olate dispersal behavior to the landscape level (*17*, *21*, *27*, *28*). Explicit tests of such models are needed (*18*). The tight fit between observed and predicted patterns of seed rain in our habitat patches provides strong support for the key assumption that small-scale behavioral responses can drive landscape-scale distributional patterns. From a conservation perspective, impacts of corridors can be predicted on the basis of behaviors that are relatively simple to measure (*29*).

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29. Funding was provided by the National Science Foundation (grants DEB 9815834 and 9907365) and by the Department of Energy–Savannah River Operations Office through the U.S. Forest Service (USFS) Savannah River under Interagency Agreement DE-AI09-00SR22188. J. Blake, E. Olson, and other members of the USFS Savannah River Institute were instrumental in the construction of the experimental landscapes. T. Chaplin, S. Daniels, E. Franklin, M. Huizinga, C. Murray, N. Perlut, J. Warr, and A. Weldon provided help in the field. A. Whitney, J. Lauderbach, T. Daley, M. Galatowitsch, and M. Rosenbaum examined seed samples. T. Okuyama and N. Seavy quantified movement behavior and helped construct the model.

Identification of a Universal Group B *Streptococcus* Vaccine by Multiple Genome Screen

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Group B *Streptococcus* (GBS) is a multiserotype bacterial pathogen representing a major cause of life-threatening infections in newborns. To develop a broadly protective vaccine, we analyzed the genome sequences of eight GBS isolates and cloned and tested 312 surface proteins as vaccines. Four proteins elicited protection in mice, and their combination proved highly protective against a large panel of strains, including all circulating serotypes. Protection also correlated with antigen accessibility on the bacterial surface and with the induction of opsonophagocytic antibodies. Multigenome analysis and screening described here represent a powerful strategy for identifying potential vaccine candidates against highly variable pathogens.

Group B *Streptococcus* (GBS) is the foremost cause of life-threatening bacterial infections in newborns (*1*). In about 80% of cases, neonatal GBS infection is acquired during delivery by direct mother-to-baby transmission of the pathogen, which colonizes the anogenital mucosa of 25 to 40% of healthy women (*2*). Despite the introduction of intrapartum antibiotic prophylaxis, in the United States GBS still causes ∼2500 cases of infection and 100 deaths annually among newborns in the first 3 months of life (*3*). About half of these cases occur in the first week after birth. Thus, it is commonly believed that effective vaccination will be the only way to reduce the incidence of GBS disease over the long term. The rationale for GBS vaccine development is supported by the observation that the risk of neonatal infection is inversely proportional to the maternal amounts of specific antibodies to the capsular polysaccharide (CPS) antigen that surrounds GBS (*4*, *5*), the implication being that protective immunoglobulin G (IgG) antibodies are transferred from the mother to the baby through the placenta.

As a first approach to vaccine development, CPS-tetanus toxoid conjugates against all nine GBS serotypes were shown to induce CPS-specific IgG that is functionally active against GBS of the homologous serotype (*6*). Clinical phase 1 and phase 2 trials of conjugate vaccines prepared with CPS from GBS types Ia, Ib, II, III, and V revealed that these preparations are safe and highly immunogenic in healthy adults (*7*). Although these vaccines are likely to provide coverage against the majority of GBS serotypes that currently cause disease in the United States, they do not offer protection against pathogenic serotypes that are more prevalent in other parts of the world (e.g., serotypes VI and VIII, which predominate among GBS isolates from Japanese women) (*8*). Hence, a universal protein-based vaccine against GBS is highly desirable. To date, a few potential protective antigens have been described. These include the tandem repeat–containing α and β antigens of the C protein complex (*9*) and Rib (*10*); surface immunogenic protein, Sip (*11*); and C5a-ase, a serine protease that inactivates complement factor C5a (*12*). However, of these proteins, only Sip and C5a-ase are conserved at the gene level in the majority of GBS isolates (*11*, *13*), and no systematic analysis on the extent of cross-protection is available.

To identify possible antigens suitable for use in a universal GBS vaccine, we compared the genome sequences of eight GBS strains belonging to serotypes Ia (515 and A909), Ib

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Table 1. Protection conferred by four antigens against six GBS strains assessed by active maternal immunization/neonatal pup challenge model. Female mice received three doses (days 1, 21, 35) of either 20 μg antigen or phosphate-buffered saline (PBS) combined with Freund's adjuvant. Mice were then mated, and the resulting offspring challenged with a dose of GBS calculated to kill 80 to 90% of the pups. Survival of pups was monitored for 2 days after challenge. Fluorescence given in -fold difference between cells stained with immune sera versus pre-immune sera. Protection values calculated as [(% dead in control − % dead in vaccine)/% dead in control] × 100. ND, not determined.

GBS strain	Type	Fluorescence immune/preimmune	Protein alive/treated	PBS alive/treated	Protection (%)	Statistical significance (P value)
			Antigen GBS 80			
515	Ia	0*	1/30	4/38	0	>0.05
7357 B	Ib	2.2	11/28	13/32	0	>0.05
DK21	II	0*	4/30	4/19	0	>0.05
COH1	III	7.5	26/29	3/29	88.5	<0.0001
2603 V/R	V	1.5	13/40	8/30	7.5	>0.05
CJB111	V	9.0	23/35	11/49	56.0	0.0001
			Antigen GBS 67			
515	Ia	10.1	19/30	5/29	55.8	0.0005
7357 B	Ib	7.8	10/20	5/34	41.2	0.01
DK21	II	8.1	27/40	9/40	58.3	0.0001
COH1	III	0*	7/30	5/30	7.6	>0.05
2603 V/R	V	2.6	5/29	7/40	2.9	>0.05
CJB111	V	11.8	29/37	1/39	77.9	<0.0001
			Antigen GBS 104			
515	Ia	0*	ND	ND		
7357 B	Ib	0	6/39	13/32	0	>0.05
DK21	II	0*	5/38	7/40	0	>0.05
COH1	III	5.6	22/40	7/33	43.0	0.0041
2603 V/R	V	0	9/30	8/30	4.1	>0.05
CJB111	V	5.8	32/48	7/26	54.3	0.0014
			Antigen GBS 322			
515	Ia	5.6	23/25	9/21	86.0	0.0004
7357 B	Ib	2.5	22/46	13/32	11.6	>0.05
DK21	II	8.4	28/40	6/24	60.0	0.0007
COH1	III	3.2	2/30	3/29	0	>0.05
2603 V/R	V	7.2	36/42	12/32	77.3	<0.0001
CJB111	V	1.4	ND	ND	ND	ND

*Gene missing in this strain.

(H36B), II (18RS21), III (COH1 and NEM316), and V (2603 and CJB111), which represent the most important disease-causing serotypes (*14*). This analysis identified a "core" genome of 1811 genes (~80% of each genome) shared by all strains and a "variable" genome of 765 genes that were not present in all strains. Computer algorithms were then used to select, within the two subgenomes, the genes encoding putative surface-associated and secreted proteins. Among the predicted surface-exposed proteins, 396 were core genes and 193 were variable genes. Of these 589 proteins, 312 were successfully expressed in *Escherichia coli* either as soluble His-tagged fusions or soluble glutathione *S*-transferase fusions.

Each purified soluble protein was next used to immunize groups of adult female mice. At the end of the immunization schedule, these were mated, and the resulting offspring (<48 hours of age) were challenged with a dose of GBS calculated to kill 80 to 90% of the pups (*14*). For conserved antigens, a virulent serotype III strain (COH1) was used for challenge; antigens that were absent from the COH1 strain were tested with one of the sequenced strains known to carry the corresponding gene. This systematic screening identified four antigens capable of significantly increasing the survival rate among challenged infant mice. One of these antigens—GBS322 (SAG0032), which

encoded the previously described Sip protein (*11*)—was part of the core genome. The other three antigens—GBS67 (SAG1408), GBS80 (SAG0645), and GBS104 (SAG0649)—were present in the variable portion of the subgenome. The proportion of mice protected against challenge with strains carrying one of these proteins varied from 43% in the case of GBS104 to 80% in the case of GBS80.

The four proteins were purified to homogeneity (*14*) and then tested in mice in the active maternal immunization–neonatal pup

Table 2. Protection against 12 GBS strains by a four-antigen combination. Experiments were performed as in Table 1 except that mice were vaccinated with a mixture of 15 μg of each protein (a total of 60 μg). Protection P values were less than 0.0001.

GBS strain	Serotype	Vaccine (alive/treated)	PBS (alive/treated)	Protection
515	Ia	39/40	6/40	97.0%
DK1	Ia	50/50	8/38	82.5%
7357B	Ib	49/60	5/46	79.4%
DK21	II	25/34	17/48	59.3%
5401	II	35/40	3/37	86.4%
3050	II	48/48	1/30	100%
COH1	III	36/36	7/40	100%
M781	III	30/40	4/39	72.0%
2603V/R	V	27/33	10/35	75.0%
CJB111	V	25/28	4/46	88.2%
JM9130013	VIII	37/39	5/40	94.2%
SMU071	VIII	44/50	18/50	81.2%
Total		445/498	88/498	87.0%

challenge model described above with the use of six GBS strains. Each antigen elicited protection against more than one strain but not against all strains (Table 1). As expected, whenever the corresponding gene was absent from the challenge strain, the antigen was not protective. However, in a few cases, protection was not conferred even though the challenge strain carried the antigen-coding gene. To test whether this is due to variability in antigen expression and/or surface exposure, we assessed antigen expression on the surface of each challenge

strain by fluorescence-activated cell sorting analysis using mouse sera specific for each of the four protective antigens. The levels of surface expression, as measured by antibody binding to viable bacteria, were variable and correlated with the protective activity of the antigen (Table 1). From the data accumulated up to this point, we estimated that an antigen was protective if antigen-specific antibody binding resulted in a >fivefold increase in fluorescence intensity over that in pre-immune controls. We then tested a combination of all four antigens in the same mouse model with the use of a panel of 12 challenge strains that represented the major pathogenic GBS serotypes and that belong to eight Multi Locus Sequence Types (MLST) (15). The combination of the four antigens was highly protective against all 12 strains (Table 2), with protection ranging from 59% to 100%—comparable to that conferred to mice vaccinated with CPS-tetanus toxoid glycoconjugates and challenged with homologous strains (16).

Lastly, we assayed the in vitro opsonophagocytic activity (17) of sera from mice immunized with the single antigens and with the four-antigen combination. Sera were incubated with the highly encapsulated GBS type V strain CJB111, which expresses all four antigens, and bacterial killing was measured in the presence of both polymorphonuclear leukocytes (PMNs) and rabbit complement. All sera promoted opsonophagocytosis and killing of GBS by PMNs
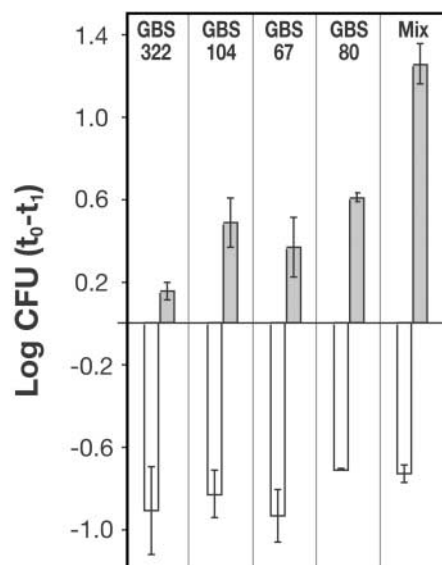

Fig. 1. Opsonophagocytic activity of sera specific for vaccine antigens. Live GBS bacteria of strain CJB111 were incubated for 1 hour with human PMNs in the presence of baby rabbit complement and specific antisera. The $\log_{10}$ of the difference between bacterial colony forming units at time = 0 and time = 1 hour are shown. Values for preimmune sera are negative because of bacterial growth during the assay. The antigens used are recorded above each bar. Shaded bars represent specific immune sera; open bars, the corresponding preimmune sera from the same animals. Error bars indicate standard deviation.

(Fig. 1), and killing was both PMN- and complement-dependent (14). However, the bacteria were most efficiently killed when opsonized with sera from mice vaccinated with the combination of four protein antigens, suggesting that the four proteins work additively as potent immunogens. Taken together, the protection in mice and the opsonophagocytic activity of the mouse sera suggest that a vaccine based on these four antigens may confer effective protection in humans also.

At least two major conclusions can be drawn from this work. First, multistrain genome analysis and screening constitute an effective new approach to identifying vaccine candidates that can provide broad protective activity when used in combination. Of the four antigens identified, none could be classified as universal because, in a fraction of GBS strains, either their coding gene was absent or their surface accessibility was negligible. Therefore, a genome screen of a single strain (18) would not have led to the identification of all four antigens but would have identified only those that, by coincidence, were sufficiently expressed in the strain used for challenge in the mouse model.

Despite the absence of universal antigens, it is clear that appropriate combinations of protective antigens—each effective against overlapping populations of isolates—can confer unexpectedly broad serotype-independent protection. In fact, the four-antigen vaccine used in this work protected mice against 12 virulent strains belonging to all nine major GBS serotypes. To estimate the strain coverage of the vaccine, we analyzed the surface expression of the four antigens on a total of 37 GBS isolates. We found that at least one of the antigens was highly accessible to antibodies (>fivefold shift in fluorescence) in 32 out of the 37 strains tested, which corresponds to 87% of circulating strains assuming that these strains sufficiently reflect the variability in the population.

A second conclusion from this work is that the extent of surface accessibility of antigens may vary from strain to strain, even if the antigens' coding genes are conserved (Table 1). Such variability may be due to differences in gene expression, antigen masking by other cellular components (e.g., CPS), protein degradation, or other factors. For instance, we found that the surface accessibility of the protein Sip was dependent on the presence of the polysaccharide capsule (table S2). In line with this, the protective antigens we identified were effective only against those strains in which the antigens were sufficiently exposed on the bacterial surface. From a practical point of view, variability in surface antigen expression highlights the importance of upfront rational selection of strains to be used in protection models. The strains should be selected not only because they carry the gene for the antigen under examination, but also in light of the amount of expression and accessibility of the antigen

itself. Between 30 and 40% of the genes of all bacteria sequenced so far belong to hypothetical or unknown families. Because our approach selects antigens independent of their function, it was likely that some protective antigens would have no assigned function. This is the case for all four protective antigens described herein. GBS322 contains a LysM domain, which is found in a variety of enzymes involved in bacterial cell-wall degradation and may have a general peptidoglycan-binding function. GBS67, GBS80, and GBS104 all contain LPXTG (Leu-Pro-X-Thr-Gly, where X is any amino acid) motifs associated with covalent linkage to the cell wall (19). Indeed, we have recently found that all three proteins are components of pilus-like structures never described before in GBS (20).

In GBS and probably other bacterial pathogens that adopt the strategy of gene variability to escape the immune system, universal protective protein antigens are unlikely to exist. However, some protein antigens are conserved in sufficiently large subpopulations of GBS that in combination they can be broadly protective. The successful use of multistrain genome analysis and screening described here for GBS provides the basis for the potential development of universal protein-based vaccines against other important and highly variable pathogens such as Group A Streptococcus and S. pneumoniae.

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21. D. Maione and I. Margarit contributed equally to this work. We thank M. Tortoli, S. Torricelli, G. Volpini, and the animal care facility at Chiron srl for expert technical assistance and G. Corsi for artworks. This work was supported, in part, by grant AI-060603 (L.C.P.) from the NIH National Institute of Allergy and Infectious Diseases. D.K. is a paid consultant for Chiron Corporation.