis were performed using the Wisconsin Package, version 10.0 (Genetics Computer Group, Madison, Wis.), Assembly LIGN, version 1.0, (Oxford Molecular Group Inc., Oxford, United Kingdom), Gene Works, version 2.5.1 (Oxford Molecular Group Inc.), and Sharedraw, version 2.0 (Pierce Software Inc., San Jose, Calif.).

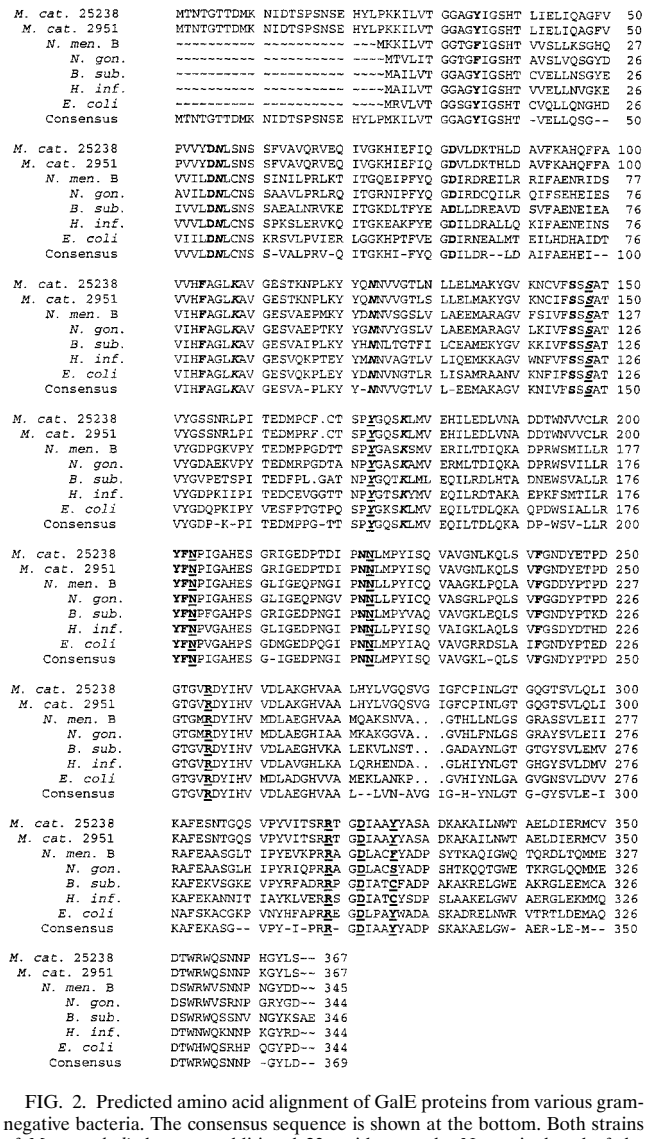

FIG. 2. Predicted amino acid alignment of GalE proteins from various gram-negative bacteria. The consensus sequence is shown at the bottom. Both strains of *M. catarrhalis* have an additional 23 residues at the N-terminal end of the protein. Boldface, amino acids that are in the binding pocket of the folded protein and that have been shown to interact with the substrate (UDP-sugar) or the cofactor (NAD^+). Residues that interact with NAD^+ are in italics, and residues that interact with UDP-sugar are underlined (15, 33, 49, 50). *M. cat.* 25238, *M. catarrhalis* ATCC strain 25238 (GenBank accession no. AF248583); *M. cat.* 2951, *M. catarrhalis* strain 2951 (GenBank accession no. AF248584); *N. men.* B, *N. meningitidis* serotype B strain FAM20 (SwissProt accession no. Q59624); *N. gon.*, *N. gonorrhoeae* (SwissProt accession no. Q05026); *B. sub.*, *B. subtilis* (SwissProt accession no. P55180); *H. inf.*, *H. influenzae* (SwissProt accession no. P24325). *E. coli* SwissProt accession no., P09147.

Nucleotide sequence accession numbers. The nucleotide sequence of *M. catarrhalis galE* is available from the GenBank database under accession no. AF248583 and AF248584.

RESULTS

Homology analysis. The Blast search protocol at the National Center for Biotechnology Information website was used to compare the *M. catarrhalis* DNA sequence with the nonredundant database (3). Three large ORFs were identified in the 6,841 bp of *M. catarrhalis* DNA that had been cloned and se-

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TABLE 2. UDP-glucose 4-epimerase activity

Bacterial strain	UDP-glucose 4-epimerase activity (nmol of NADH/min/μg of total protein) ± SD in:	
	Log phase	Stationary phase
2951 (wild type)	13.640[a] ± 3.104	0.0073[b] ± 0.0180
2951 *galE*	0.079[c] ± 0.176	0.0006 ± 0.0010

[a] $P < 0.0001$ versus log-phase 2951 *galE*.
[b] $P < 0.0001$ versus log-phase wild type.
[c] $P = 0.9490$ versus stationary-phase 2951 *galE*.

quenced. The *galE* sequence of *M. catarrhalis* contained 1,094 bp (Fig. 1D). Figure 2 shows the degree of similarity among two *M. catarrhalis* strains and four other bacterial species. The predicted amino acid sequence had 59% identity and 70% similarity over 334 amino acid residues to GalE of *Bacillus subtilis*. There was 53% identity and 68% similarity to GalE of *N. meningitidis*, 55% identity and 67% similarity to *H. influenzae* GalE, and 52% identity and 66% similarity to *N. gonorrhoeae* GalE.

A large ORF (1,836 bp) reading in the opposite orientation and ending 82 bp immediately 3′ to *galE* had homology to the gene encoding glucosamine fructose-6-phosphate aminotransferase, *glmS* (Fig. 1D). The predicted amino acid sequence had 57% identity and 70% similarity over 611 amino acids residues to the product of *glmS* from *Thiobacillus ferrooxidans*.

The product of a third 1,104-bp partial ORF at the 5′ end of the *M. catarrhalis* sequence showed homology to UDP-glucose dehydrogenase (Fig. 1D). The homology of the predicted amino acid sequence was 30% identity and 49% similarity to a putative enzyme described in *Burkholderia pseudomallei*.

Mutagenesis of the UDP-glucose 4-epimerase gene (*galE*). Strain 2951 *galE* mutant was constructed by excision of a 429-bp DNA fragment from the *galE* gene in pMCgalE by partial digestion with *Bam*HI followed by complete digestion using *Bsg*I (Fig. 1E). Blunt ends were produced by using T4 DNA polymerase as previously described (47). A spectinomycin resistance gene (*aad*) was ligated into the blunt-ended pMCgalE. Colonies were selected by growth on Luria-Bertani agar containing ampicillin and spectinomycin. The location and orientation of the spectinomycin resistance gene within *galE* were confirmed by diagnostic restriction endonuclease digestions and direct DNA sequencing. This plasmid was designated pMCgalEBBR.

M. catarrhalis strain 25238 was resistant to transformation by homologous recombination with *Xba*I-restricted DNA from pMCgalEBBR. We tested three other *M. catarrhalis* strains, and only one (*M. catarrhalis* 2951) was transformable with this restricted plasmid DNA. To insure that the *galE* genes were comparable, the *galE* gene from strain 2951 was first amplified by PCR using primers made according to the sequences of the 5′ and 3′ ends of the strain 25238 *galE* gene. A PCR product of the expected size was obtained and cloned into pTAV1 (Table 1) and transformed into *E. coli* XL1-Blue MRF′. Colonies were screened by diagnostic restriction endonuclease digestions of plasmid preparations. A plasmid having the correct diagnostic restriction endonuclease fragments was sequenced. It was found that there was divergence at 16 nucleotide residues, which translated to four amino acid differences between the products of *galE* of strain 25238 and strain 2951. A comparison of the predicted amino acid sequences of GalE from strain 25238 and strain 2951 with a consensus GalE sequence showed that none of the amino acid changes involved active sites in the enzyme (Fig. 2).

Comparison of GalE activity in *M. catarrhalis* strain 2951 and 2951 *galE* mutant. The levels of UDP-glucose 4-epimerase activity were measured by a two-step assay of whole-cell extracts obtained from bacteria in both the exponential and stationary growth phases (Table 2). GalE activity from lysates of *galE* mutant bacteria harvested during either the exponential or stationary growth phase was at the lower detectable limits of the assay. Lysates from strain 2951 harvested during the exponential growth phase demonstrated high levels of GalE activity (average of 13.640 nmol of NADH generated per min per μg of total protein). Lysates from strain 2951 obtained at stationary growth phase had activity that was not detectable and that was no different from the activity in the lysate of strain 2951 *galE* at either exponential or stationary growth. Increases in A_{340} that occurred upon addition of UDP-glucose (0.45 mM final concentration) to these lysates showed that they did not contain inhibitors of the UDP-glucose dehydrogenase used in the second step of the assay.

Characterization of the LOS epitope recognized by MAb 4G5 and reactivity with strain 2951 and 2951 *galE*. Western blot analysis showed that MAb 4G5 reacted to the LOS of *M. catarrhalis* strain 7169, the strain used for immunization of the donor mice. Flow cytometry confirmed that the LOS 4G5 epitope was expressed on the surface of strain 7169. Subsequent blots confirmed that the LOS from 22 other *M. catarrhalis* strains also expressed the MAb 4G5 epitope. These data demonstrated that the LOS epitope defined by MAb 4G5 is conserved on a diversity of clinical isolates from different geographic regions, from both adults and children and from various body sites and fluids. In addition, the *M. catarrhalis* isolates that represent the strains used to define the three major LOS serotypes also reacted with MAb 4G5. MAb 4G5 bound to the LOS of strain 2951 but did not react with LOS from strain 2951 *galE*.

We performed Western blot assays with MAb 4G5 using proteinase K lysates from 8 strains of *H. influenzae*, 11 strains of *N. meningitidis*, 12 strains of *N. gonorrhoeae*, 2 strains of *Neisseria lactamica*, and 2 strains of *Neisseria cinerea*. The only strain recognized by MAb 4G5 that was not a *Moraxella* species was the gonococcal pyocin-derived mutant, 1291b. The LOS of the 1291b mutant terminates in a structure that is immunochemically identical to the P^k antigen (Galα1-4Galβ1-4Glc) found on human cells (26, 37). These data indicate that MAb 4G5 reacted with the Galα1-4Galβ1-4Glc structure that is found as a terminal structure on all three *M. catarrhalis* LOS serotypes. It also indicated that this structure was no longer present on the LOS of strain 2951 *galE*. The mass-spectrometric analysis presented below confirmed the results of these MAb studies.

Mass-spectrometric analysis of *M. catarrhalis* strain 2951 and 2951 *galE* LOSs. The structures of the major LOSs for the three serotypes A (Fig. 3), B, and C have been previously reported (12–14). To assign the serotype of strain 2951 LOS

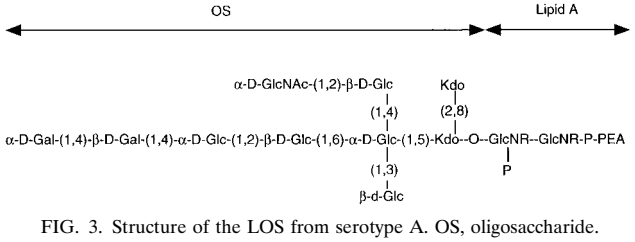

FIG. 3. Structure of the LOS from serotype A. OS, oligosaccharide.

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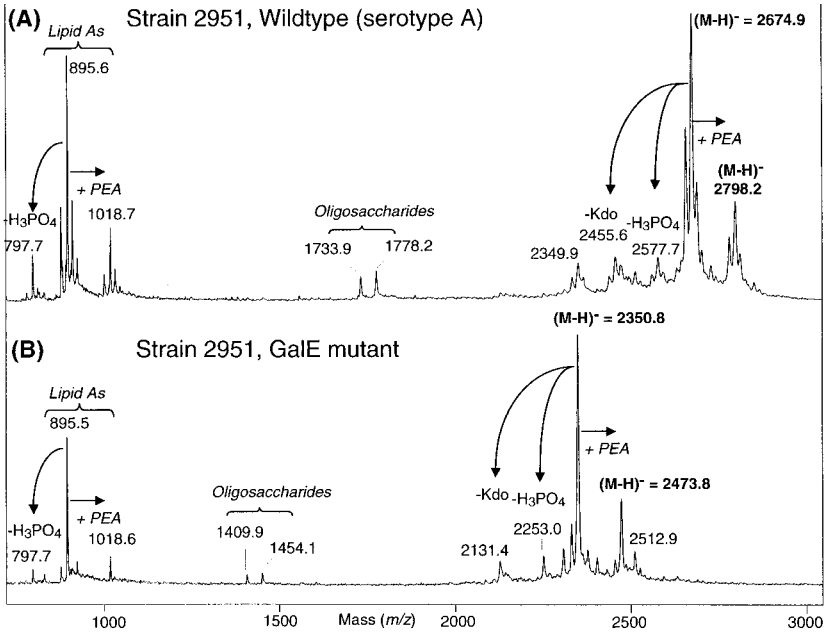

FIG. 4. Linear MALDI-time of flight spectra of the parental wild-type *M. catarrhalis* strain 2951 (A) and the isogenic *galE* mutant (B). The observed molecular ions for the individual LOS species appear as unresolved isotope clusters whose centroids correspond to the average mass. In addition to the peaks described in the text, peaks due to prompt ion fragmentations are also present due to β-elimination of phosphoric acid from the parent $(M\text{-}H)^-$ peaks (*m/z* 2,578.3, *m/z* 2,253.4) and lipid A fragments (*m/z* 797.7) and loss of a Kdo (*m/z* 2,456.3, *m/z* 2,131.8), as are peaks resulting from facile loss of CO_2 from a Kdo residue of the oligosaccharides (*m/z* 1,734.2 and 1410.1). Fragmentations of these types have been discussed in detail elsewhere (19). Masses shown in Fig. 5 are the theoretically predicted masses based on the structures shown. The experimental masses listed in the two spectra above may differ but are within the expected experimental mass accuracy (± 0.1%).

and define the structure of the *galE* mutant LOS, several MS experiments were carried out on the LOSs from both strains.

To determine the precise molecular masses of the LOS glycoforms, LOSs from both the parental strain 2951 and the 2951 *galE* were converted into the water-soluble O-deacylated form and analyzed in the negative-ion mode by MALDI-time of flight MS. MALDI-MS spectra of the O-deacylated LOS preparations are shown in Fig. 4. In both spectra, a single dominant deprotonated molecular ion peak, $(M\text{-}H)^-$, was observed at *m/z* 2,674.9 and *m/z* 2,350.5 for the wild-type and mutant strains, respectively. A less abundant second peak 123 Da higher in mass, corresponding to the presence of an additional phosphoethanolamine (PEA) moiety (i.e., *m/z* 2,798.2 and *m/z* 2,473.8 for the wild-type and mutant strains, respectively) was also detected. These masses are consistent with a composition of $Hex_7HexNAcKdo_2$-lipid A for the wild type and $Hex_5HexNAcKdo_2$-lipid A for the *galE* mutant, with elements of LOSs from both strains being partially replaced with PEA. These compositions are consistent with the previously published structure of a serotype A LOS (38) for the parental strain and with the expected loss of two galactose residues (Galα1 4Galβ1) on the nonreducing terminus of the largest oligosaccharide branch to form the truncated *galE* mutant LOS. The presence of oligosaccharide and lipid A "prompt fragments" (i.e., fragments generated from facile decomposition of the intact LOS species prior to acceleration) in these spectra adds further support to these assignments (Fig. 5). For example, lipid A peaks present in both spectra at *m/z* 895.6 and 1,018.7 are those expected based on the previously published lipid A structure (38) with the exception of a partial substitution of PEA. Likewise, the oligosaccharide fragments at *m/z* 1,778.2 (wild type) and *m/z* 1,453.1 (*galE* mutant) are consistent with the monosaccharide compositions as stated above.

Since the oligosaccharide structures are known to be highly branched, we examined these in more detail using a successive series of tandem MS (MS^n) experiments. The MS^n experiments were all carried out using an ion trap instrument operating under negative ion electrospray ionization conditions (data not shown). The original full-scan mass spectrum of the

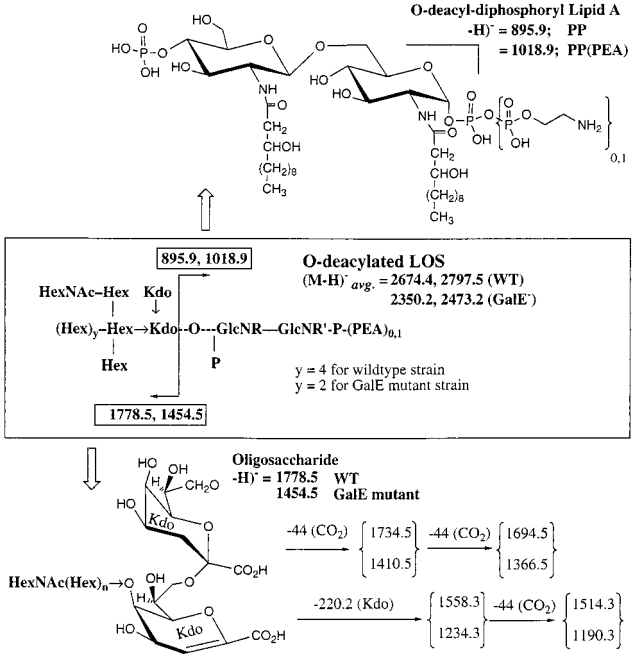

FIG. 5. Schematic of prompt fragmentation processes observed for O-deacylated LOS by negative-ion MALDI-MS. All masses shown are average masses based on those calculated for both the wild-type and *galE* mutant LOSs. Hex, hexose; HexNAc, *N*-acetylhexosamine; Kdo, 2-deoxy-3-keto-octulosonic acid. See the legend for Fig. 4 and text for further discussion.

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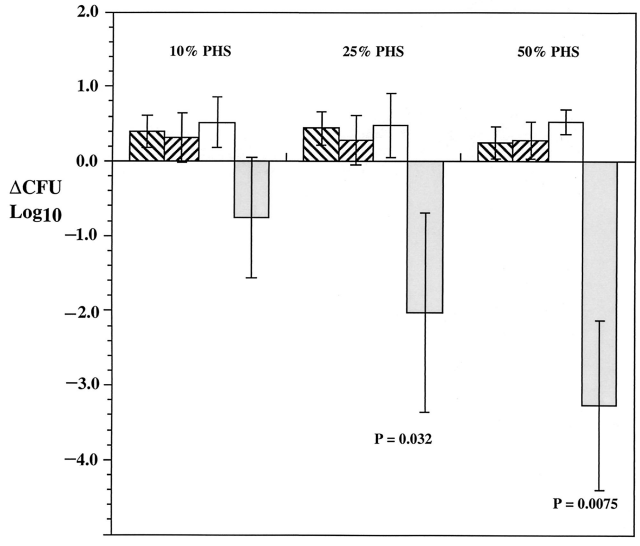

FIG. 6. Sensitivity to serum complement-mediated bactericidal activity for *M. catarrhalis* strain 2951 and 2591 *galE* mutant after 30 min. ▧, results obtained with *M. catarrhalis* 2951 and heat-inactivated PNHS; ▨, results obtained with *M. catarrhalis* 2951 *galE* and inactivated PNHS; □, results obtained with *M. catarrhalis* 2951 and PNHS; ■, results obtained with *M. catarrhalis* 2951 *galE* and PNHS. *P* values, comparisons between survival of strain 2951 and that of 2951 *galE* in PNHS. Each result is the mean from four separate experiments ± 1 standard deviation.

O-deacylated LOS from the *galE* mutant strain showed four major peaks with monoisotopic masses at *m/z* 1,452.3 (singly charged oligosaccharide fragment), *m/z* 1,174.0 (doubly charged parent mass), *m/z* 895.4 (singly charged lipid A fragment), and *m/z* 508.2 (doubly charged lipid A-PEA fragment; m = 1,016.4). The peak corresponding to the oligosaccharide at *m/z* 1,452.3 was selected, and a tandem experiment (MS^2) was performed yielding a peak at *m/z* 1,232.3 that indicated the loss of Kdo (220 Da). Further fragmentation of this peak (MS^3) yielded several peaks corresponding to loss of CO_2 (*m/z* 1,188.4) followed by the further loss of hexose (*m/z* 1,026.3), hexose and HexNAc (*m/z* 823.3), and two hexoses and HexNAc (*m/z* 660.8). These data are all consistent with the serotype A structure. Moreover, since the mass difference observed for the LOS and the oligosaccharide fragments between the wild-type and the mutant strains correspond to two hexose residues (ΔM, 324 Da), the *galE* mutant strain would be predicted to produce a structure identical to that of a previously reported heptasaccharide containing a minor glycoform of serotype C (12). This structure differs from the LOS of serotype A only by the absence of a galactose disaccharide linked α1 α4 on the nonreducing terminus of the largest branch.

Comparison of bactericidal activity of PNHS against *M. catarrhalis* strain 2951 and the 2951 *galE* mutant. Figure 6 shows the change in serum bactericidal activity of PNHS against strain 2951 and 2951 *galE*. *M. catarrhalis* strain 2951 was resistant to serum-mediated killing at all serum concentrations tested (Fig. 6). In contrast, incubation of strain 2951 *galE* in 50 or 25% PNHS resulted in a greater-than-2-$\log_{10}$-unit reduction in CFU ($P = 0.0075$ and $P = 0.0352$, respectively). Survival of the mutant strain was also impaired in 10% PHS, but substantial variability (range in log reduction, 0.42 to 1.30) decreased the statistical significance ($P = 0.0910$) of the results.

DISCUSSION

The presence of antibody and complement in various body environments constantly poses a challenge for the colonization and spread of bacteria. Resistance to these factors is frequently a requirement for pathogenesis. Multiple studies have demonstrated that resistance to complement-mediated killing is an important virulence factor for *M. catarrhalis* infections. Hol et al. reported in two studies that 156 of 179 (87%) and 124 of 200 (62%) clinical isolates from adult patients with pulmonary infections were serum resistant (23, 24). Recently, Verduin et al. have reported that 89% of *M. catarrhalis* strains isolated from adults with lower respiratory disease are completely or partially resistant to the bactericidal action of serum (54). In contrast, it has been reported that 58% of *M. catarrhalis* isolates carried in the pharynges of asymptomatic, healthy children are serum sensitive and do not appear to cause disease (23, 24, 54).

There are multiple reports that implicate various bacterial factors potentially involved in serum resistance. Verduin and coworkers have reported that resistant *M. catarrhalis* strains either bind or bind and inactivate a terminal complement component or intermediate involved in the formation of the membrane attack complex (53). Other investigators have demonstrated that complement resistance may involve an HMW outer membrane protein (OMP) or ubiquitous surface proteins (UspA1 and UspA2) (22, 30; C. M. Verduin, H. J. Bootsma, C. Hol, A. Fleer, M. Jansze, K. L. Klingman, T. F. Murphy, and H. van Dijk, Abstr. 95th Gen. Meet. Am. Soc. Microbiol. 1995, abstr. B137, p. 189, 1995; C. M. Verduin, M. Jansze, J. Verhoef, A. Fleer, and H. van Dijk, Clin. Exp. Immunol., abstr. 143, p. 50, 1994). Further studies have suggested that this HMW OMP binds human vitronectin, which subsequently inhibits complement activity (Verduin et al., Abstr. 95 Gen. Meet. Am. Soc. Microbiol. 1995; Verduin et al., Clin. Exp. Immunol.).

M. catarrhalis mutants defective in expression of UspA1 or UspA2 have been constructed and analyzed for sensitivity to human serum (1, 39). Whereas UspA1 is primarily involved in attachment, the putative function of UspA2 appears to be associated with the resistance of *M. catarrhalis* to the bactericidal activity of normal human sera. In comparative studies, the *M. catarrhalis* mutant defective in UspA2 expression was shown to be extremely sensitive to killing by normal human serum, whereas the wild type and the *uspA1* mutant were completely resistant (1). Analysis using HMW OMP-specific MAb 9E9 (30) of strain 2951 and strain 2951 *galE* indicates that the HMW OMP reacts with both strains in a Western blot (data not shown).

More recently, a study by Verduin and coworkers analyzed 75 strains of *M. catarrhalis* with various degrees of complement susceptibility by pulsed-field gel electrophoresis and automated ribotyping (54). These studies divided the complement-sensitive and complement-resistant strains into two groups. Therefore, these investigators conclude that *M. catarrhalis* complement resistance represents a separate lineage in the species.

The role of the LOS in the serum resistance of *M. catarrhalis* is less clear. Ninety-five percent of the *M. catarrhalis* clinical isolates can be grouped into three serotypes based on the reaction of their LOS (52) with a polyclonal rabbit antibody raised against whole bacteria. The chemical structures corresponding to these three serotypes have been defined (25). Sixty-one percent of clinical isolates belong to serogroup A. *M. catarrhalis* strain 2951 LOS is type A based on mass spectrometric analysis.

In contrast to normal nonimmune serum, convalescent se-

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rum from patients with *Moraxella* infection was found to be bactericidal to the patient's own isolate (9) and to contain anti-LOS antibodies (20). However, antibodies to LOS in a patient's convalescent serum do not appear to be serotype specific (44). In addition, there does not appear to be any linkage between the serotype and the site of infection or severity of disease (44).

The 2951 *galE* mutant is sensitive to complement-mediated killing in normal human serum, whereas the parent strain is resistant. These data suggest that resistance to serum complement-mediated killing may be related to the presence of the two terminal galactose residues on the LOS. These residues form part of a structure that has been shown to be immunochemically identical to the P^k (Galα1-4Galβ1-4Glc) antigen found on a number of human cells including erythrocytes, as well as gastrointestinal, ureteral, and bladder epithelial cells (7, 29, 35). It would appear that the *Moraxella* Galα1-4Galβ1-4Glc structure may act as a human self-antigen and that antibodies to it are not present in normal human serum. The increased sensitivity to the bactericidal effects of normal human serum that occurs in the *galE* mutant suggests that removal of this epitope exposes a LOS antigen with which naturally occurring human antibodies can react resulting in complement-mediated lysis. Mutations in *galE* genes of other bacteria have been shown to alter the pathogenic potential of these strains. *N. meningitidis* group B strain B1940 was made serum sensitive by the introduction of this mutation (56). A *galE* mutation introduced into *N. meningitidis* strain NMB by Kahler et al. produced a serum-sensitive mutant in a capsule-positive bacterium (27). *Pasteurella multocida* with a *galE* mutation showed reduced virulence when tested by intraperitoneal inoculation in mice (17).

Resistance to killing by normal human serum has been shown to be important in the pathogenesis of the closely related species *N. gonorrhoeae* and *N. meningitidis*. Strains of *N. gonorrhoeae* isolated from disseminated gonococcal infections are typically serum resistant. In contrast, mucosal gonococcal isolates are serum sensitive, as typically studied in vitro. Previous studies have indicated that the LOS structure is an important factor in both of these phenotypes (4, 5).

It is of interest that the LOS of serum-sensitive gonococci undergoes sialylation in vivo, a modification that allows the isolate to become serum resistant and that seems critical in the pathogenesis of mucosal infection. Unlike the LOS from these gonococci, *M. catarrhalis* serotype A LOS lacks the lactosamine (Galβ1-4 GlcNAc) sialylation site. Only serotype C LOS contains this potential sialylation site (12). Studies in our laboratory confirm that serotype A LOS of *M. catarrhalis* does not undergo sialylation at an alternative site (data not shown). Hence, the intact LOS carbohydrate chain appears to confer protection against complement-mediated killing.

The resistance of bacteria to killing by normal human serum is the result of a complex array of bacterial surface factors. These studies suggest that in *M. catarrhalis* as with other pathogens LOS is one of these factors (4, 5).

ACKNOWLEDGMENTS

This research was supported by Public Health Service Research grants AI45728, AI44642 (M.A.A.), AI46469 (A.A.C.), and AI31254 (B.W.G.). The MALDIMS was performed with instrumentation kindly provided by PE Biosystems, and the ESI MS was performed at the mass spectrometry facility at the University of California, Berkeley (Department of Chemistry). The University of Iowa DNA facility is supported in part by the Diabetes Endocrinology Research Center with National Institutes of Health grant DK25295 and by the College of Medicine.

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Editor: J. T. Barbieri

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