Preventing Bacterial Infections with Pilus-Based Vaccines: the Group B Streptococcus Paradigm

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We recently described the presence of 3 pilus variants in the human pathogen group B streptococcus (GBS; also known as Streptococcus agalactiae), each encoded by a distinct pathogenicity island, as well as the ability of pilus components to elicit protection in mice against homologous challenge. To determine whether a vaccine containing a combination of proteins from the 3 pilus types could provide broad protection, we analyzed pili distribution and conservation in 289 clinical isolates. We found that pilus sequences in each island are conserved, all strains carried at least 1 of the 3 islands, and a combination of the 3 pilus components conferred protection against all tested GBS challenge strains. These data are the first to indicate that a vaccine exclusively constituted by pilus components can be effective in preventing infections caused by GBS, and they pave the way for the use of a similar approach against other pathogenic streptococci.

Streptococcus agalactiae, also known as group B streptococcus (GBS), is a gram-positive pathogen with 9 serotypes distinguished on the basis of capsular polysaccharide (CPS) composition. Nontypeable strains have also been isolated and currently account for 8%–14% of the isolates in the United States and Europe [1, 2]. GBS can cause severe invasive infections in older persons [3, 4] and pregnant women and life-threatening infections in newborns and young infants [5].

It has been shown that babies can acquire maternal opsonizing antibodies through the placenta [6, 7], and maternal anti-CPS IgG concentrations in infants are inversely correlated with their risk of developing early-onset disease (age, 0–6 days) and late-onset disease (age, 7–89 days). Therefore, vaccination of women of childbearing age appears to be an attractive strategy for preventing GBS morbidity and mortality in newborns. Clinical studies performed using CPS conjugated to tetanus toxoid have shown that these vaccines are safe and capable of eliciting high serum concentrations of anti-capsular IgGs [8, 9]. However, considering that a 9-glycoconjugate vaccine would not protect against the increasing number of nontypeable isolates [10], attempts are in progress to develop vaccines based on conserved protein antigens [11].

Recently, we have shown that a combination of selected surface-exposed proteins can become the basis of universal GBS vaccines [12]. These protective antigens included proteins subsequently found to constitute pilus-like structures [13].

Pili appear to play a key role in the adhesion and attachment of gram-negative and gram-positive pathogens to host cells. For instance, it has been proposed that the pili of gram-negative bacteria promote the initial association with host cells, which is then followed by a more intimate attachment mediated by other adhesins [14]. In S. agalactiae, Streptococcus pneumoniae, and Streptococcus pyogenes (also known as group A streptococcus), piliated strains were shown to adhere better than their pilus-negative isogenic mutants to epithelial cells [15–18], to form microcolonies and biofilm [18], and to promote transepithelial migration (Soriani et al., unpublished data).
With the idea of eliciting immune responses that counteract the essential biological role of pili, components of pili have been tested as vaccine candidates against different pathogens. In the 1970s, Brinton et al. [19] and Morgan et al. [20] showed that pili-based vaccines induced protection in humans against gonococcus and against enterotoxigenic Escherichia coli. However, sequence variability has so far been the major obstacle to the development of pilus-based vaccines.

Here, we investigate whether, unlike attempts for other pathogens, a broadly protective vaccine constituted solely of GBS pilus proteins can be developed to prevent GBS infections. In GBS, the 2 pili encoded by pilus island 1 (PI-1) and pilus island 2a (PI-2a) and localized in 2 distinct loci (PI-1 and PI-2) were first identified [13]. The overall gene organization of the 2 islands consists of 3 genes that encode LPXTG motif–carrying proteins and correspond to the major pilus subunit (i.e., backbone protein [BP]) and the 2 ancillary proteins (AP1 and AP2) and 2 sortase genes that are involved in the assembly of pilus. BP and AP1 of both pilus islands were shown to elicit opsonophagocytic antibodies that protected mice in the active maternal immunization model [12]. More recently, PI-2a was shown to have an allele, pilus island 2b (PI-2b), that displayed a similar genetic organization but varied substantially in gene sequence [15, 21]. The protective activity of the backbone and the ancillary proteins of PI-2b was not investigated. Furthermore, no extensive analysis of pilus distribution and variability in GBS has been reported at the time of writing.

In the present article, we show that (1) as in the case of PI-1 and PI-2a, 2 pilus components of PI-2b elicit protection against GBS challenge in mice; (2) on the basis of PCR analysis of 289 clinical isolates, all GBS strains carry at least 1 of the 3 pilus islands; (3) on the basis of DNA sequence analysis of 186 isolates, pilus were well conserved within each of the islands; and (4) a vaccine containing 1 component of each pilus is capable of providing a high level of protection against a large panel of virulent strains. Overall, these data are the first to show that highly protective pilus-based vaccines can be developed to prevent GBS infection, and they form the basis for the use of pili in vaccines against other gram-positive pathogens.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** GBS isolates were collected from patients with invasive GBS infection or asymptomatic colonization by the Center for Disease Control and Prevention (Atlanta, GA) during 2000–2003, Baylor College of Medicine (Houston, TX) during 2002–2005, and the Istituto Superiore di Sanità (Rome, Italy) during 1992–2006. The capillary precipitin method of Lancefield was used for serotyping. Strains 2603 V/R (serotype V) and COH1 (serotype III) were obtained from Dr. Dennis Kasper (Harvard Medical School [Boston, MA]). Strains 515 (serotype Ia), CJB111 (serotype V), and H36B (serotype Ib) were obtained from Dr. Carol Baker (Baylor College of Medicine). Bacteria were grown at 37°C in Todd Hewitt broth (Difco Laboratories) or in trypticase soy agar supplemented with 5% sheep blood.

**DNA isolation, PCR amplification, and sequencing.** Genomic DNA was isolated by a standard protocol for gram-positive bacteria, using a NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer’s instructions. Genes were amplified using the primers external to the coding sequences, which are listed in the appendix (table A1), which appears only in the electronic edition of the Journal. The nucleotide sequences of PCR products were determined using a BigDye Terminator V3.1 kit (Applied Biosystem) in an ABI Prism 3700 Analyzer (Applied Biosystems). The nucleotide sequences have been submitted to the Genbank database under accession numbers EU929085–EU930009.

**Sequence alignments and phylogenetic analysis.** Sequence identity was measured by pairwise BLAST with Vector NTI Suite 9 (Informax), with gaps included. Sequence alignments were performed using ClustalW (1.83, GCG Wisconsin Package, version 11.1), and phylogenetic trees were inferred by the neighboring-distance-based method and bootstrapped 1000 times.

**Cloning, expression, and purification of recombinant proteins.** Recombinant proteins were expressed in *E. coli* BL21DE3 as 6His-tagged fusion proteins by cloning the corresponding genes in pET24b+ (Novagen), and they were purified by affinity chromatography. Primers were designed to amplify the coding regions without the signal peptide and the 3’ terminal sequence starting from the LPXTG motif.

**GBS 2603 V/R** was used as the source of DNA for cloning the sequences coding for PI-1 proteins (SAG0645, SAG0646, and SAG0649) and PI-2a proteins (SAG1408, SAG1407, and SAG1404). Strains 515 and CJB111 were used as the source of PI-2a backbone proteins (SAL1486 and SAM1372), and strain H36B was used for the ancillary protein 1 of pilus 2a (AP1-2a; SAI1512). GBS COH1 was used to express ancillary protein 1 of pilus 2b (AP1-2b), backbone protein of pilus 2b (BP-2b), and ancillary protein 2 of pilus 2b (AP2-2b).

**Mouse immunization and flow cytometry analysis.** Purified recombinant GBS proteins were used for intraperitoneal immunization of CD-1 outbred mice (Charles River) as described elsewhere [21]. Protein-specific immune responses (i.e., the total immunoglobulin level) in pooled sera were monitored by ELISA. Immune sera showed titers of greater than 1:50,000 for the homologous antigen and titers of 1:10–50 for heterologous antigens. GBS strains in the exponential phase of growth were analyzed by FACS as described elsewhere [12], using preimmune or specific immune sera. A pool of 4 monoclonal antibodies raised against AP-1 was used instead of mouse immune serum; a pool of 2 unrelated monoclonal antibodies was used as a control. To analyze the surface exposure of the PI-2a backbone protein, antisera specific for the 2603, 515, and CJB111 variants were used.
In vitro and in vivo protection assays. Opsonophagocytosis analysis using sera from immunized mice was conducted as described elsewhere [12]. A maternal immunization/neonatal pup challenge model of GBS infection was created using CD-1 mice as previously described [12]. In brief, groups of 4 – 8 CD-1 female mice (age, 6 – 8 weeks) were immunized on days 1, 21, and 35 with 20 μg of a single antigen or 45-μg combinations of 3 antigens (15 μg/antigen) formulated in complete Freund’s adjuvant (for the priming dose) and incomplete Freund’s adjuvant (for each boosting dose). Mice were bred 3 days after the final immunization. Within 48 h after birth, pups were challenged intraperitoneally with a dose lethal enough to kill 90% of mice (corresponding to 10^3–10^6 cfu, depending on the strains), after which surveillance for death was performed for 7 days. Because most deaths occurred ≤ 48 h after challenge, survival analysis was limited to this 2-day period. Protection was calculated as follows: (the proportion of control mice that died minus the proportion of vaccinated mice that died) divided by the proportion of control mice that died, multiplied by 100.

Statistical analysis was performed using the Fisher exact test. All animal studies were performed according to guidelines of the Istituto Superiore di Sanità.

RESULTS

PI-2b pilus antigens are protective in mice. We previously demonstrated that 2 of the 3 pilus components encoded by PI-1 and PI-2a induce protective immunity against GBS infection in mice and that the levels of protection correlate with antigen surface exposure as measured by FACS analysis [12, 21]. To investigate whether the structural components of PI-2b also elicit protection in vivo, we analyzed the recombinant proteins BP-2b, AP1-2b, and AP2-2b, using the mouse active maternal immunization/neonatal pup challenge model [12]. BP-2b and AP1-2b conferred significant levels of protection against 3 challenge strains in which antigens were well expressed and exposed on the bacterial surface (>5-fold shift in fluorescence) (table 1). No protection was observed when mice were challenged with CJB111, a strain not carrying the PI-2b island. As previously observed for islands PI-1 and PI-2a, the AP2 protein, judged by FACS analysis to be unexposed on the bacterial surface, did not confer protection (unpublished data).

From these and previously published data, we concluded that BP and AP-1 proteins from all 3 pili elicit protective immunity in mice against GBS strains expressing sufficiently high levels of the corresponding proteins (see also below).

All clinical isolates carry at least 1 of the 3 pilus islands. We next investigated pili distribution in GBS clinical isolates obtained from the Centers for Disease Control and Prevention, Baylor College of Medicine, and the Istituto Superiore di Sanità and classified by disease type and serotype (table A2, which appears only in the electronic edition of the Journal). A total of 289...
isolates underwent PCR analysis to detect PI-1, PI-2a, and PI-2b islands, using primers that were selected on the basis of the genome sequences of 8 GBS strains [22] and specifically annealed to the genomic regions coding for the backbone protein and the 2 ancillary proteins of each island. The results of PCR analysis, shown in figure 1, revealed that at least 1 of the 3 islands was present in each of the investigated strains. More specifically, all 289 strains carried either PI-2a (in 73% of strains) or PI-2b (in 27%). On the other hand, the PI-1 locus was empty in 28% of the strains. The most common pilus organization (detected in 133 strains [46%]) consisted in the concomitant presence of PI-1 and PI-2a, whereas the least frequent situation (detected in 4 strains [1.4%]) involved the presence of PI-2b alone.

When island distribution was analyzed with respect to disease and age groups (figure 1A), no difference was found between strains from early-onset disease and those from late-onset disease. In contrast, a statistically significant difference was observed between strains from infants and strains from adults ($P < .001$); in infants, there was a greater number of strains bearing both PI-1 and PI-2b, and a large proportion of strains from adults carried both PI-1 and PI-2a. No specific distribution correlated with any of the 3 different geographic areas where the isolates had been collected (data not shown).

Interestingly, a correlation was observed between the presence of a particular combination of PIs and the CPS type (figure 1B). Most serotype Ia isolates (91%) contained only the PI-2a island, whereas the majority of type Ib (85%), type V (96%), and nontypeable (71%) strains carried PI-1 and PI-2a. All but one of the serotype III isolates carried a 2-pilus combination, of which 30% contained PI-1 and PI-2a, and 70% carried PI-1 and PI-2b. Finally, serotype II strains were always associated with PI-2a, and the 4 available serotype IV isolates carried PI-2b alone.

**Pilus proteins in each island are conserved.** The finding that all GBS isolates carried 1 or 2 of the 3 pilus islands prompted us to investigate the sequence conservation in the backbone protein and the 2 ancillary proteins encoded by each island. To this end, the PCR products of each pilus structural gene from all 186 isolates were sequenced to determine the extent of sequence variation. In all strains, the backbone protein sequence was well conserved, with no major insertions or deletions detected. However, some variation was observed in the 2 ancillary proteins, with a few amino acid changes detected in each of the 186 isolates. These changes were mapped onto the phylogenetic trees shown in figure 1C and 1D, which illustrate the evolutionary relationships among the different GBS isolates based on the backbone protein and ancillary protein sequences.

**Figure 2.** Schematic representation of sequence variability of pilus-coding genes among 186 group B streptococcus clinical isolates from the Centers for Disease Control and Prevention and the Istituto Superiore di Sanità. A, B, and C, Gene conservation in isolates containing pilus island (PI)-1, PI-2a, and PI-2b, respectively. Grey arrows represent the sortase genes present in each island. Sequences with 100% identity are shown in the same color, whereas variants showing $<90\%$ sequence identity are indicated in different colors. Single mutations are represented with vertical bars, and the numbers above each bar indicate the position/substitution of the mutated residue. D, Phylogenetic trees inferred from the alignments by the neighbor-joining-distance-based method of variants of backbone protein of pilus 2a (BP-2a) and ancillary protein 1 of pilus 2a (AP1-2a). Numbers at the nodes indicate bootstrap values.
of the Centers for Disease Control and Prevention and Istituto Superiore di Sanità strains were sequenced. A summary of this analysis is presented in figure 2. The 3 genes coding for the structural proteins of PI-1 were well conserved, and their products differed by very few amino acids (figure 2A).

For instance, a group of 37 isolates (mostly serotype V) showed only 1 substitution in the backbone protein, whereas 8 serotype II strains carried a point mutation resulting in a frameshift and premature termination after Thr360. Similarly, AP1 contained only 3 amino acid replacements, present only in serotype III isolates. Finally, AP2 was completely conserved. The high conservation of AP2 was found in the other 2 islands, as well.

A good degree of conservation was also observed for the BP and AP1 of island PI-2b (figure 2C). In 40 isolates that carried PI-2b, BP shared 100% identity, whereas AP1 clustered into 2 alleles that differed with respect to 7 amino acids and the presence of a variable 181–amino acid region.

PI-2a was the most variable of the 3 islands, even though it was still relatively well conserved (figure 2B). Backbone protein of pilus 2a (BP-2a) could be classified into 2 major families, one including 4 alleles and the other including 3 alleles (named on the basis of the reference strains), with sequence identities ranging from 48% to 98%. A similar clustering of 7 alleles was found when comparing AP1-2a sequences, with identities ranging from 87%–98% (figure 2D).

A correlation was observed between capsular serotypes, particular PI combinations, and specific PI-2a alleles (figures 1 and 2B). For instance, serotype Ia strains predominantly carried only PI-2a allele 515. The presence of PI-2a variant DK-21 was restricted to serotype II strains that were not carrying PI-1. Finally, serotype II strains carrying PI-1 and PI-2a were associated with variants CJB111 or 2603 V/R, and serotype III isolates nearly always contained PI-1 together with the PI-2a 2603 V/R variant.

In conclusion, these data illustrate that, with the exception of the BP in island PI-2a, the sequences of the 3 pilus structural proteins were well conserved.

**Pili are well expressed in most GBS isolates.** Having shown that all GBS isolates carry 1 or 2 pilus islands and that sequences within each island are conserved, we next investigated the expression level of pili in all 289 isolates, using FACS analysis and antibodies against the BP and AP1 subunits of the 3 islands. The outcome of this analysis is instrumental in predicting how many of the strains carrying a specific pilus island would be neutralized upon immunization with the corresponding pilus subunits. In fact, we previously observed that a fluorescence intensity shift of ≥5-fold consistently correlated with high protection levels in the active maternal immunization model [12].

**Figure 3.** Analysis of pili expression in group B streptococci, showing the correlation between strains that were PCR positive for pilus islands (PIs) and strains for which flow cytometry detected surface-exposed (FACS positive) and highly surface-exposed (HSE) pilus structural components. FACS-positive strains displayed a 2-fold greater fluorescence intensity than those stained with preimmune sera, whereas HSE strains had a 5-fold greater fluorescence intensity than those stained with preimmune sera. The mean fold-increases in fluorescence (±SD) are reported at the top of the columns.

<table>
<thead>
<tr>
<th>Antigen, antigen variant, challenge strain</th>
<th>Allelic variant</th>
<th>Protection, % of mice</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BP-2a</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CJB111 variant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CJB111 (V)</td>
<td>CJB111</td>
<td>80.4</td>
<td>&lt;.001</td>
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<tr>
<td>515 (la)</td>
<td>515</td>
<td>0</td>
<td>.04</td>
</tr>
<tr>
<td>3050 (II)</td>
<td>2603</td>
<td>2.2</td>
<td>&gt;.99</td>
</tr>
<tr>
<td>515 variant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CJB111 (V)</td>
<td>CJB111</td>
<td>7.6</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>515 (la)</td>
<td>515</td>
<td>50.6</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>3050 (II)</td>
<td>2603</td>
<td>0</td>
<td>&gt;.99</td>
</tr>
<tr>
<td>2603 variant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CJB111 (V)</td>
<td>CJB111</td>
<td>0</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>515 (la)</td>
<td>515</td>
<td>30</td>
<td>.004</td>
</tr>
<tr>
<td>3050 (II)</td>
<td>2603</td>
<td>92.1</td>
<td>&lt;.001</td>
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<tr>
<td><strong>AP1-2a</strong></td>
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<tr>
<td>2603 variant</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CJB111 (V)</td>
<td>CJB111</td>
<td>69.6</td>
<td>&lt;.001</td>
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<tr>
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<td>515</td>
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<td>.018</td>
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<td>H36B variant</td>
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<td>&lt;.001</td>
</tr>
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<td>DK21 (II)</td>
<td>H36B</td>
<td>60.2</td>
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* Serotype is indicated in parentheses.

† Strains used as reference for pilus sequence variants.
The results of FACS analysis are shown in figure 3 and in table A3, which appears only in the electronic edition of the Journal. In general, whenever PI-2a and PI-2b islands were present, the level of pilus surface exposure was greater than the 5-fold fluorescence intensity shift threshold. In contrast, only 31% of the strains containing the PI-1 island expressed the pilus antigens at high levels. However, for most of the 44 clinical isolates with low PI-2a or PI-2b pilus expression, the PI-1 island was highly expressed. Overall, only 26 of 289 isolates had expression levels less than the 5-fold threshold value, and only 18 strains were FACS negative. Interestingly, 49 strains appeared to simultaneously express 2 different pili at levels that correlate with protection in the mouse model. In conclusion, all strains carried at least 1 of the islands, and 94% expressed pili on their surface.

A vaccine constituted by a combination of pilus components provides broad protection in the mouse model. On the basis of the foregoing data, a universal vaccine against GBS infection should be achievable by combining specific components of each of the pilus islands. However, the existence of 7 PI-2a alleles poses the question of how many variants should be included to provide protection against strains carrying this island. We tested the cross-protection activity of the 2 AP1-2a variants (2603 and H36B) and 3 of the 6 BP-2a variants (2603, 515, and CJB111) that together represent >80% of the sequenced genes.

### Table 3. Neonatal protection conferred by pilus-based vaccine formulations against 9 group B streptococcus (GBS) strains expressing different combinations of pili.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>PI component(s) detected by PCR</th>
<th>Fluorescence, by vaccine component</th>
<th>Protection, % of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>A909</td>
<td>Ia</td>
<td>PI-1 plus PI-2b</td>
<td>BP-1 (PI-1) 0</td>
<td>0 10.6</td>
</tr>
<tr>
<td>515</td>
<td>Ia</td>
<td>PI-2a</td>
<td>AP1-2a (PI-2a) 0</td>
<td>10.1</td>
</tr>
<tr>
<td>3050</td>
<td>II</td>
<td>PI-1 plus PI-2a</td>
<td>BP-2b (PI-2b) 0</td>
<td>0</td>
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<tr>
<td>5401</td>
<td>II</td>
<td>PI-1 plus PI-2a</td>
<td>1.7 11.3</td>
<td>0</td>
</tr>
<tr>
<td>COH1</td>
<td>III</td>
<td>PI-1 plus PI-2b</td>
<td>7.5 0</td>
<td>7.7</td>
</tr>
<tr>
<td>M732</td>
<td>III</td>
<td>PI-1 plus PI-2b</td>
<td>3.5 0</td>
<td>9.7</td>
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<tr>
<td>CJB111</td>
<td>V</td>
<td>PI-1 plus PI-2a</td>
<td>9.0 11.8</td>
<td>0</td>
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<td>JM9130013</td>
<td>VIII</td>
<td>PI-1 plus PI-2b</td>
<td>14.4 1.2</td>
<td>11.9</td>
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</table>

**NOTE.** PI, pilus island.

* Data are the fold-shift in fluorescence in cells stained with immune sera versus those stained with preimmune sera. Vaccine contained single proteins or a combination of the 3 subunits from each of the pilus islands (i.e., BP-1, AP1-2a, and BP-2b). Targeted PI components are specified in parentheses.

* b P < .001, by the Fisher exact test.

* c Gene was missing in this strain.

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**Figure 4.** Opsonophagocytic activity elicited by immunization with pilus subunits. Mice were immunized with single proteins or a combination of the 3 subunits from each of the pilus islands (i.e., BP-1, ancillary protein 1 of pilus 2a [AP1-2a], and BP-2b). The opsonophagocytic activity of each serum was tested in the presence of human PMNs and baby rabbit complement by use of 3 group B streptococcus (GBS) strains, each expressing a different pilus combination. The log₁₀ difference between GBS colony-forming units at time 0 (10⁴ CFU) and time 1 h are shown. Values for preimmune sera are negative because of bacterial growth during the assay. The antigens used are recorded above each bar. Black shaded bars represent specific immune sera; grey bars, corresponding preimmune sera from the same animals; and whisker bars, SD. As shown, only serum from mice immunized with the 3-subunit combination was capable of mediating killing of the 3 GBS strains (P < .001). The marginal growth inhibition observed with nonspecific sera could have been due to cross-reactivity at the 1:30 dilution used in this assay.
coding for BP-2a. Each soluble purified protein was assessed in the mouse model, using as a challenge strains that expressed either a homologous or a heterologous variant. Interestingly, AP1 conferred protection against all strains tested, irrespective of the cluster to which they belonged. In contrast, all BP-2a proteins analyzed were able to protect only pups challenged with strains carrying the allelic variant used to immunize their mothers, and protection was not observed against strains expressing a heterologous allele (table 2).

On the basis of the data described above, we created a vaccine containing the backbone proteins from PI-1 and PI-2b, as well as ancillary protein 1 from PI-2a, and tested the vaccine in mice challenged with a panel of strains representative of the major currently circulating serotypes. As shown in table 3, significant protection was obtained against all strains, and protection correlated with the capacity of the vaccine to elicit opsonophagocytic antibodies against strains specifically expressing 1 of the 3 pili at high levels (figure 4).

**DISCUSSION**

We recently have shown that pilus components of *S. agalactiae* are capable of eliciting high titers of opsonophagocytic antibodies that protect mice both in the active and passive immunization models. The development of a pilus-based GBS vaccine could become a reality, provided that pili are sufficiently conserved among strains. To establish the level of conservation, 289 clinical isolates were tested for the presence of the 3 pilus islands recently identified in GBS.

The first important finding of our analysis is that all isolates contained at least 1 (often 2) islands, confirming the key role played by pili in GBS pathogenesis. At present, no sufficient data are available to establish whether the presence of a specific pilus pattern is associated with particular biological and/or pathological features. With the exception of the combination of PI-1 and PI-2b, which was rarely observed in strains from adults but was relatively frequent in strains from infants, no remarkable association was found between the presence or absence of a particular PI and the type of disease or carriage. This is similar to what has been previously observed for other genetic traits, such as capsular serotypes [23], virulence factors [24], and pathogenicity islands [25], if one excludes the association of serotype III strains with early- and late-onset meningitis in infants.

Interestingly, CPS serotypes were genetically linked to pilus islands, and the same allele was shared by different capsular serotypes. This suggests that CPS serotypes have evolved after pilus divergence.

Not only do all GBS strains carry pili, but the sequences of the 3 pilus subunits appear to be remarkably well conserved. PI-2a is the only island to show some extent of variability. This is particularly true for BP, which diverges into 7 variants, with homology ranging from 48% to 98%. This difference is sufficient to prevent cross-protection in animals immunized with the protein of a specific allele and challenged with strains carrying a different PI-2a allele. On the other hand, differences among the 7 AP1 alleles of PI-2a are limited, and AP1 alleles are cross-protective.

The high degree of conservation of GBS pili was unexpected, when one considers the variability observed in other pathogenic bacteria. One possible explanation is that pili expression may be regulated such that they appear on the bacterial surface only transiently, thus avoiding the selective pressure of the host immune system. However, no substantial regulation was detected in GBS grown in a chemostat under different conditions [26] or isolated from the blood of infected mice (Margarit et al., unpublished data). A second explanation for pilus conservation is that GBS occupies environmental niches (e.g., lower gastrointestinal and vaginal mucosa) that are relatively inert from an immunological standpoint. In fact, a considerable proportion of women heavily colonized with GBS have low CPS IgG serum concentrations, and these women pose a high risk of early-onset infection to their newborns. On the other hand, in *S. pyogenes*, which colonizes the immunologically reactive nasopharynx and tonsils, 9 pilus islands and ~20 serologically distinct alleles have been identified (Falugi et al., unpublished data) [27, 28].

The most noteworthy of our results is that when pilus components from the 3 islands are combined, a vaccine conferring protection against 94% of contemporary GBS strains circulating in the United States and Italy can be formulated. This provides a rationale for the development of a universal GBS vaccine that is potentially capable of preventing GBS disease in all groups. Furthermore, because the role of pili is to promote bacterial adhesion to host tissue, it is tempting to speculate that pilus-based vaccines might also elicit antibodies capable of preventing GBS colonization. Because of the absence of reliable animal models for GBS colonization, this attractive possibility can only be tested in future human studies.

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**References**


