INFECTION AND IMMUNITY, July 2002, p. 3551–3556 Vol. 70, No. 7
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.7.3551–3556.2002
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Identification of the Lipooligosaccharide Biosynthesis Gene *lic2B* as a Putative Virulence Factor in Strains of Nontypeable *Haemophilus influenzae* That Cause Otitis Media

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Received 20 December 2001/Returned for modification 28 February 2002/Accepted 4 April 2002

Nontypeable (NT) strains of *Haemophilus influenzae* are an important cause of acute otitis media (OM). The pathogenic process by which NT *H. influenzae* strains cause OM is poorly understood. In order to identify specific virulence factors important for OM pathogenesis, genomic subtraction of the NT *H. influenzae* middle ear isolate G622 against *H. influenzae* strain Rd was conducted and the resulting subtraction products were used to screen a panel of *H. influenzae* isolates. Subtraction identified 36 PCR fragments unique to strain G622, which were used in a preliminary screen of 48 middle ear isolates and 46 nasopharyngeal and throat isolates to identify genes found more frequently among middle ear isolates. These experiments identified a PCR fragment with high homology to the lipooligosaccharide biosynthesis gene *lic2B* (originally identified in an *H. influenzae* type b strain) among 52% of the middle ear isolates and 9% of nasopharyngeal and throat isolates. The *lic2B* gene cloned from NT *H. influenzae* strain G622 was 99% identical at the amino acid level to that of the *H. influenzae* type b strain RM7004. The *lic2B* gene was used to screen a larger panel of *H. influenzae* isolates including the original 48 middle ear isolates, 40 invasive type b isolates, 90 NT *H. influenzae* throat isolates from children attending day care, and 32 NT *H. influenzae* nasopharyngeal clinical isolates. The *lic2B* gene was found 3.7 times more frequently among middle ear isolates than in throat isolates from children attending day care. These data suggest that a specific NT *H. influenzae* gene is associated with OM.

Haemophilus influenzae is a gram-negative bacterium that colonizes the upper respiratory tract of humans. In addition to asymptomatic colonization, *H. influenzae* also causes significant infection. Encapsulated *H. influenzae* serotype b strains cause invasive diseases such as bacteremia, septic arthritis, and meningitis. Widespread vaccination with type b polysaccharide conjugate vaccines has greatly reduced the incidence of severe disease due to *H. influenzae* type b; these vaccines, however, do not protect against infection with non-type b encapsulated or nontypeable (NT) *H. influenzae* strains.

Although infections with NT *H. influenzae* are rarely fatal, these strains cause significant morbidity and are isolated from up to half of middle ear aspirates from children with otitis media (OM) (6). OM is an infection of the middle ear resulting in middle ear effusion, fever, irritability, and inflammation of the tympanic membrane and is the most common bacterial infection in infants and young children (17). It is generally assumed that middle ear infection occurs when bacteria colonizing the nasopharynx enter the middle ear space through the Eustachian tube (21). Epidemiologic factors known to be associated with the development of OM include a genetic predisposition, preceding viral respiratory infection, attendance of day care, lack of breast feeding, and young age (1, 2, 28). While these factors have been well studied, the specific bacterial virulence factors important for the invasion of the middle ear have not been well defined.

Multiple studies have shown that NT *H. influenzae* strains are very diverse (4, 10, 23), and children may be colonized with multiple strains at one time (26), leading us to question whether all strains of *H. influenzae* are capable of causing OM or if only a particular subset of strains is associated with infection. Animal models have shown that some NT *H. influenzae* strains are more virulent for OM than others and that the increased virulence may be related to the amount and character of lipooligosaccharide (LOS) produced (20). Long et al. demonstrated that the majority of NT *H. influenzae* middle ear isolates belonged to biotype I, compared to isolates from healthy individuals, which were rarely biotype I (19). Furthermore, multilocus enzyme electrophoresis analysis of 80 NT *H. influenzae* isolates provided evidence that the genetic diversity of OM strains is lower than the overall diversity of strains from healthy carriers (29).

While these studies suggest that a subset of nasopharyngeal NT *H. influenzae* strains may cause OM, the specific virulence genes associated with OM remain unknown. We used a molecular epidemiologic approach (33) involving genomic subtraction followed by a dot blot screen of a panel of *H. influenzae* isolates to identify virulence genes that might play a role in OM. These experiments were based on the hypothesis that genes found more frequently among NT *H. influenzae* middle ear isolates than among NT *H. influenzae* throat isolates from healthy children would be important for OM pathogenesis.

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MATERIALS AND METHODS

***H. influenzae* collections.** Bacterial strains used in this study included *H. influenzae* strain Rd (8), 48 middle ear NT *H. influenzae* isolates, 32 NT *H. influenzae* nasopharyngeal isolates from ill children with respiratory symptoms for which *H. influenzae* may or may not have been the cause of infection, 90 throat isolates from children attending day care, and 40 *H. influenzae* type b invasive strains. With the exception of *H. influenzae* strain Rd, the isolates used in this study were collected from sites in Minnesota (9); Ann Arbor, Mich. (26); Battle Creek, Mich. (26); and Bardstown, Ky. (18), between 1980 and 2001.

Biotyping of *H. influenzae* isolates. The biotype of *H. influenzae* strains was defined based on the production of indole, urease, and ornithine decarboxylase as described by Kilian (16).

Differential cloning by subtraction PCR. The *H. influenzae* middle ear strain G622 was used as the tester strain for subtractive hybridization experiments. This strain was chosen from a collection of 17 middle ear strains (18) because of the large number of bands held in common with other middle ear strains as determined by pulsed-field gel electrophoresis (data not shown). *H. influenzae* strain Rd was used as the driver strain and was chosen for initial subtraction experiments because the genomic sequence of this *H. influenzae* strain has been fully determined (8).

Genomic subtraction was conducted by using a commercially available kit from Clontech (PCR-Select bacterial genome subtraction kit; Palo Alto, Calif.) based on the suppressive subtractive hybridization method (5, 11). DNA sequences unique to the middle ear strain G622 were identified by subtraction according to the manufacturer's instructions with two modifications. First, plasmid DNA unique to strain G622 was isolated with a Qiagen miniprep kit and added to the driver DNA pool. This step was added because the equalization step within the genomic subtraction is imperfect, and previous subtractions with *H. influenzae* have resulted in an overrepresentation of cryptic plasmid sequences among the subtraction products (data not shown). A small, 3-kb plasmid was identified within strain G622 and was added to the driver DNA in order to suppress its copy number in the final pool of tester specific sequences. Second, genomic tester, G622 plasmid DNA, and *H. influenzae* Rd driver DNA were digested with the restriction enzyme *Alu*I from Gibco BRL (Rockville, Md.) instead of *Rsa*I. Secondary PCR products identified by subtractive hybridization (sPCR) fragments were cloned into the plasmid TOPO vector pCR2.1 from Invitrogen (Carlsbad, Calif.). One hundred seventy clones were selected for further analysis.

Determination of tester and driver specificity. The sPCR inserts were amplified from the 170 clones by using T7 and M13 reverse primers (35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min) followed by a nested PCR with primers provided with the subtraction kit (25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 2 min). To confirm tester specificity, nested PCR products were blotted onto a Hybond N^+ membrane (Amersham Pharmacia Biotech, Piscataway, N.J.) with a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Hercules, Calif.) and probed separately with *Alu*I-digested tester and driver genomic DNA labeled with the ECF random labeling module (Amersham Pharmacia Biotech). The hybridization signal was detected by using the fluorescein-based ECF detection system (Amersham Pharmacia Biotech) and the STORM 860 PhosphorImager from Molecular Dynamics (Sunnyvale, Calif.). DNA sequence analysis of the tester-specific sPCR fragments was performed at the University of Michigan Molecular Biology Core facility on an Applied Biosystems Model 3700 DNA sequencer.

Screening of *H. influenzae* isolates. The presence or absence of each sPCR fragment within an *H. influenzae* isolate was determined by dot blot hybridization as previously described (34) with the exception that *H. influenzae* strains were grown in 800 μl of brain heart infusion broth supplemented with NAD and hemin. Each blot was hybridized with unique, tester-specific, sPCR fragments labeled as described above. Images were analyzed with ImageQuaNT version 5.0 (Molecular Dynamics), and the signal was expressed as a percentage of the signal obtained for strain G622 (positive control) after correcting for the background. All isolates were screened independently with each sPCR probe at least twice, and discrepancies were resolved by Southern blot hybridization (22).

Cloning and sequencing of *lic* genes. The *lic2A* gene was amplified from strain G622 with the primers lic2AF, 5′ ATG AGT GCT ATT GAA AAT ATT GTC ATT 3′, and lic2AR, 5′ CTA CAT AAA ACG AAC AAT TTC TTT ACC TTG C 3′ (35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min). The complete *lic2B* gene was amplified with the primers lic2BF, 5′ T AAG TAT GAT CCT CAA ATG CAT 3′, and lic2BR, 5′ CCA TTA ACA ATA TCA AGA AGA TAT C 3′, and the PCR conditions outlined above. The *lic2A-lic2B* intergenic region was amplified with lic2AF and lic2BF primers and the PCR conditions outlined above, with the exception that the extension time was increased to 4 min. Sequencing was conducted at the University of Michigan Molecular Biology Core facility as described above.

Statistical analyses. Prevalence ratios were calculated as the ratio of the proportion of isolates with the gene in the collection of interest to the proportion with the gene in the healthy carriers (referent group). Differences in the proportions among each collection were calculated by the χ^2 test. Statistical calculations were done in SAS (SAS Institute, Cary, N.C.).

Nucleotide sequence accession number. The sequence determined in this work was deposited in the GenBank database under accession no. AY091470.

TABLE 1. Biotypes of *H. influenzae* isolates

Collection[b] (*n*)	Biotype[a]													
	I		II		III		V		VI		VII		VIII	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Healthy (89)[c]	7	8	39	44	15	17	11	12	0	0	12	13	5	6
Clinical (32)	6	19	13	41	5	16	5	16	2	6	0	0	1	3
Middle ear (48)	11	23	23	48	12	25	1	2	0	0	1	2	0	0
Type b (40)	35	88	5	13	0	0	0	0	0	0	0	0	0	0
Total (209)	59	28	110	53	32	15	17	14	2	1	13	6	6	3

[a] No biotype IV strains were found in our collection.
[b] Collections represent NT *H. influenzae* isolates unless they are specifically designated as type b.
[c] One isolate from healthy carriers was lost.

RESULTS

Biotype results. In order to test the association of biotype of *H. influenzae* strains with OM virulence, we biotyped our *H. influenzae* collection (Table 1). The distribution of biotypes among NT *H. influenzae* OM strains differed from that of the NT *H. influenzae* strains from healthy children in day care (Fisher's exact test, *P* value of 0.0031). The prevalence of NT *H. influenzae* biotype I strains among middle ear strains was 2.9 times higher (11 out of 48 strains compared to 7 out of 89 strains) than that among NT *H. influenzae* strains from healthy children in day care (*P* value, 0.013).

Genomic subtraction. Forty sPCR clones ranging in size between 248 and 825 bp were shown to be tester specific and underwent DNA sequence analysis. Of these, four were duplicates of the same sPCR fragment, leaving 36 distinct sPCR clones. A search of the GenBank database was conducted for each of the clones, one of which provided no match to currently known sequenced genes or proteins (Table 2). Seventeen of the sPCR clones showed similarity to phage-associated proteins. These included open reading frames from known *H. influenzae* bacteriophage HP-1 (7) and HP-2. Two sPCR fragments with high similarity to LOS-encoding genes were also identified, one isolated from *Haemophilus ducreyi* (24) and the other from the *H. influenzae* type b strain RM7004 (14). We also identified sPCR fragments with 96% similarity at the amino acid level to the high-molecular-weight C protein (HMWC) and 61% amino acid identity to the high-molecular-weight A protein (HMWA) (30). HMWA is thought to mediate adherence to respiratory epithelial cells, and HMWC is thought to help localize the adhesin to the outer membrane. The sPCR fragments with similarity to *HmwA* and *HmwC* genes were not analyzed further in this study. Others have shown that these adhesins are present in up to 80% of NT *H. influenzae* strains (18, 25, 30).

TABLE 2. Dot blot hybridization results for 48 middle ear isolates and 46 nasopharyngeal or throat isolates probed with 34 unique sPCR fragments

sPCR probe	No. (%) of type[a]		% Amino acid identity and potential function
	ME	NP	
Phage-related proteins			
A6O2	3 (6)	4 (9)	26; putative bacteriophage protein of *Salmonella enterica*
A6O8	18 (38)	16 (35)	41; C5 methylase MarMP of *Mycoplasma arthritidis* bacteriophage MA
A6O25	4 (8)	6 (13)	37; putative bacteriophage protein of *Salmonella enterica*
A6O26	4 (8)	6 (13)	39; replication O protein of phage lambda
A6N38	8 (17)	9 (20)	23; putative bacteriophage protein of *Salmonella enterica*
A6N42	3 (6)	4 (9)	37; Fels-1 prophage transcriptional regulator of *Salmonella enterica* serovar Typhimurium
A6O42	3 (6)	4 (9)	33; replication O protein of phage H19B
A6O43	8 (17)	9 (20)	33; Orf82 of *Pseudomonas* phage D3
A6N44	1 (2)	2 (4)	43; putative phage-related protein of *Yersinia pestis*
A6N60	7 (15)	7 (15)	50; putative bacteriophage late gene regulator of *Salmonella enterica*
A6O62	15 (31)	18 (39)	46; replication initiation gene A protein of *H. influenzae* phage HP-1
A6O70	22 (46)	22 (48)	84; putative methylase of bacteriophage Tuc2009
A6N81	—[b]	—[b]	47; regulatory phage antirepressor of phage 434
A6O83	8 (17)	12 (26)	46; phage-related protein XF2294 of *Xylella fastidiosa*
A6O111	13 (27)	21 (46)	84; Orf32 of *H. influenzae* phage HP-2
A6O120	15 (31)	13 (28)	40; CP4-57 integrase-like protein of *H. influenzae*
A6O129	23 (48)	27 (59)	40; phage-related protein of *Bacillus halodurans*
Known *Haemophilus* genes			
A6N17	11 (23)	7 (15)	51; HINDVIP methyltransferase of *H. influenzae* Rd
A6O22	19 (40)	16 (35)	50; LOS biosynthesis enzyme LBGB of *H. ducreyi*
A6N36	25 (52)	4 (9)	95; lipopolysaccharide biosynthesis *lic2B*-encoded protein of *H. influenzae*
Other			
A6O18	4 (8)	3 (7)	31; hypothetical protein of *E. coli* O157:H7
A6O24	15 (31)	14 (30)	25; vasopressin-activated calcium-mobilizing receptor of *Homo sapien*
A6O28	22 (46)	17 (37)	31; conserved protein of *Streptococcus coelicolor*
A6N29	5 (10)	7 (15)	27; transmembrane protein of *Methanothermobacter thermautotrophic*
A6N40	6 (15)	7 (15)	33; conserved hypothetical protein of *Xylella fastidiosa* XF0161
A6O55	2 (4)	1 (2)	48; hypothetical proteins of *Listeria innocua*
A6O73	5 (10)	6 (13)	35; sodium potassium pump of *Drosophila melanogaster*
A6N95	4 (8)	6 (13)	No match
A6O95	4 (8)	6 (13)	33; glucan endo-1,3-β-D-glucosidase of *Tritium aestivium*
A6O98	4 (8)	5 (11)	27; hypothetical protein of *Mesorhizobium loti*
A6O127	10 (21)	12 (26)	25; dual-specificity phosphatase of *Arabidopsis thaliane*
A6O132	7 (15)	2 (4)	37; DNA repair MMS21 of *Saccharomyces cerevisiae*
A6O139	8 (17)	9 (20)	54; hypothetical 9.8-kDa protein of *E. coli* K-12
A6O143	3 (6)	1 (2)	56; conserved hypothetical protein of *Ureaplasma urealyticum*

[a] ME, middle ear; NP, nasopharyngeal or throat.
[b] This probe exhibited high levels of nonspecific binding, perhaps due to the presence of similar sequences among the *H. influenzae* strains.

Frequency of *lic2b* in *H. influenzae* strains. Thirty-four of the sPCR probes that were shown to be both unique and tester specific were used to screen a collection of 48 NT *H. influenzae* middle ear isolates and 46 NT *H. influenzae* strains isolated from either the throat or the nasopharynx. One of the clones, number A6N36, hybridized more frequently with middle ear isolates (25 out of 48 isolates) than with NT *H. influenzae* throat or nasopharyngeal isolates (4 out of 46 isolates) (Fig. 1). Probe A6N36 is a 357-bp fragment with 96% similarity at the nucleotide level to a lipopolysaccharide biosynthesis gene, *lic2B*, originally described for the *H. influenzae* type b strain RM7004 (13, 14). Because of the apparent link of sPCR probe A6N36 with middle ear isolates, additional *H. influenzae* isolates were screened to confirm the association. Additional *H. influenzae* isolates from 90 healthy children in day care, 32 NT *H. influenzae* clinical isolates, and 40 *H. influenzae* type b strains were screened. Probe A6N36 hybridized more frequently with *H. influenzae* type b strains, NT *H. influenzae* middle ear isolates, and NT *H. influenzae* clinical isolates than with isolates from healthy carriers (Table 3).

Cloning of the *lic2A-lic2B* cluster. Because the *lic2B* gene was originally described for a type b strain as part of a gene cluster, we attempted to determine whether the gene within our NT tester strain was present in a similar gene cluster. PCR with the lic2AF primer and the lic2BF primer amplified a 3.7-kb PCR fragment that was subsequently cloned into the TOPO vector pCR2.1, and DNA sequence was obtained. The *lic2B* gene, identified in NT *H. influenzae* strain G622, encodes a protein that is 99% identical at the amino acid level to that found in the type b strain. The sequence and arrangement of the *lic2A-lic2B* gene cluster are virtually identical to those found in the type b strain (14) The middle ear isolate G622 contains proteins that are 94, 93, and 96% identical to the *lic2A*-, orf 3-, and *ksgA*-encoded proteins from the type b strain RM7004, respectively (14). Like the majority of *H. influenzae* isolates surveyed, the *lic2A* gene from our middle ear strain

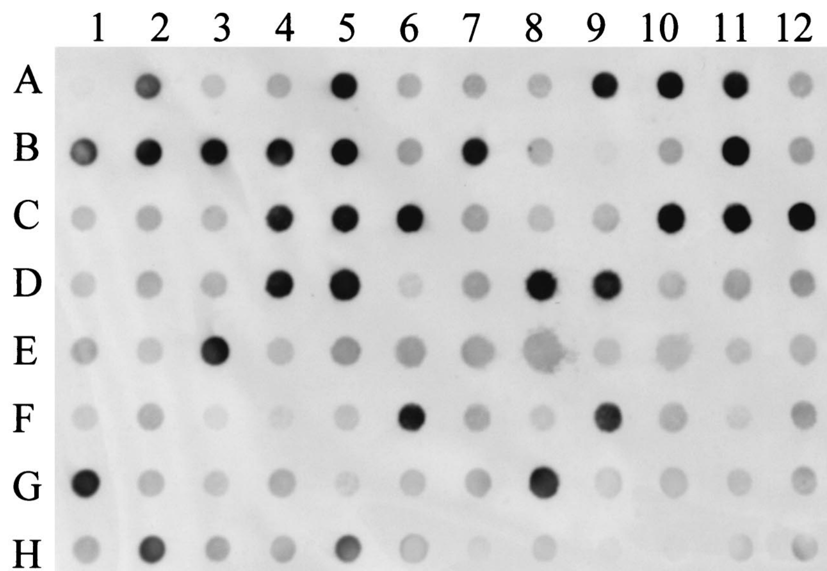

FIG. 1. Dot blot hybridization of sPCR fragment A6N36 with 48 *H. influenzae* middle ear and 46 throat or nasopharyngeal isolates. Dot A1 is blank, dot A5 represents the tester strain G622, and dot E5 represents the driver strain Rd. The top half of the blot represents middle ear isolates (except dots D1, D2, and D3, which are throat isolates), and the bottom half of the blot represents nasopharyngeal and throat isolates with the exception of dots F6, G8, G10, and H5, which are middle ear strains.

contains 19 CAAT repeats (14), indicating that it can undergo phase variation. The complete *lic2A* gene was amplified from strain G622 and used to probe our collection of *H. influenzae* isolates. The majority of *H. influenzae* strains contain the *lic2A* gene (Table 3). The *lic2B* gene was present only in strains that also contained the *lic2A* gene.

DISCUSSION

We hypothesized that some NT *H. influenzae* strains have virulence characteristics that enhance their ability to cause OM. This paper provides evidence that a specific gene, *lic2B*, is found significantly more frequently among NT *H. influenzae* strains that cause OM than among those found in the throats of healthy children. This suggests not only that OM is a disease of opportunity but that special characteristics may be required for throat isolates to invade the middle ear. A potentially analogous model is urinary tract infections caused by *Escherichia coli*. Urinary tract isolates may originate in the bowel flora, but only a subset of gastrointestinal isolates is found among those causing urinary tract infections (15).

Long et al. previously showed that 67% of NT *H. influenzae* strains isolated from children with OM were biotype I (19); however, only 14 NT *H. influenzae* strains were examined in this study. Almost half (48%) of the middle ear isolates belonged to biotype II, but, the higher prevalence of biotype I strains among our middle ear isolates than among isolates from healthy children in day care further supports our hypothesis.

The results from our genomic subtraction experiments identified the *lic2B* gene as potentially important for OM pathogenesis. The *lic2B* gene was originally identified in the type b strain RM7004. While the precise function of this gene is not known, *lic2B* is 58.9% identical at the nucleotide level to *lic2A* (12). The *lic2A* gene is thought to encode a galactosyltransferase that adds the terminal galactose on Galα1-4Gal structures of LOS. *H. influenzae* LOS is composed of a lipid A portion which is anchored into the membrane, linked by a single 2-keto-3-deoxyoctulosonic acid and three molecules of heptose and an outer core of glucose and galactose.

The structure of *H. influenzae* LOS has been implicated in virulence. Expression of phosphorylcholine through the phase-variable *lic1* locus has been shown previously to enhance nasopharyngeal colonization and the development of OM in the chinchilla model of OM (27). Transformation of a virulence-deficient LOS mutant of *H. influenzae* type b with plasmids

TABLE 3. Distribution of *lic2A* and *lic2B* genes by *H. influenzae* collection[a]

Collection[b] (*n*)	*lic2A*				*lic2B*			
	n	%	PR	*P*	*n*	%	PR	*P*
Healthy (90)	69	77	Referent		13	14	Referent	
Clinical (32)	32	100	1.30	0.002	13	39	2.93	2.8×10^{-3}
Middle ear (48)	47	98	1.27	0.001	25	52	3.71	2.6×10^{-5}
Type b (40)	40	100	1.30	8.8×10^{-4}	35	88	6.29	$<1 \times 10^{-7}$

[a] The prevalence ratios (PR) and χ^2 *P* values of the *lic2A* and *lic2B* genes relative to their presence in throat isolates from healthy children (referent) are shown.
[b] Collections represent NT *H. influenzae* isolates unless they are specifically designated as type b.

from an *H. influenzae* type b genomic library resulted in transformants with structurally altered LOS molecules that exhibited increased virulence in an animal model for invasive *H. influenzae* type b disease (3). Observed intrastrain variations in pathogenicity of an NT *H. influenzae* strain have been postulated to be due to changes in LOS (20).

Several of the surface-exposed epitopes of *H. influenzae* strains undergo high-frequency phase variation, thought to be important for survival in different microenvironments within the host (31). The *lic1A*, *lic2A*, and *lic3A* genes each contain multiple copies of the tetrameric repeat 5′-CAAT-3′ within the 5′ end of the coding sequence. The number of copies of the repeat can vary due to slipped-strand mispairing, which places the initiation codons in or out of frame with respect to the open reading frame, resulting in important LOS variations.

A mechanism for the influence of *lic2A* on virulence has also been proposed (32). Galα1-4Gal is found on the surface of human glycolipids, and it has been suggested that the decoration of the bacterial cell surface may allow these cells to evade antibody-mediated killing (32). Furthermore, Galα1-4Gal may not be expressed during carriage but may be important in pneumonia (32). The *lic2A* gene was expressed in five of five isolates from patients with pneumonia and was not expressed in four of five isolates from individuals without pneumonia.

Unlike the *lic2A* gene, *lic2B* does not contain a repeat region and does not undergo phase variation. The *lic2A* gene was present in the vast majority of *H. influenzae* strains, and the *lic2B* gene was present in 14 to 88% of *H. influenzae* strains, depending on the collection surveyed. *lic2B* was never found in an *H. influenzae* strain where *lic2A* was absent. While the *lic2A* gene is present in the vast majority of *H. influenzae* strains, the *lic2b* gene provides a stronger marker (prevalence ratio, 3.79) for differentiating between middle ear strains and those strains obtained from healthy children. This gene is also found more frequently among NT *H. influenzae* clinical isolates from sick children (prevalence ratio, 2.93) than among isolates from healthy children. This difference in prevalence is also striking considering the relatively even distribution of other genomic subtraction products among the middle ear and throat isolates.

In summary, *lic2B*, originally identified in *H. influenzae* type b strain RM7004 (13, 14), was present in more than half of NT *H. influenzae* middle ear isolates and only 14% of isolates from children attending day care. *lic2B* was also present in the majority of type b isolates screened (88%). Although *lic2B* may be important for the pathogenesis of OM, we cannot rule out the possibility that *lic2B* is a marker for other virulence determinants. Future mechanistic studies should shed light on this question.

ACKNOWLEDGMENTS

We thank May Patel for assistance with the biotyping of *H. influenzae* strains and both Rand Farjo and Jennifer St. Sauver for the isolation and identification of *H. influenzae* strains from the day care studies. We also thank Graham Krasan, Stan Block, and Joseph W. St. Geme III for the use of their OM strains.

This work was supported by an award from the National Institute of Allergy and Infectious Diseases (RO1-AI25630 to J.G.), the C. S. Mott Children's Hospital Fund for Research (J.G.), and the Deafness Research Foundation (to M.M.P.). M.M.P. was supported by supplement NIAID-CO36919 (to J.G.).

REFERENCES

1. **Berman, S.** 1995. Current concepts: otitis media in children. N. Engl. J. Med. **332:**1560–1565.
2. **Casselbrant, M. L., E. M. Mandel, P. A. Fall, H. E. Rockette, M. Kurs-Lasky, C. D. Bluestone, and R. E. Ferrell.** 1999. The heritability of otitis media: a twin and triplet study. JAMA **282:**2125–2130.
3. **Cope, L. D., R. Yogev, J. Mertsola, J. L. Latimer, M. S. Hanson, G. H. McCracken, Jr., and E. J. Hansen.** 1991. Molecular cloning of a gene involved in lipooligosaccharide biosynthesis and virulence expression by *Haemophilus influenzae* type B. Mol. Microbiol. **5:**1113–1124.
4. **Dhooge, I., M. Vaneechoutte, G. Claeys, G. Verschraegen, and P. Van Cauwenberge.** 2000. Turnover of *Haemophilus influenzae* isolates in otitis-prone children. Int. J. Pediatr. Otorhinolaryngol. **54:**7–12.
5. **Diatchenko, L., Y. F. Lau, A. P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E. D. Sverdlov, and P. D. Siebert.** 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc. Natl. Acad. Sci. USA **93:**6025–6030.
6. **Eskola, J., and T. Kilpi.** 2000. Potential of bacterial vaccines in the prevention of acute otitis media. Pediatr. Infect. Dis. J. **19S:**S72–S78.
7. **Esposito, D., W. P. Fitzmaurice, R. C. Benjamin, S. D. Goodman, A. S. Waldman, and J. J. Scocca.** 1996. The complete nucleotide sequence of bacteriophage HP1 DNA. Nucleic Acids Res. **24:**2360–2368.
8. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenny, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirely, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
9. **Gilsdorf, J. R., H. Y. Chang, K. W. McCrea, and L. O. Bakaletz.** 1992. Comparison of hemagglutinating pili of *Haemophilus influenzae* type b with similar structures of nontypeable *H. influenzae*. Infect. Immun. **60:**374–379.
10. **Gomez-De-Leon, P., J. I. Santos, J. Caballero, D. Gomez, L. E. Espinosa, I. Moreno, D. Pinero, and A. Cravioto.** 2000. Genomic variability of *Haemophilus influenzae* isolated from Mexican children determined by using enterobacterial repetitive intergenic consensus sequences and PCR. J. Clin. Microbiol. **38:**2504–2511.
11. **Gurskaya, N. G., L. Diatchenko, A. Chenchik, P. D. Siebert, G. L. Khaspekov, K. A. Lukyanov, L. L. Vagner, O. D. Ermolaeva, S. A. Lukyanov, and E. D. Sverdlov.** 1996. Equalizing cDNA subtraction based on selective suppression of polymerase chain reaction: cloning of Jurkat cell transcripts induced by phytohemagglutinin and phorbol 12-myristate 13-acetate. Anal. Biochem. **240:**90–97.
12. **High, N. J., M. E. Deadman, D. W. Hood, and E. R. Moxon.** 1996. The identification of a novel gene required for lipopolysaccharide biosynthesis by *Haemophilus influenzae* RM7004, using transposon Tn916 mutagenesis. FEMS Microbiol. Lett. **145:**325–331.
13. **High, N. J., M. E. Deadman, and E. R. Moxon.** 1993. The role of a repetitive DNA motif (5′-CAAT-3′) in the variable expression of the *Haemophilus influenzae* lipopolysaccharide epitope α Gal(1–4)β Gal. Mol. Microbiol. **9:**1275–1282.
14. **High, N. J., M. P. Jennings, and E. R. Moxon.** 1996. Tandem repeats of the tetramer 5′-CAAT-3′ present in *lic2A* are required for phase variation but not lipopolysaccharide biosynthesis in *Haemophilus influenzae*. Mol. Microbiol. **20:**165–174.
15. **Johnson, J. R.** 1991. Virulence factors in *Escherichia coli* urinary tract infection. Clin. Microbiol. Rev. **4:**80–128.
16. **Kilian, M.** 1991. *Haemophilus*, p. 463–470. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
17. **Klein, J. O.** 1997. Role of nontypeable *Haemophilus influenzae* in pediatric respiratory tract infections. Pediatr. Infect. Dis. J. **16:**S5–S8.
18. **Krasan, G. P., D. Cutter, S. L. Block, and J. W. St. Geme III.** 1999. Adhesin expression in matched nasopharyngeal and middle ear isolates of nontypeable *Haemophilus influenzae* from children with acute otitis media. Infect. Immun. **67:**449–454.
19. **Long, S. S., M. J. Teter, and P. H. Gilligan.** 1983. Biotype of *Haemophilus influenzae*: correlation with virulence and ampicillin resistance. J. Infect. Dis. **147:**800–806.
20. **Melhus, A., A. Hermansson, A. Forsgren, and K. Prellner.** 1998. Intra- and interstrain differences of virulence among nontypeable *Haemophilus influenzae* strains. APMIS **106:**858–868.
21. **Murphy, T. F., J. M. Bernstein, D. M. Dryja, A. A. Campagnari, and M. A. Apicella.** 1987. Outer membrane protein and lipooligosaccharide analysis of paired nasopharyngeal and middle ear isolates in otitis media due to nontypable *Haemophilus influenzae*: pathogenic and epidemiologic observations. J. Infect. Dis. **156:**723–731.

22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Smith-Vaughan, H. C., K. S. Sriprakash, A. J. Leach, J. D. Mathews, and D. J. Kemp.** 1998. Low genetic diversity of *Haemophilus influenzae* type b compared to nonencapsulated *H. influenzae* in a population in which *H. influenzae* is highly endemic. Infect. Immun. **66:**3403–3409.
24. **Stevens, M. K., J. Klesney-Tait, S. Lumbley, K. A. Walters, A. M. Joffe, J. D. Radolf, and E. J. Hansen.** 1997. Identification of tandem genes involved in lipooligosaccharide expression by *Haemophilus ducreyi*. Infect. Immun. **65:** 651–660.
25. **St. Geme, J. W., III, V. V. Kumar, D. Cutter, and S. J. Barenkamp.** 1998. Prevalence and distribution of the *hmw* and *hia* genes and the HMW and Hia adhesins among genetically diverse strains of nontypeable *Haemophilus influenzae*. Infect. Immun. **66:**364–368.
26. **St. Sauver, J., C. F. Marrs, B. Foxman, P. Somsel, R. Madera, and J. R. Gilsdorf.** 2000. Risk factors for otitis media and carriage of multiple strains of *Haemophilus influenzae* and *Streptococcus pneumoniae*. Emerg. Infect. Dis. **6:**622–630.
27. **Tong, H. H., L. E. Blue, M. A. James, Y. P. Chen, and T. F. DeMaria.** 2000. Evaluation of phase variation of nontypeable *Haemophilus influenzae* lipooligosaccharide during the development of otitis media in the chinchilla model. Infect. Immun. **68:**4593–4597.
28. **Uhari, M., K. Mantysaari, and M. Niemela.** 1996. A meta-analytic review of the risk factors for acute otitis media. Clin. Infect. Dis. **22:**1079–1083.
29. **van Alphen, L., D. A. Caugant, B. Duim, M. O'Rourke, and L. D. Bowler.** 1997. Differences in genetic diversity of nonencapsulated *Haemophilus influenzae* from various diseases. Microbiology **143:**1423–1431.
30. **van Schilfgaarde, M., P. van Ulsen, P. Eijk, M. Brand, M. Stam, J. Kouame, L. van Alphen, and J. Dankert.** 2000. Characterization of adherence of nontypeable *Haemophilus influenzae* to human epithelial cells. Infect. Immun. **68:**4658–4665.
31. **Weiser, J. N., J. M. Love, and E. R. Moxon.** 1989. The molecular mechanism of phase variation of *H. influenzae* lipopolysaccharide. Cell **59:**657–665.
32. **Weiser, J. N., and N. Pan.** 1998. Adaptation of *Haemophilus influenzae* to acquired and innate humoral immunity based on phase variation of lipopolysaccharide. Mol. Microbiol. **30:**767–775.
33. **Zhang, L., B. Foxman, S. D. Manning, P. Tallman, and C. F. Marrs.** 2000. Molecular epidemiologic approaches to urinary tract infection gene discovery in uropathogenic *Escherichia coli*. Infect. Immun. **68:**2009–2015.
34. **Zhang, L., B. W. Gillespie, C. F. Marrs, and B. Foxman.** 2001. Optimization of a fluorescent-based phosphor imaging dot blot DNA hybridization assay to assess *E. coli* virulence gene profiles. J. Microbiol. Methods **44:**225–233.

Editor: A. D. O'Brien