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ArcA-Regulated Glycosyltransferase Lic2B Promotes Complement Evasion and Pathogenesis of Nontypeable Haemophilus influenzae

Sandy M. S. Wong,1 Frank St. Michael,3 Andrew Cox,3 Sanjay Ram,2 and Brian J. Akerley1*

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Signaling mechanisms used by Haemophilus influenzae to adapt to conditions it encounters during stages of infection and pathogenesis are not well understood. The ArcAB two-component signal transduction system controls gene expression in response to respiratory conditions of growth and contributes to resistance to bactericidal effects of serum and to bloodstream infection by H. influenzae. We show that ArcA of nontypeable H. influenzae (NTHI) activates expression of a glycosyltransferase gene, lic2B. Structural comparison of the lipooligosaccharide (LOS) of a lic2B mutant to that of the wild-type strain NT127 revealed that lic2B is required for addition of a galactose residue to the LOS outer core. The lic2B gene was crucial for optimal survival of NTHI in a mouse model of bacteremia and for evasion of serum complement. The results demonstrate that ArcA, which controls cellular metabolism in response to environmental reduction and oxidation (redox) conditions, also coordinates genes that are critical for immune evasion, providing evidence that NTHI integrates redox signals to regulate specific countermeasures against host defense.

Haemophilus influenzae is a Gram-negative bacterium that colonizes the human nasopharyngeal mucosa and can disseminate to other sites to cause otitis media, upper and lower respiratory tract infections, septicemia, and meningitis (37, 50). It frequently infects the lungs of individuals with chronic obstructive pulmonary disease (51, 52, 65) and cystic fibrosis (20, 49). The introduction in 1990 of an effective vaccine against the capsular polysaccharide of encapsulated H. influenzae type b (Hib) strains has decreased the incidence of systemic infections caused by Hib strains in developed countries (9). However, the vaccine is not effective against nonencapsulated, nontypeable H. influenzae (NTHI). NTHI predominantly causes respiratory tract infections and otitis media but occasionally can enter the bloodstream to cause meningitis (11, 15, 54, 55). Prior to introduction of the Hib vaccine, NTHI was not a major cause of invasive disease; however, in the post-Hib vaccine era, the incidence of invasive infections due to NTHI has increased and is shifting from infants to older populations (14, 68).

The factors contributing to invasive disease, likely involving host susceptibility and strain-specific virulence genes, are not well understood. A correlation was observed between disease severity during invasive NTHI infections (bacteremia or meningitis) and the degree of resistance of the corresponding NTHI isolate to bactericidal effects of human serum in vitro, suggesting that increased resistance to complement enhances virulence (23). The complement system plays an important role in adaptive and innate immune defenses against H. influenzae infection in both humans and animal models (16, 17, 59, 72, 74). Three major pathways, i.e., classical, lectin, and alternative, that differ in their mode of activation on the pathogen surface (60, 72) can initiate complement deposition. Each pathway involves a cascade of proteolytic cleavage steps that activate subsequent factors, leading to antimicrobial activities that include target cell lysis, inflammation, opsonization-promoting phagocytosis, and activation of the bactericidal mechanisms of macrophages and neutrophils.

The lipopolysaccharide (LPS) glycolipid of the outer leaflet of the Gram-negative bacterial outer membrane mediates evasion of the complement system and is essential in animal models of invasive infection by H. influenzae (7, 16, 29, 41). In H. influenzae and in many other human respiratory tract pathogens, the LPS is termed lipooligosaccharide (LOS) because it lacks the repetitive polysaccharide O-antigen side chain present in the LPS of other Gram-negative bacteria (50). The LOS structure varies between strains, yet several features are conserved. H. influenzae LOS consists of lipid A, an inner core usually composed of a single 3-deoxy-D-manno-octulosonic acid linked to three heptose residues, and an outer core usually containing a short heteropolymer of glucose and galactose residues in different configurations extending from the heptosyl residues of the inner core. The outer core may additionally be modified with sialic acid, N-acetylgalactosamine, and phosphorylcholine (31, 62).

During pathogenesis, bacteria sense and respond to environmental signals to appropriately express critical virulence factors or to repress those that would otherwise detract from efficient infection, such as structures recognized by host immune pathways. H. influenzae has been shown to modify its LOS in response to environmental aeration conditions by increasing levels of phosphorylcholine displayed on the LOS outer core as oxygen levels decrease (75), a response that may

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2 Published ahead of print on 28 February 2011.
allow NTHI to differentially express LOS structures for evasion of immune effectors present in environments in the host such as airway mucosal surfaces versus invasion into deeper tissues or in the bloodstream. Mechanisms by which \textit{H. influenzae} senses and responds to such reduction/oxidation (redox) signals to regulate LOS synthesis have not been identified; however, \textit{H. influenzae} possesses a redox-responsive regulatory system, the ArcAB two-component signaling system (TCS), that is biochemically and functionally similar to that of \textit{Escherichia coli} (19, 44). Under low-oxygen conditions, ArcB senses the redox status of the quinone pool and autophosphorylates, leading to activation of ArcA by phosphoryl transfer (4, 18, 43). Phosphorylated ArcA transcriptionally activates or represses diverse target genes, including genes of the tricarboxylic acid cycle and genes involved in other aspects of respiratory or fermentative metabolism (12, 39, 40, 77). Under high-oxygen conditions, ArcAB activity is greatly decreased. In \textit{H. influenzae}, ArcA has been demonstrated to be important for serum resistance and pathogenesis in mouse models of bacteremia (12, 77); however, ArcA-regulated virulence genes have not been identified, and the mechanism of ArcA-mediated serum resistance is unknown.

In the current study, we investigated mechanisms by which the \textit{H. influenzae} ArcAB two-component signaling system influences NTHI pathogenesis. Strain-specific differences in ArcA-dependent serum resistance led us to investigate whether ArcA-regulated LOS genes unique to invasive strains are involved in this virulence phenotype. We found that ArcAB controls expression of genes involved in the production of the outer core of the LOS, lic2B and lic2C, in NTHI strains. The LOS structure specified by Lic2B and its role in inhibition of complement interactions were characterized to provide insight into the molecular mechanism of NTHI pathogenesis. Together the results indicate that the redox-responsive ArcA regulator controls transcription of LOS glycosyltransferase genes that are important for pathogenicity and evasion of complement, suggesting that \textit{H. influenzae} utilizes redox signaling to appropriately modify its immune evasion strategies in different environments encountered during infection.

\section*{MATERIALS AND METHODS}

\textbf{Media and \textit{Haemophilus influenzae} growth conditions.} The nontypeable \textit{Haemophilus influenzae} (NTHI) clinical isolates NT127 and PittGG were grown at \(35^\circ\text{C} \pm 1.5^\circ\text{C}\) in brain heart infusion supplemented with 10 \(\mu\text{g}/\text{mL}\) NAD and 10 \(\mu\text{g}/\text{mL}\) hemin (sBHI) on agar plates or in sBHI broth cultures. NT127 was isolated from the blood of a 6-month-old patient with meningitis (provided by Garth D. Ehrlich), which also was isolated from the blood of a 6-month-old patient with meningitis (provided by Garth D. Ehrlich). NT127 lacks the lex2A locus, as described previously (25), for subsequent transformation with pXT10 vector.

\textbf{Plasmid and \textit{H. influenzae} strain construction.} Standard molecular biology methods were used for plasmid construction (2). All primer sequences are listed in Table 1. Strains and plasmids used in this study are listed in Table 2. An arcA mutation in NT127 was created by amplification of a 3.56-kb PCR product using primers ArcA2466 and ArcA5582 from \textit{H. influenzae} Rd strain RAA6, which contains a nonpolar, in-frame deletion of the \textit{arcA} protein-coding sequences (19), and transformation of NT127 with the resulting product. Km\(^\prime\) transformants were selected on sBHI agar containing Km to create strain NTlic2B. Strains NT127 and NTlic2B, containing empty vector sequences at the partial \(xyl\) locus, were generated as follows. A 1.4-kb PCR product was amplified from NT127 with primers JhspC2-Pei and xylAorfout, and an 8.2-kb PCR product was amplified from \(p\)T10 (76) with primers tetR-in1 and xylB-3ORF3. These two products were used as templates in a PCR stitching reaction to generate a 8.2-kb PCR product for transformation into NT127 and NTAA. Tet\(^\prime\) transformants undergoing homologous recombination between the 8.2-kb product at arcC2 and the \(xyl\) locus were selected on sBHI agar containing Tet. An arcA complementing strain containing a wild-type copy of \textit{arcA} in the \(xyl\) locus was created as follows. The 1.4-kb PCR product described above and a 7.73-kb PCR product amplified from \(p\)TAA (19) with primers AAtetR-in1 and xylB-3ORF3 were used as templates in a PCR stitching reaction to generate a 9.1-kb PCR product used for transformation into NTAA. Tet\(^\prime\) transformants were selected on sBHI agar containing Tet to create the \textit{arcA} complementing strain NTAAc. Nonpolar, in-frame deletion mutations of \textit{lic2B} in NT127 were created by replacement of the protein-coding sequences with the \textit{aucC1} gentamicin resistance cassette to create NTlic2B by PCR stitching as follows. A 1.023-kb PCR product containing the \(5\)' flanking region of \(lic2B\) was amplified from NT127 with primers 1kb5' JBlic2B and JBlic2B-5' out. A 536-bp fragment containing the \textit{aucC1} gentamicin resistance gene was amplified with primers aucC15' and aucC13' from pBSL182 (1). The 1032-bp, 1050-bp, and 536-bp products were stitched in a PCR with primers 1kb5' JBlic2B and JBlic2B-5' out. The resultant 2,832-bp product was introduced into three independent cultures of NT127, and Km\(^\prime\) transformants were selected on sBHI agar containing Gm to create independent isolates of strain NTlic2B containing a precise replacement of the \(lic2B\) coding sequence with those of \textit{aucC1}. Similarly, a \(lic2B\) deletion mutation in PittGG was created by transforming this strain with the same 2,559-bp PCR product (see above) and selecting for Gm\(^\prime\) transformants on sBHI agar containing Gm to create strain PttGlClic2B.

A nonpolar, in-frame deletion of \(lic2C\) in NT127 was created by replacement of the protein-coding sequences with the \textit{aucC1} gentamicin resistance cassette to create NTlic2C by PCR stitching as follows. A 1.023-kb PCR product containing the \(5\)' flanking region of \(lic2C\) was amplified from NT127 with primers 1kb5' JBlic2C and JBlic2C-5' out. A 1.036-bp PCR product containing the \(3\)' flanking region of \(lic2C\) was amplified from NT127 with primers 1kb3' JBlic2C and 1kb3' JBlic2C. The 1,023-bp, 1,036-bp, and 536-bp products containing the \textit{aucC1} gentamicin resistance cassette (see above) were combined by sequence overlap expansion PCR with primers 1kb5' JBlic2C and 1kb3' JBlic2C. The resultant 2,565-bp product was introduced into NT127, and Km\(^\prime\) transformants were selected on sBHI agar containing Gm to create strain NTlic2C.

A nonpolar, in-frame deletion of \(lex2A\) in NT127 was created by replacement of the protein-coding sequences with a kanamycin resistance gene to create NTlic2A by PCR stitching as follows. A 1,019-bp PCR product containing the \(5\)' flanking region of \(lex2A\) was amplified from NT127 with primers 1kb5' JBlex2A and JBlex2A-5' out. A 1,025-bp PCR product containing the \(3\)' flanking region of \(lex2A\) was amplified from NT127 with primers 1kb3' JBlex2A and 1kb3' JBlex2A. The resultant 2,832-bp product was introduced into NT127, and Km\(^\prime\) transformants were selected on sBHI agar containing Km to create strain NTlex2A.

The \(lic2B\)-complemented strain NTlic2Bcomp was generated by using our exchange vector \(p\)XT10 (76) containing the wild-type copy of \(lic2B\) for homologous recombination into the \(xyl\) locus. Briefly, a 1,081-bp product containing the \(lic2B\) gene and its promoter region was amplified from NT127 with primers lic2Bomp5' and lic2Bomp3. The 1,081-bp PCR product was digested with SapI and cloned into the SapI sites of \(p\)T10 to create pllic2Bcomp. Because strain NT127 lacks \(xylFGH\), which are needed for recombination with \(p\)XT10, we generated a Km\'-marked derivative of NT127 that contains the complete \(xyl\) locus, as described previously (25), for subsequent transformation with \(p\)XT10 and its derivatives. The Km\'-marked NT127 derivative was transformed with the 2,559-bp PCR product containing the \(lic2B\) mutation (see above) to create a \(lic2B\) mutant in this background. This resultant strain was then transformed with \(p\)XT10 and the complementing plasmid, pllic2Bcomp, to generate Ntllic2BV and Ntllic2Bcomp, respectively. The Km\'-marked NT127 derivative was also transformed with \(p\)XT10 to create a parent strain, NTV, carrying the empty cloning vector (25). All strain constructions were verified by PCR amplification across the inserted recombination region with primers specific for flanking sequences. Genes introduced for complementation of mutations were verified by DNA sequence analysis.

\textbf{Serum bactericidal assays.} Serum bactericidal testing was performed as described previously (48). Briefly, \(2,000 \text{ CFU}\) of bacteria were grown anaerobi-
TABLE 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jba5pC2-Pci</td>
<td>5'-AAAGATCTACATGTTAAAAACACCCATTGGACTTGGTTT</td>
</tr>
<tr>
<td>xylAorFout</td>
<td>5'-CATACACATTTCCTCCTGATTTG</td>
</tr>
<tr>
<td>tetR-in1</td>
<td>5'-GAGAAAGATTGTAGCCTTGAGTGATGTCAGGAAATTTG</td>
</tr>
<tr>
<td>xylB-3OF3</td>
<td>5'-TTTAATATTTAGTAAATGTTTGT</td>
</tr>
<tr>
<td>AAT-AetR-in1</td>
<td>5'-GAGGAAAGATTGTAGCCTTGAGTGATGTCAGGAAATTTG</td>
</tr>
<tr>
<td>JBa2b-5'out</td>
<td>5'-GCTGTCGCTGAACACATTGGTAGTATGGAAATTTG</td>
</tr>
<tr>
<td>JBlb2c-5'out</td>
<td>5'-AGTACCGCGACCATAATTCACCTAGACGTGTAAGT</td>
</tr>
<tr>
<td>1kb3' JBlb2c</td>
<td>5'-ATGGTAGGAAATACGGTGTAGCTTGGT</td>
</tr>
<tr>
<td>1kb3' JBlb2c</td>
<td>5'-ATGGTAGGAAATACGGTGTAGCTTGGT</td>
</tr>
<tr>
<td>JBlb2c-25'out</td>
<td>5'-GAAGAGCTGTCGAAACACATTTGTTTATTAATG</td>
</tr>
<tr>
<td>JBlb2c-3'out</td>
<td>5'-TTGAAATATCCTAGCGAATCTAAAACTG</td>
</tr>
<tr>
<td>ic2bcomp5</td>
<td>5'-CGCGGATCGCTTCTTCTAAATGGAAGATATAGTATGTTGAA</td>
</tr>
<tr>
<td>ic2bcomp3</td>
<td>5'-CGCGGATCGCTTCTTCTAAATGGAAGATATAGTATGTTGAA</td>
</tr>
<tr>
<td>ic2b-5'</td>
<td>5'-ATGCATATAAATGCGTTATATTG</td>
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<tr>
<td>JBlb2cint3'2</td>
<td>5'-CTACATTTACCTTGTCTTTCCTTCG</td>
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<tr>
<td>JBlb2cint3'2</td>
<td>5'-CTACATTTACCTTGTCTTTCCTTCG</td>
</tr>
<tr>
<td>JBlb2cri2</td>
<td>5'-CATAATAAACAATATTATTTAATG</td>
</tr>
<tr>
<td>JBlb2cri3</td>
<td>5'-CATAATAAACAATATTATTTAATG</td>
</tr>
<tr>
<td>JBlb2cint3'2</td>
<td>5'-CATAATAAACAATATTATTTAATG</td>
</tr>
<tr>
<td>JBlb2cri5</td>
<td>5'-AATATATTTGATGGCGTATTG</td>
</tr>
<tr>
<td>JBlb2cri3</td>
<td>5'-AATATATTTGATGGCGTATTG</td>
</tr>
<tr>
<td>JBlb2cri2</td>
<td>5'-AATATATTTGATGGCGTATTG</td>
</tr>
<tr>
<td>lic2BPE</td>
<td>5'-CTGGGTATTTACATTTATACATC</td>
</tr>
<tr>
<td>lic2BPE</td>
<td>5'-CTGGGTATTTACATTTATACATC</td>
</tr>
</tbody>
</table>

* (Labeled at the 5' end with 6-carboxyfluorescein (FAM)).

gually (DBB BBL GasPak Plus anaerobic system; Fisher Scientific) or aerobically (10 ml in a 500-ml flask with shaking at 250 rpm) to mid-log phase and incubated with or without normal human serum (NHS) (Innovative Research) at concentrations specified for each experiment in a final reaction mixture volume of 150 μl. Dilutions of NHS-treated and untreated samples were plated on sBHI agar plates at 0 and 30 min. In all cases, similar numbers of bacteria were recovered from treated and untreated samples at 0 min. Survival was calculated as the ratio of the number of CFU recovered at 30 min to the number of CFU recovered from untreated samples. To isolate the role of the alternative pathway, 10 mM serum (Quidel) was performed as described above to selectively block activation of complement factors C3 and C4 and serum antibodies IgM.

Briefly, 10^8 CFU/ml of log-phase NT127 parent NTV, JBlb2c deletion mutant NT127Δc2, and lic2B-2-complemented strain NTlic2BComp suspended in Hanks balanced salt solution (HBSS) containing 0.15 mM CaCl_2 and 1 mM MgCl_2 (Invitrogen) were incubated with 5% pooled NHS in a final volume of 100 μl. Dilutions of NHS-treated and untreated samples were plated on sBHI agar plates and incubated at 37°C for 30 min, followed by detection with fluorescein isothiocyanate (FITC)-conjugated polyclonal antibodies specific to C3c (Biodesign Int., Suco, ME), C4 (Abcam), IgM (Sigma), and IgG (Sigma).

Reverse transcriptase quantitative PCR (RT-qPCR). Quantification of relative mRNA expression of lic2B, JBlb2c, JBlb2c, and rpoA from strains NT127, NT127V, NTAA, NTAAV, NTAAc1, and NTAAc2 with RNA samples from four independent cultures was performed using qSYBR green Supermix (BioRad Laboratories) in quantitative real-time PCR measured with the DNA Engine Opticon II system (MJ Research). Total RNA was obtained from cultures grown anaerobically in sBHI to an optical density at 600 nm (OD_{600}) of 0.2 to 0.4. RNA was isolated using TRIzol reagent (Invitrogen), treated with DNase I (Ambion), and phenol extracted. Briefly, 6 μg of DNAse I-treated total RNA from the above-mentioned strains was used as the template in the cDNA synthesis using random primers (New England BioLabs) and SuperScript II reverse transcriptase (Invitrogen). One-tenth of the reverse transcriptase reaction products was used as the template in the qPCR for amplification using 5' and 3' primer pairs for lic2B (lic2B-5 and JBlb2Cint3'2), rpoA (rpoA-3 and lic2BPE), and 258rep3-2 (JBlb2c-5 rep and JBlb2c-3 rep), lpc (JBlb2c-5' and 258rep3-2), and rpoA (HEB802-5' and JBlb2c-3'). Genomic NT127 DNA ranging from 80 ng to 100 ng was used as the template in the qPCRs with the same primer set to generate a standard curve. Real-time cyinder cycles were as follows: 95°C for 3 min; 39 cycles of 96°C for 20 s, 55°C for 30 s, and 72°C for 30 s; and one cycle of 72°C for 7 min. Fluorescence was read at 72, 76, 78, 80, 81, and 82°C and normalized to the housekeeping gene rpoA, which encodes the alpha subunit of RNA polymerase. Control real-time PCRs performed in parallel with mock cDNA reactions generated without reverse transcriptase to verify specific amplification yielded values below the level of detection. Product sizes were confirmed by agarose gel electrophoresis.

Primase extension analysis of NT11 mRNA. Briefly, 10 μg of DNAse I-treated total RNA from wild-type NT127 was used as the template in a 20-μl cDNA synthesis reaction using 2 pmol of a 5' 6-carboxyfluorescein (FAM)-labeled primer, lic2BPE, located 70 bp 3' of the ATG initiation codon of lic2B and SuperScript II reverse transcriptase (Invitrogen). Control reactions were performed in parallel with mock cDNA reactions generated without reverse transcriptase to verify specific amplification. DNA fragment analysis of the FAM-labeled cDNAs was conducted at the University of Illinois at Urbana-Champaign Sequencing Core using an ABI Prism 3730xl Analyzer with ROX 500 dye-labeled size standards. Fragment sizes of the cDNA extension products were calculated with Peak Scanner software (Applied Biosystems, Foster City, CA) with a resolution of ±2 bp.
acetone, and 2/H11003. Briefly, freeze-dried cells were washed with organic solvents (1 g) followed by washing with 10 ml of water and repelleting for 15 min. The supernatant gets were not identified.

complementary construct (expressed via lic2B promoter) in the xyl locus, replacing xylA; Gm^Tet

expressed via lic2B promoter.}

Murine bacteremia model. *H. influenzae* was grown to logarithmic phase (OD_600 = 0.3) as 20-ml cultures in 50-ml shake flasks at 35°C. Four-week-old C57BL/6 mice were inoculated by the intraperitoneal (i.p.) route at a dose of 2 \times 10^8 CFU of wild-type NT127 or its isogenic, nonpolar lic2B mutant NTlic2B derivatives. For evaluating the effects of the lic2B mutation, each mouse was inoculated with one of three lic2B deletion mutants generated in independent transformations with the deletion construct as described above. Blood (5 μl) was collected by tail vein sampling at 4, 8, and 22 h postinfection and serially diluted in BHI for CFU determination. Experiments were conducted with approval and in accordance with guidelines of the University of Massachusetts Institutional Animal Use and Care Committee.

Structural analysis of LOS. *H. influenzae* wild-type NT127 and lic2B mutant NTlic2B were grown at 35°C to an OD_600 of ~1 to 2 for ~15 h (with shaking at 80 rpm) in 4 liter Fernbach flasks containing 2.5 liters of sBHI for a total of 10 liters of culture per strain. Bacterial pellets were collected by centrifugation followed by washing with 10 ml of HBSS and repelleted. Bacteria were killed by phenol treatment (2%) with the final concentration for 3 h at room temperature with gentle rotation followed by washing with 10 ml of water and repelleting for lyophilization. LOS was isolated and purified as described previously (67). Briefly, freeze-dried cells were washed with organic solvents (1% ethanol, 2% acetonitrile, and 2% light petroleum ether) and extracted by the hot phenol-water method. The aqueous phase was treated with DNase and RNase at 37°C for 4 h, followed by proteinase K treatment at 37°C for 4 h, with the resulting small peptides removed by dialysis. The retentate was freeze-dried and then brought to 70°C, 5 volumes) was gradually added to destroy excess hydrazine, and the precipitated LOS-OH was centrifuged, redissolved in water, and lyophilized. The core oligosaccharides (OS) were isolated by treating the LOS with 1% acetic acid (10 mg/ml, 100°C, 1.5 h), with subsequent removal of the insoluble lipid A by centrifugation (5,000 \times g). The lyophilized OS samples were subsequently further purified on a Bio-Gel P-2 column and analyzed by capillary electrophoresis-mass spectrometry (CE-MS) and nuclear magnetic resonance (NMR) spectroscopy as described previously (67). Sugars were determined as their alditol acetate derivatives and linkage analysis conducted following methylation analysis by gas-liquid chromatography-mass spectrometry (GLC-MS) as described previously (67).

Western blot analysis. *H. influenzae* strains were grown anaerobically to logarithmic phase (OD_600 = 0.2 to 0.3) as 3-ml cultures at 35°C. A total of 10^8 bacteria suspended in HBSS containing 0.15 mM CaCl_2, and 1 mM MgCl_2, were incubated with 5% pooled NHS in a final volume of 100 μl for 30 min at 37°C. Whole-cell lysates were separated by NuPAGE (Novex 4 to 12% Bis-Tris) and immunoblotted with anti-iC3b monoclonal antibody (MAB) G-3E (a gift from Kyoko Iida, University of Tsukuba, Japan) (32). Bound primary antibody was visualized using the appropriate secondary antibodies conjugated to alkaline phosphatase as described previously (6). As a loading control, the bottom third of the same blot (proteins that migrated faster than 50 kDa) was stained with Coomassie blue (CB). The ImageJ64 gel analysis image processing program (http://rsbweb.nih.gov/ij/) was used to quantify the relative densities of bands in the anti-iC3b Western blot and CB-stained gel. The relative density of total intensity within bands ranging from ~120 kDa to ~67 kDa in sample lanes was determined after subtracting the background area corresponding to the same size range in the buffer-only control lane, followed by normalization to the relative density of the 40-kDa band in the corresponding lane of the CB-stained portion of the gel.

RESULTS

Based on the observation that arcA mutants of *H. influenzae* type b were more sensitive than wild-type strains to killing by human serum, it was previously postulated that ArcA-regulated genes encoding cell surface structures may act as potential targets of humoral immune components in serum, such as complement (12); however, such potential ArcA-regulated targets were not identified.

In contrast to the Hib study, our previous results detected no difference in the serum resistances of an arcA mutant and the wild type in the *H. influenzae* Rd strain background (77). Because LOS structure influences serum resistance levels and the LOS outer core sugar extensions differ between *H. influenzae* strains such as Rd (62) and Hib (46, 47), we postulated that LOS structures found in pathogenic clinical isolates but absent in Rd may account for the differential serum sensitivity between the arcA mutants of different strains. This hypothesis led

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and/or relevant features</th>
<th>Reference and/or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT127</td>
<td>Nontypeable <em>H. influenzae</em> clinical isolate</td>
<td>26</td>
</tr>
<tr>
<td>PittGG</td>
<td>Nontypeable <em>H. influenzae</em> clinical isolate</td>
<td>G. Ehrlich (69)</td>
</tr>
<tr>
<td>NT127V</td>
<td>NT127 xyA^4882::arc4R, Tet’, empty cloning vector</td>
<td>This study</td>
</tr>
<tr>
<td>NTAA</td>
<td>NT127 ΔarcA::aphI, Km^Tet</td>
<td>This study</td>
</tr>
<tr>
<td>NTAAV</td>
<td>NT127 ΔarcA::aphI, yxlA^4882::arc4R, arcA mutant carrying empty cloning vector sequence from pX1T10; Km^Tet</td>
<td>This study</td>
</tr>
<tr>
<td>NTAAAC1</td>
<td>NT127 ΔarcA::aphI xyA^4882::arc4A, arcA mutant complemented with arcA expressed via arcA promoter replacing xylA; Km^Tet</td>
<td>This study</td>
</tr>
<tr>
<td>NTAAAC2</td>
<td>Same genotype as NTAAAC1; isolate from an independent transformation</td>
<td>This study</td>
</tr>
<tr>
<td>NTlic2B</td>
<td>NT127 Δlic2B::aacC1; Gm^Tet</td>
<td>This study</td>
</tr>
<tr>
<td>NTlic2C</td>
<td>NT127 Δlic2C::aucC1; Gm^Tet</td>
<td>This study</td>
</tr>
<tr>
<td>NTlic2A</td>
<td>NT127 Δlic2A:: aphI; Km^Tet</td>
<td>This study</td>
</tr>
<tr>
<td>PittGGlic2B</td>
<td>NT127 Δlic2B::aacC1; Gm^Tet</td>
<td>This study</td>
</tr>
<tr>
<td>NTV</td>
<td>NT127 xyA^4882::arc4A, contains empty cloning vector sequence from pX1T10; xylHGF introduced into genome (see Materials and Methods); Tet’</td>
<td>26</td>
</tr>
<tr>
<td>NTlic2BV</td>
<td>NTV Δlic2B::aacC1; lic2B mutant carrying empty cloning vector sequence from pX1T10; Gm^Tet</td>
<td>This study</td>
</tr>
<tr>
<td>NTlic2BComp</td>
<td>NT127 Δlic2B::aacC1 xyA^4882::lic2B; lic2B mutant containing lic2B complementing construct (expressed via lic2B promoter) in the xyl locus, replacing xylA; Gm^Tet</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
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<tr>
<td>pX1T0</td>
<td>Delivery vector for chromosomal integration and expression at the xyl locus of <em>H. influenzae</em>; contains xylF, xylB, xyA^4882 and tetracycline resistance cassette; tetAR</td>
<td>80</td>
</tr>
<tr>
<td>plic2Bcomp</td>
<td>pX1T0 carrying lic2B expressed from lic2B promoter</td>
<td>This study</td>
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TABLE 2. Strains and plasmids used in this study


FIG. 1. Role of ArcA in resistance to the bactericidal effects of serum and binding to iC3b. (A and B) The NTHI parent strain NT127V (arcA+), the arcA deletion mutant NTAAV (ΔarcA), and the arcA-complemented strain NTAAC1 (ΔarcA, arcA+) were grown anaerobically (A) and aerobically (B) to mid-log phase and exposed to pooled normal human serum (NHS) (Innovative Research) at the indicated concentrations for 30 min, followed by plating on sBHI to enumerate CFU. The survival ratios are plotted as the percentage of CFU obtained from serum-treated/un-treated samples. Survival levels of the arcA mutant differed significantly (∗) from those of both the parental and complemented strains at 3% NHS under anaerobic conditions (P < 0.001 and P < 0.05, respectively, by one-way analysis of variance [ANOVA] with Bonferroni’s multiple-comparison test). In all cases, differences between the parent and complemented strains were not statistically significant. No statistically significant differences were detected among the aerobic samples in panel B. (C) iC3b binding to NT127. Western blots of wild-type NT127, NT127 containing an empty cloning vector in the xyl locus (NT127V), an arcA deletion mutant (NTAAV), and arcA mutant containing an empty vector in xyl (NTAAV), and two independent isolates containing arcA complemented in trans at the xyl locus (NTAAC1 and NTAAC2) (lanes 2–7, respectively) are shown. Bacteria grown anaerobically to logarithmic phase were incubated with 5% pooled NHS for 30 min at 37°C. Whole-cell lysates were separated by NuPAGE (Novex 4 to 12% Bis-Tris) and immunoblotted with anti-iC3b MAb. For a loading control, the bottom third of the same blot is stained with Coomassie blue. The arrow indicates the iC3bα′ chain. Lane 1 contains molecular size markers, lane 8 contains pure iC3b made from fluid-phase C3b, and lane 9 contains bacteria incubated in the absence of serum. Relative total densities of the bands in the region between ~120 kDa and ~67 kDa from lanes 2 to 7 normalized to that of the 40-kDa band from the same corresponding lane in the Coomassie blue-stained portion of the gel are 0.5, 0.6, 2.1, 1.4, 0.5, and 0.8, respectively.

us to investigate a possible ArcA-mediated serum resistance phenotype in NTHI and to address whether arcA-regulated LOS biosynthesis genes participate in defense against complement-mediated killing. Transcriptional analysis of LOS genes present in Hib and in pathogenic NTHI strains but absent in Rd identified ArcA-mediated regulation of a putative LOS glycosyltransferase gene, lic2B. The role of lic2B in LOS biosynthesis was then characterized by structural analysis of the wild-type NTHI strain in comparison to a mutant in which lic2B was deleted. Because complement resistance is an important factor in invasive infection (63), the role of lic2B was then evaluated in a murine model of bacteremia. To obtain insight into the molecular mechanisms of immune evasion by NTHI, interactions of the lic2B mutant with specific complement components were assessed.

ArcA is required for serum resistance and evasion of complement by NTHI. To determine whether ArcA controls serum resistance in NTHI, we tested the effect of a range of concentrations of normal human serum on the viability of an arcA mutant derived from an NTHI clinical isolate, strain NT127. Exposure of the anaerobically grown arcA mutant to 3% serum resulted in ~60% killing of the mutant but did not affect viability of the parent, and survival was restored to near-parental levels in the arcA complemented strain (Fig. 1A). In contrast, serum resistance of the aerobically grown arcA mutant did not differ significantly from that of the parental strain at this serum concentration (Fig. 1B), consistent with greater activity of ArcA under low-oxygen conditions. That this difference was associated with a difference in complement binding was supported by evidence that deletion of arcA resulted in ~3-fold-increased binding to iC3b, a cleavage product of complement component factor C3b, compared to that of the parent (Fig. 1C, lanes 4 and 5 versus lanes 2 and 3) based on densitometry of the region of the Western blot between ~120 kDa and ~67 kDa (see Materials and Methods). The Western immunoblot analysis of iC3b associated with serum-treated bacterial samples revealed a “doublet” (~67 kDa) in which the upper band likely represents LOS covalently bound to the iC3bα′ chain and the lower band the free (or “released”) iC3b α′ chain; the α′ chain of iC3b bound to NTHI may undergo additional cleavage that may account for its lower molecular mass compared to that of purified soluble iC3bα′ chain (lane 8) (16). Both bands were absent in samples containing untreated bacteria (lane 9). Complementation of the arcA mutant (lanes 4 and 5) with a copy of arcA inserted in trans at the xyl locus in two independent isolates (lanes 6 and 7) decreased levels of binding to the iC3b MAb back to parental levels (lanes 2 and 3). As expected, the NT127 parental (lane 3) and arcA mutant (lane 5) strains containing the empty cloning vector used in generating the complementing construct exhibited iC3b MAb binding similar to those of their respective wild-type (lane 2) and arcA mutant (lane 4) derivatives.

ArcA modulates mRNA levels of LOS glycosyltransferase genes. In contrast to results with NTHI (Fig. 1), our previous studies indicated that the serum resistance of an Rd arcA mutant did not differ from that of the wild type (77). Because LOS structures represent major factors in complement evasion by NTHI, we hypothesized that ArcA may regulate LOS biosynthesis genes in NT127 that are not present in the Rd genome. The genome of NT127 (GenBank accession no. AC540000000.1) reveals genes that are absent in Rd and have 97 to 98% predicted amino acid identity to characterized LOS glycosyltransferase genes. These include lic2C (26, 30), lex2A and lex2B (21, 33), and a gene with 98% identity to the...
predicted glycosyltransferase gene, lic2B (26). To evaluate potential regulation of LOS genes by ArcA, transcriptional analysis of lic2B, lic2C, and lex2A, genes likely to participate in modifying the LOS outer core, was done by RT-qPCR in NT127, its arcA mutant, and the arcA mutant that was complemented with arcA. Figure 2A shows the results obtained with two independently derived strains of each genotype used in a total of four independent experiments conducted in parallel. The lic2B gene exhibited up to ~6-fold-decreased expression in arcA mutant isolates relative to parental or complemented strains (Fig. 2A, left panel). Furthermore, expression of lic2C, which is located immediately downstream of lic2B, was also ArcA activated (Fig. 2A, right panel). The lic2C gene is needed for addition of the proximal glucose residue in an α1-3 linkage to heptose II of the LOS inner core (30). In contrast, expression levels of lex2A, which is cotranscribed with lex2B (21), and lgtC (data not shown) did not exhibit regulation by ArcA. Primer extension analysis mapped a transcriptional start site to a distance of ~26 bp upstream of the putative lic2B ATG start codon (Fig. 2B). Located 87 bp upstream of the start site is a potential ArcA binding site with an 8/10 match to the 10-bp consensus binding site of ArcA (39). Promoter regions for lic2B were compared among available NTHI genome sequences by ClustalW alignment. Of the 18 NTHI strains for which partial or complete genome sequences are available, 8 strains contain single copies of lic2B with 96 to 100% predicted amino acid identity to Lic2B of NT127. The lic2B promoter regions were 95 to 96% identical between strains, and the 10-bp ArcA motif and its position relative to −10/−35 promoter consensus sequences were identical in all eight NTHI strains and in Hib.

lic2B and lic2C mutants exhibit decreased serum resistance.

Because arcA is required for serum resistance in NT127 and was observed to regulate the expression of lic2B and lic2C, we examined the roles of these genes in serum resistance. Nonpolar deletion mutations precisely removing the complete open reading frames of lic2B or lic2C were generated in strain NT127. In serum bactericidal assays conducted with cultures grown anaerobically, with two independently derived clones for each mutation, lic2B and lic2C mutants yielded marked and statistically significant decreases in survival compared to the wild type, reaching differences in 3% NHS of 9-fold and 14-fold between the wild type and the lic2B and lic2C mutants, respectively (Fig. 3A). In contrast, nonpolar deletion of the lex2A gene, which is not regulated by ArcA and is thought to be required for addition of the second β-glucose residue extending from Hep I of the inner core (21), did not confer an appreciable defect in serum resistance (Fig. 3A). Complementation of the lic2B mutation restored viability of the lic2B mutant to parental levels (Fig. 3B), reaching marked and statistically significant differences in 3% and 5% NHS (~4-fold and ~80-fold differences between the lic2B mutant and the parent; equivalent differences were seen between the lic2B mutant and complemented strain).

To determine whether lic2B is required for serum resistance in an additional clinical NTHI isolate, we examined this phenotype in PittGG, a strain first isolated from the external ear discharge of a child with otitis media (7, 66) and for which a draft genome sequence is available. PittGG was previously shown to
exhibit a greater degree of virulence than 10 other clinical NTHI isolates, producing the most rapid and severe local and systemic disease in a chinchilla model of otitis media (7). In our serum bactericidal assay PittGG appeared to be more serum resistant than NT127, and a deletion mutant NTlic2B, NThlic2C, and NTlex2A (NTlic2B, NTlic2C, and NTlex2A, respectively). Each deletion mutant is represented by isolates obtained from two independent transformations. Differences were statistically significant (*) between the parent and the lic2B and lic2C mutants at 2% and 3% NHS (mixed-model ANOVA for repeated measures with Bonferroni’s multiple-comparison test). (B) NTV (lic2B*), lic2B deletion mutant, NThlic2BV (lic2B), and lic2B-complemented strain NTlic2BComp (lic2B, lic2B*). The lic2B mutant exhibited a statistically significant decrease in survival (*) compared to both the parental and complemented strains at 2%, 3%, and 5% NHS. Statistical significance for panels B and C was evaluated by one-way ANOVA with Bonferroni’s multiple-comparison test. (C) Wild-type NTHI strain PittGG and lic2B deletion mutant PittGGlic2B (lic2B*), represented by isolates from three independent transformations. Differences were statistically significant (*) between the parent and the lic2B mutant at 10% and 20% NHS.

FIG. 3. Serum resistance defect of NTHI lic2B and lic2C mutants. Wild-type strains and LOS mutants were grown anaerobically to mid-log phase and exposed to pooled NHS at the indicated percentages for 30 min, followed by plating to enumerate CFU. Survival ratios are plotted as the percentage of CFU obtained from serum-treated/untreated samples (A) Wild-type NTHI strain NT127, and deletion mutants NTHlic2B, NThlic2C, and NThlex2A (NTlic2B, NTlic2C, and NTlex2A, respectively). Each deletion mutant is represented by isolates obtained from two independent transformations. Differences were statistically significant (*) between the parent and the lic2B and lic2C mutants at 2% and 3% NHS (mixed-model ANOVA for repeated measures with Bonferroni’s multiple-comparison test). (B) NTV (lic2B*), lic2B deletion mutant, NThlic2BV (lic2B), and lic2B-complemented strain NTlic2BComp (lic2B, lic2B*). The lic2B mutant exhibited a statistically significant decrease in survival (*) compared to both the parental and complemented strains at 2%, 3%, and 5% NHS. Statistical significance for panels B and C was evaluated by one-way ANOVA with Bonferroni’s multiple-comparison test. (C) Wild-type NTHI strain PittGG and lic2B deletion mutant PittGGlic2B (lic2B*), represented by isolates from three independent transformations. Differences were statistically significant (*) between the parent and the lic2B mutant at 10% and 20% NHS.
LD-Hep in an approximate ratio of 1:1:2:1. Trace amounts of 4-substituted and 3-substituted Glc were also observed (Table 5). Conversely, for the lic2B mutant, methylation analysis on the nonfractionated core OS revealed only terminal Glc, 4-substituted Glc, and terminal LD-Hep in an approximate ratio of 2:1:1. Linkage analysis in combination with the compositional and mass spectrometry analyses therefore suggested that, in comparison to the parent LOS, the mutant elaborates a truncated molecule lacking a galactose residue, presumably 4-linked to a glucose residue.

In order to elucidate the precise locations and linkage patterns of the OS from the wild-type and mutant strains, NMR studies were performed on the OS fraction that gave the most resolved and homogeneous spectrum. The assignment of 1H resonances of the inner core oligosaccharides for each strain were achieved by correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY) experiments with reference to the published data for the structurally related oligosaccharides from H. influenzae strains Eagan (47), Rd (62), and SB33 (10) and revealed that the conserved inner core structure (Hep I-III and Glc I) was present. Similarly, the assignment of 13C resonances of the samples was achieved by virtue of 13C-1H heteronuclear single-quantum correlation (HSQC) and 13C-1H HSQC-TOCSY experiments (data not shown). Apart from the conserved inner core residues (data not shown), an anomic proton was observed for an α-glucose residue at 5.28 ppm in both the wild-type and lic2B mutant core OS (Table 6). The chemical shifts for the spin systems of this residue in these two strain backgrounds suggested that for the mutant strain the α-glucose residue was not substituted, whereas in the parent strain it appeared to be 4-substituted. An inter-NOE connectivity from the anomic proton of this glucose residue to a resonance at 4.07 ppm was identified. This resonance was assigned as the proton at the 3 position of the Hep II residue by virtue of 13C-1H HSQC and 13C-1H HSQC-TOCSY experiments (data not shown). Furthermore a β-galactose residue was identified in only the parent strain core OS, based on characteristic spin systems in TOCSY experiments, with H-1 (4.48 ppm), H-2 (3.55 ppm), H-3 (3.67 ppm), and H-4 (3.92 ppm) resonances being identified (Table 6). An inter-NOE connectivity from the anomic proton of this galactose residue to a resonance at 3.74 ppm was identified. This resonance was assigned as the proton at the 4 position of the α-glucose residue by virtue of 13C-1H HSQC and 13C-1H HSQC-TOCSY experiments (data not shown). This assignment is also consistent with the methylation analysis data, which identified a 4-linked Glc residue and a terminal galactose residue in the wild-type strain. Together these data are consistent with lic2B-dependent addition of a β-galactose at position 4 of the α-glucose extension on Hep II. A model of the NT127 and lic2B mutant LOS is depicted in Fig. 4.

lic2B is required for NTTH survival in a mouse model of bacteremia. Because an arcA mutation confers survival and virulence defects in the murine bloodstream (12, 77), we tested whether lic2B was also required in vascular infection in mice. Wild-type NT127 was compared to three independently generated lic2B mutants. After intraperitoneal inoculation, bacteria were recovered at similar levels from blood at 4 h for both the wild type and the lic2B mutants. At subsequent times, marked differences were observed. At 8 h postinoculation the CFU recovered for the lic2B mutants had declined significantly (~83-fold; P < 0.01) compared to that recovered for the wild type, and at 22 h postinoculation they differed by at least 12-fold, a trend consistent with the 8-h results (Fig. 5).

Role of Lic2B in resistance to the classical pathway of complement. H. influenzae surface structures, including LOS and outer membrane proteins, have been implicated in diverse mechanisms of complement resistance that are not fully understood (24). Therefore, it was of interest to investigate the influence of lic2B on NTTH interactions with elements of the complement system. The majority of the bactericidal effect of serum on H. influenzae in vitro requires activity of the classical pathway (73). The classical pathway is initiated by the C1 complex (C1q, C1r, and C1s), a component specific to this pathway with C1q binding to a variety of targets, including antigen-antibody complexes on the pathogen surface, to initiate the stepwise cascade of complement activation (71). To determine if the lic2B-dependent modification of the LOS confers resistance to the classical pathway, we tested the bactericidal effect of C1q-depleted human serum on the lic2B mutant. Viability of the lic2B mutant was similar to that of the wild-type strain in C1q-depleted human serum, and supplementation with human C1q protein restored killing of the mutant to a greater degree than for the wild type, whose survival was at least 146-fold that of the mutant (Fig. 6A). This result indicated a specific role for the classical pathway in killing the lic2B mutant in complement-dependent bactericidal assays.

Additional support for this conclusion was obtained by examining the viability of the lic2B mutant in bactericidal assays with NHS containing EGTA and MgCl2, which selectively blocks the classical and mannose binding lectin pathways but not the alternative pathway (Fig. 6B and C). In the absence of EGTA/MgCl2, we observed ~60% killing of the lic2B mutant at 2% NHS, over 90% killing of the mutant at 3% NHS, and no appreciable killing of the wild type at either concentration.
which classical complement components are influenced by position, or C4b inactivation. To begin to address the step at antibody binding, C1 engagement, C4 activation, C4b de-pathway of complement may occur at a variety of steps, including killing of the lic2B pathway is required for complement-dependent killing of the with the C1q-dependent killing data confirm that the classical not involved in bactericidal activity against NTHI.

The contribution of the lic2B gene in resisting the classical pathway of complement may occur at a variety of steps, including antibody binding, C1 engagement, C4 activation, C4b deposition, or C4b inactivation. To begin to address the step at which classical complement components are influenced by lic2B, we evaluated total binding of C3, C4, and serum antibodies (IgG and IgM) to the NT127 parent, lic2B mutant, and complemented strain (Fig. 6D). As observed with the arcA mutant, the lic2B mutant bound increased levels of C3 and C4 compared to the parent strain. Complementation reduced deposition of C3 and C4 to levels similar to those seen with the parental strain. We did not detect a difference in interaction with IgG and IgM between the two strains, suggesting that differences in the amount of antibody binding do not account for the serum resistance or differences in complement C4 or C3 deposition. These results suggest that the lic2B-encoded LOS structure confers complement resistance by interfering with the classical complement pathway at a step after antibody binding but at or preceding C4b deposition.

**DISCUSSION**

The results of this study provide insight into two previously unresolved aspects of *H. influenzae* pathogenesis. First, the redox-responsive regulatory protein ArcA was previously implicated in serum resistance and virulence of *H. influenzae*; however, the ArcA-regulated genes or factors that could account for either of these phenotypes were unknown. Second, the lic2B gene was considered likely to contribute to the pathogenic properties of NTHI strains based on epidemiological data, yet the molecular function of lic2B and the mechanism by which it may participate in virulence had not been defined. In
this report we demonstrate that ArcA positively regulates transcription of lic2B, which we determined to be responsible for a galactose addition to the LOS outer core and serum resistance in NTHI. Consistent with its role in resistance to serum complement, lic2B promotes survival of NTHI in the mammalian bloodstream. Complement has been implicated in host defense in other sites of disease, such as the middle ear during infectious otitis media or during lung inflammation (16, 17, 59, 72, 74). Therefore, these results indicate a potential mechanism whereby strains expressing lic2B may exhibit enhanced virulence in the human host.

Signal transduction in response to environmental cues is used by bacterial pathogens to appropriately coordinate gene expression during stages of colonization or pathogenesis. For obligate pathogens such as *H. influenzae* that have evolved to grow exclusively within the mammalian host, an economical strategy would be to coordinate control physiological adaptations together with virulence-associated responses. The ArcAB signal transduction system appears to mediate both of these activities together with virulence-associated responses. The current study shows that ArcA also activates LOS biosynthesis genes, including lic2B (Fig. 2), which is required for serum resistance (Fig. 3) and NTHI survival in the bloodstream model (Fig. 5). The niche in which ArcA may function during natural infection is not known; however, ArcA-mediated activation of the virulence gene lic2B suggests that NTHI encounters a low-oxygen environment leading to ArcA activity at some point during colonization or pathogenesis in which lic2B is required.

The lic2B gene has been thought to be a glycosyltransferase for many years; however, its biochemical function and potential role in virulence have remained inferential in that loss of the gene is associated with LOS truncation (26), but the precise contribution of lic2B to the structure was not known (46). Its distribution in clinical isolates suggested that lic2B may contribute to disease, as Pettigrew and colleagues found it to be present more frequently among 48 middle ear isolates of NTHI than among 46 nasopharyngeal and throat isolates in healthy children, suggesting a potential importance of lic2B in otitis media (56). However, a study of 72 isolates by Erwin and colleagues did not reveal an increased prevalence of lic2B in invasive isolates (15). It is likely that numerous genes contribute to pathogenesis of genetically distinct strains at different sites of infection, and more complete knowledge of each gene’s relative mechanistic contribution to pathogenesis is necessary.
to evaluate the significance of such clinical correlations. Our results demonstrate lic2B-dependent addition of a galactose extension from the glucose residue on the penultimate heptose (Hep II) of the LOS in strain NT127. Based on previous studies, the glucose on Hep II is likely added by lic2C (30), the gene immediately downstream of lic2B in a probable operon. The lic2B mutant also appeared to be deficient in a minor N-acetyhexose moiety, which likely represents a terminal N-acetylgalactosamine added by the product of lgtD (28). Although the lack of this structure in the lic2B mutant could result from difficulty in detecting less-abundant species in the mass spectrometry analysis, it is also possible that addition of N-acetylgalactosamine requires the galactose residue added by lic2B. Sialic acid can also be added to the LOS and may depend on lic2B; however, the medium for these experiments was not supplemented with the precursor required for sialylation of NTHI, CMP-N-acetylneuraminic acid, and sialic acid was not detected by our structural analysis of NT127. Therefore, although N-acetylgalactosamine and sialic acid have been implicated in mediating serum resistance, lic2B appears to be capable of generating the scaffold for additional LOS modifications that influence virulence.

Resistance to complement-mediated killing mediated by lic2B provides a mechanism that may account for the association between the presence of this gene and increased severity of otitis media, and it likely contributes to lung pathogenesis as well. Active complement is found in effusions isolated from patients with otitis media (53), and complement has been implicated as playing a role in defense against NTHI in the middle ear in a chinchilla model of otitis media (16). Complement is also likely to play a role in the context of inflammation in the lung. Although the healthy lung does not appear to contain abundant levels of serum complement factors, disease states such as chronic obstructive pulmonary disease (COPD) or infection with influenza virus, both predisposing conditions that exacerbate by subsequent H. influenzae infection, have been shown to result in increased levels of complement proteins in the lungs (45). In addition to their roles in the lytic pathway, complement components C1q, C3b, and C4b act as opsonins that can bind and target bacteria for destruction by phagocytes (61). Our results in Fig. 6 indicate that, relative to the wild type, the lic2B mutant bound more readily to complement components C3 and C4, and the presence of the C1q protein, the initiator of the classical complement pathway, was required for the bactericidal effect, indicating involvement of this complement factor in killing of the lic2B mutant in our serum bactericidal assays. Therefore, the ability of NTHI to inhibit binding of C1q or subsequent interactions with other complement proteins could protect the pathogen from recognition and killing by mucosal phagocytes, and it will be of interest to determine whether lic2B may contribute to respiratory tract infection by inhibiting such interactions.

Overall, the results of our study indicate a role for the redox-responsive regulator ArcA in positive control of NTHI LOS biosynthesis genes that are required for serum resistance and invasive infection. ArcA-deficient mutants of other bacte-

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