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Group B Streptococcal Opacity Variants

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Colony opacity variants were detected for type III group B streptococci (GBS). Transparent colonies predominate in the parent GBS, with occasional colonies having opaque portions. Two stable opaque variants (1.1 and 1.5) were compared with three transparent clones (1.2, 1.3, and 1.4). All grew well on blood agar and on GC medium, but variant 1.1 failed to grow on Todd-Hewitt medium. Scanning and transmission electron microscopy demonstrated that colony opacity correlated with bacterial aggregation status, with opaque variants forming longer and more organized chains. Opaque-transparent switches were observed in both directions for most variants, with transparent to opaque noted most frequently, but 1.5 did not switch at all. Switching of the opacity phenotype was observed both in vitro and in neonatal mice. Relationships between colony opacity and several cell surface phenomena were explored. (i) Opaque variant 1.1 had two surface proteins (46 and 75 kDa) that were either unique or greatly overexpressed. (ii) Variant 1.1 was deficient in type III polysaccharide, while 1.5 lacked group B antigen. Diminished capsular polysaccharide of variant 1.1 was reflected in reduced negative electrophoretic mobility and in increased buoyant density. (iii) Transparent variant colonies growing closest to a penicillin disk were opaque, but colonial variants did not differ in their sensitivity to penicillin. These data indicate that GBS can exist in both opaque and transparent forms, with opaque appearance occurring by multiple routes. Opaque variants grow poorly on Todd-Hewitt medium generally used for isolation of GBS, so any possible relationships between opacity variation and pathogenesis of GBS infection are unknown.

Bacterial colony opacity depends on the light-scattering characteristics of the colony, which, in turn, reflects its shape and the organization of cells making up the colony (24). Differences in opacity have been correlated with both tissue tropism and virulence of diverse bacteria, including *Neisseria gonorrhoeae* (14), *Streptococcus pyrogenes* (group A) (22), *Haemophilus influenzae* (13, 30), and Francisella tularensis (4, 19). This association may exist because states of interbacterial aggregation in some ways echo the interactions between bacteria and host cells.

Diverse biochemical mechanisms influence colony opacity. In gonococci, opacity correlates with both specific outer membrane proteins (Opa or protein II) (25, 26) and lipooligosaccharide phenotype (27). Opacity in group A streptococci was associated with the expression of M type 12 protein (22) in one study but not in others (15, 28, 33). Light and electron microscopic studies revealed that opaque streptococci form elongated chains (15, 28, 33). Virulent *H. influenzae* organisms are opaque, are encapsulated, and can phase shift to the nonopaque, unencapsulated phenotype (13, 30). *F. tularensis* colony opacity may be associated with the expression of capsule (19).

Group B streptococci (GBS) can cause fatal septic infections in both neonates (1) and adults (21). However, GBS may also exist as noninvasive forms that colonize mucosal surfaces. Although virulence factors have been reported for GBS (3, 8), the cell differences that dictate colonization versus invasive disease have not been defined and are usually attributed to host determinants rather than bacterial factors. While studying GBS grown on lucid solid medium, we noted occasional transparent colonies with one or more opaque regions. From these we isolated two stable opaque variants and compared them, morphologically and biochemically, with three transparent isolates from the same parental stock of type III GBS. The findings are that GBS exists in at least three different morphologic forms that change from one to another. The role of GBS opacity variation in disease pathogenesis is being explored.

MATERIALS AND METHODS

Bacterial strains and culture media. Type III GBS strain Fu was isolated from a human neonate with bacterial sepsis. Serotyping was performed with sera prepared by the method of Lancefield and with monoclonal anti-GBS antibodies (16). *Staphylococcus aureus*, CAMP strain, was obtained from Community Hospital, Missoula, Mont. The following bacterial culture media were used: (i) GC typing agar, containing Trypticase Peptone (3.75 g/liter; BBL, Cockeysville, Md.), Thiotone E Peptone (7.5 g/liter; BBL), soluble starch (1 g/liter; Baker Chemical Co., Phillipsburg, N.J.), IsoVitaleX Enrichment (1% [vol/vol]; BBL), and agar (10.5 g/liter; Difco Laboratories, Detroit, Mich.) in phosphate-buffered 0.5% saline (PBS), pH 8.0; (ii) blood agar, which has fresh defibrinated sheep blood (5% [vol/vol]), Bacto-Tryptose (20 g/liter; Difco), and agar (15 g/liter; Difco) in 0.5% saline, pH 7.3; and (iii) Todd-Hewitt agar, made with Todd-Hewitt mixture (30 g/liter; Difco) and agar (15 g/liter) in distilled water. Broth cultures were made by omitting the agar. Penicillin G potassium was obtained from Squibb-Marsam Pharmaceuticals (Princeton, N.J.).

Determination of bacterial morphology. Bacteria were cultured for 18 h at 37°C on GC typing agar. Colony morphology

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was examined with a binocular dissecting microscope (Nikon model SFZ-10) with obliquely transmitted light. Colonies were photographed with a 35 mm camera and Kodak TMAX ASA 400 film. Gram staining was performed by suspending a single colony in 1 ml of PBS, spreading 30 μl on a microscope slide, air drying, and staining, using solutions supplied by Sigma Chemical Co. (St. Louis, Mo.). Stained slides were photographed through an oil immersion objective.

Transmission electron microscopy (TEM) was performed on colonies fixed in 2.5% glutaraldehyde–4% paraformaldehyde–0.5% tannic acid in PBS. Colonies were flooded with fixative, gently dislodged, aspirated by pipette, placed in microcentrifuge tubes, and allowed to fix for 2 h at room temperature. The fixed colonies were pelleted and washed twice with PBS containing 0.2 M sucrose and then twice with tap water. The samples were stained with aqueous uranyl acetate and fixed as described previously (17). To determine if there was a difference in the thicknesses of the extracellular matrix (cell wall, capsule, etc.) of the different variants, we measured the distance between the cell membranes of adjacent cells that were divided by a complete septum. Distances were measured on photographs (8 by 10 in.) of transmission electron micrographs and represented a magnification of ×33,000. Conversion of photographic measurements to actual distances was accomplished by using a standardized grid. Measurements were made from the outer aspects of the electron-dense border for 25 pairs of cells at three places along the interbacterial septum. Scanning electron microscopy (SEM) was performed on whole colonies as described elsewhere (12). Colonies were fixed in 3% glutaraldehyde and then 2% osmium tetroxide in 0.1 M sodium cacodylate buffer. The fixed colonies were washed, dehydrated, mounted, and coated with 10-nm gold-palladium. The specimens were examined in a model 35 CF scanning microscope (JEOL, Peabody, Mass.).

Percoll density sedimentation. The buoyant density of GBS was measured on a Percoll (Pharmacia LKB Biotechnology, Uppsala, Sweden; solution density, 1.129 g/ml) gradient. A 35-ml linear gradient from 100% H_2O to 100% Percoll was poured in tubes (1 by 3.5 in. [ca. 2.5 by 8.9 cm]). GBS were suspended in 0.5 ml of H_2O and layered on the top of the gradient. The tubes were spun in an SW-27.1 rotor at 3,000 × *g* for 16 h by the protocol of Hakansson et al. (9). Densities at different levels of the gradient were determined by weighing 100-μl aliquots of the gradient.

Measurement of EPM. GBS were suspended in PBS (pH 6.5) and loaded into the sample cell of a DELSA 440 (Doppler Electrophoretic Light Scattering Apparatus; Coulter Electronics, Hialeah, Fla.). Electrophoretic measurements were carried out at 25°C and 15.0 mA for 36 s of total elapsed time (2 s on, 0.5 s off) with a frequency of 500 Hz and frequency shift of 250 Hz. Cell electrophoretic mobility (EPM) measurements were made with photoelectric cells located at four different angles (8.6, 17.1, 25.6, and 34.2°) relative to the plane of the incident laser beam. The values reported represent averages of results from the four different detectors (with results taken from each detector three or four times in each experiment). The data are reported as EPM in micrometers of cell migration per second per volt per centimeter (μm-cm/V-s).

In vivo passage of GBS. RML outbred mice were infected with GBS at 1 to 3 days of age. Preliminary experiments determined the maximal dose of each GBS variant that would allow the mice to survive for 2 days postinfection. Mice were injected intraperitoneally with GBS in 10 μl of PBS. Mice were sacrificed 1 and 2 days later. The peritoneal cavity was lavaged with 1 ml of PBS. Brains were removed, washed several times in PBS, and homogenized in 1 ml of PBS. Serial dilutions were made and plated on GC typing agar.

Protein analyses. GBS were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis by mutanolysin digestion, using a modification of the protocol of Rubens et al. (18). GBS were washed in PBS and resuspended to an optical density at 600 nm of 0.9. Seven milliliters of suspension was pelleted and resuspended in 1.0 ml of 0.3 M potassium phosphate (pH 7)–40% sucrose containing 25 U of mutanolysin (Sigma; 5,000 U/mg). The cells were digested overnight at 37°C with tumbling. The digestion mixture was spun at 10,000 × *g* and separated into supernatant and pellet (resuspended in 1 ml of H_2O). Samples for SDS-PAGE were mixed with an equal volume of lysing buffer (0.5 M Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), boiled for 5 min, and run on 12.5% acrylamide minigels (gel apparatus model 200; Hoefer Scientific, San Francisco, Calif.), using a Tris-glycine-SDS running buffer. Molecular size standards were Rainbow Markers (Amersham, Arlington Heights, Ill.). ^{125}I surface labelling of intact bacteria was performed using Iodogen (Pierce, Rockford, Ill.)-coated tubes according to the manufacturer's protocol. Amino-terminal protein sequencing was performed as follows. Proteins derived from mutanolysin extracts were separated by SDS-PAGE and then transferred to polyvinylidene-fluoride membranes (Immobilon P; Millipore) by the method of Towbin et al. (29), except that the transfer buffer was used at one-half strength (23). The transferred proteins were then stained with Coomassie brilliant blue R-250. The band corresponding to the 46-kDa protein was excised and placed in the reaction cartridge of an Applied Biosystems model 470A gas-phase sequenator equipped with an on-line phenylthiohydantoin amino acid analyzer. The blotted protein was subjected to 30 cycles of Edman degradation, using the modified program 01CPVD described by Speicher (23).

Immunoassays. The specificity of monoclonal antibodies to GBS and the enzyme-linked immunosorbent assay (ELISA) used to measure binding of antibodies to intact GBS have been described previously (16). The amount of surface-associated type III or group B antigen on the bacteria was measured by competitive inhibition of antibody binding to the purified carbohydrate antigen. Fifty milligrams of lyophilized bacteria was suspended in 5 ml of 30 mM sodium phosphate buffer (pH 7.0) containing 40% (wt/vol) sucrose and 2 mg of mutanolysin (Sigma) per ml. After end-over-end rotation at 37°C for 2 h, the suspension was pelleted at 12,000 × *g* for 20 min. Serial dilutions of the supernatant (100 μl) were mixed with diluted rabbit antiserum raised against type III GBS organisms (for the type III capsular polysaccharide ELISA) or with diluted rabbit antiserum raised against strain COH31-15, an unencapsulated mutant strain of GBS (for the group B antigen ELISA). One-hundred-microliter aliquots of the extract-antiserum mixtures were transferred to ELISA wells coated either with type III capsular polysaccharide, purified from a type III GBS strain as described previously (31) (type III ELISA), or with depolymerized group B antigen covalently coupled to bovine serum albumin (generously donated by Francis Michon and Harold Jennings, National Research Council of Canada, Ottawa). ELISAs were performed as described previously (18).

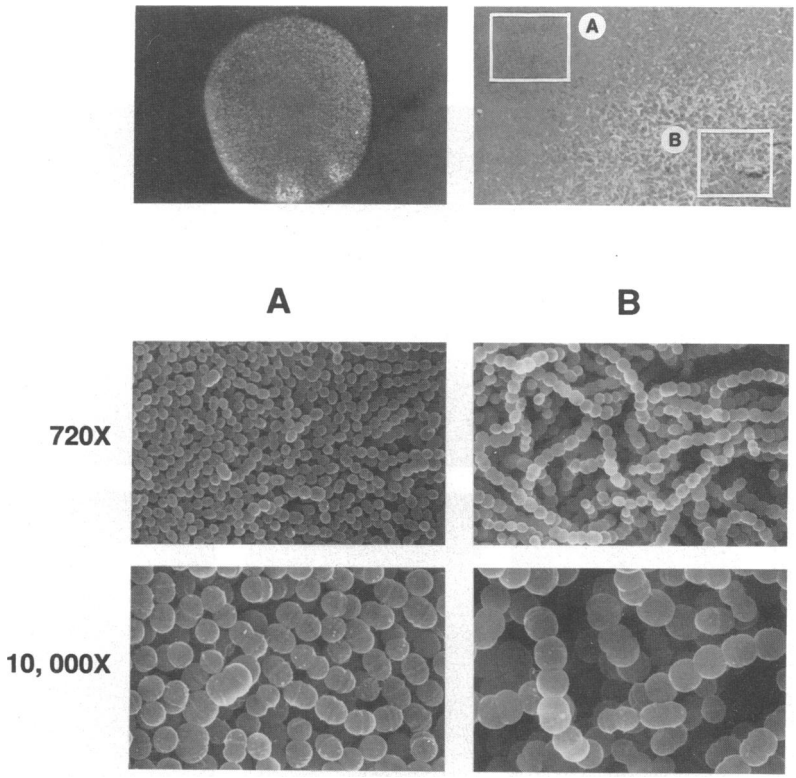

FIG. 1. Transparent GBS colony with two opaque sectors. The top left panel shows the colony as viewed under a dissecting microscope with oblique light. Two opaque sectors may be seen at 5 and 6 o'clock. The top right panel shows a low-power scanning electron micrograph of a portion of the same colony, containing the transition between opaque and transparent regions. The boxed areas correspond to transparent (A) and opaque (B) regions. Further enlargements of sections A and B are shown in the lower panels. (A) Transparent bacteria have a homogeneous distribution and form short chains; (B) opaque GBS have an irregular surface and long chains.

RESULTS AND DISCUSSION

Identification and isolation of opaque forms of GBS. GBS type III strain Fu was isolated from a neonate with sepsis and was passaged an unknown number of times in Todd-Hewitt broth prior to this study. When these bacteria were plated onto GC typing agar and grown overnight, the majority of colonies were transparent (nonopaque) and dull blue. Occasional colonies had white, opaque segments (Fig. 1). As observed by SEM, the bacteria in these opaque portions formed longer chains while those in transparent portions formed shorter chains and gave a more regular, homogeneous colony surface. Stable opaque variants were isolated from mixed colonies of GBS by serial subculture. Approximately 25% of the colonies contained opaque regions on initial subculture. The most opaque colony was then chosen, and the process was repeated. After approximately 10 repetitions, cultures were obtained that were reliably 100% opaque. Two independent opaque variants were derived, designated 1.1 and 1.5. Three transparent sublines of GBS strain Fu were established in parallel, 1.2, 1.3, and 1.4. The identity of all variants as type III GBS was established by the CAMP test (5) and their reactivities with monoclonal antibodies specific for group or type antigens.

Growth of GBS opacity variants. Growth parameters for the variant GBS were studied in GC typing medium. Fewer colonies or CFU (a streptococcal CFU is a chain or fragment of a chain, not a single bacterium) per milliliter were obtained for the opaque than for the transparent variants (Table 1). Possible explanations include (i) decreased viability of opaque GBS, (ii) longer chains of opaque GBS and therefore more cells per CFU, or (iii) a smaller size for transparent GBS, allowing more to be present at a given optical density. The data that follow suggest that all three explanations are relevant. Vigorous sonication to disrupt the streptococcal chains did not increase the colony counts. The growth rates in broth for both opaque variants lagged behind those for the nonopaque GBS, with 1.5 growing slowest and having a lower plateau level than the others (data not shown).

All variant GBS grew well on blood agar and gave the same pattern of weak beta-hemolysis. The transparent variants, 1.2, 1.3, and 1.4, all grew as well on Todd-Hewitt agar as they did on GC or blood agar. Opaque variant 1.5 yielded

TABLE 1. Characteristics of GBS opacity variants

Variant	Opacity[a]	No. of colonies per ml (10^6)[b]	Interbacterial distance (nm)[c]
1.1	OP^+	104 ± 12	17.89 ± 0.50
1.2	OP^-	480 ± 26	17.26 ± 0.64
1.3	OP^-	550 ±24	ND
1.4	OP^-	664 ± 22	ND
1.5	OP^+	58 ± 18	12.44 ± 0.78

[a] Colony opacity as observed under a dissecting microscope with oblique light. OP^+, opaque; OP^-, nonopaque or transparent.

[b] GBS were suspended at an optical density at 600 nm of 0.9 and then serially diluted and plated.

[c] Distance in nanometers between outer electron-dense border of two adjoining bacteria separated by a completely formed septum. Data are means ± standard errors. ND, not done.

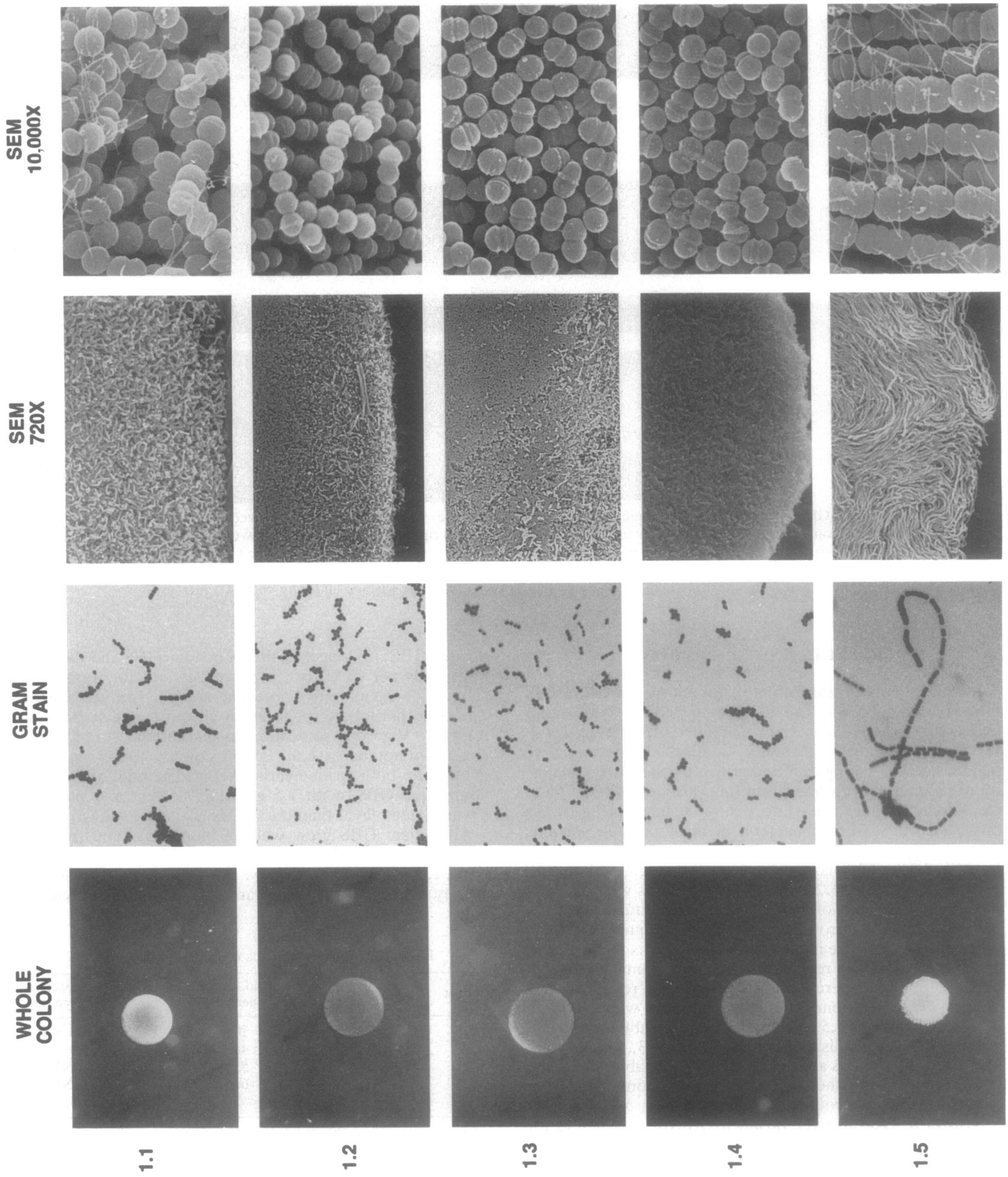
SEM 10,000X
SEM 720X
GRAM STAIN
WHOLE COLONY
1.1
1.2
1.3
1.4
1.5

TABLE 2. Buoyant densities of GBS variants[a]

Expt	Specific gravity (g/ml)			
	1.1	1.2	1.3	1.5
1	1.094	1.072	1.064	ND[b]
2	1.082	1.052	1.047	1.055
3	1.099	1.062	1.037	1.061

[a] GBS variants were spun on H_2O-Ficoll density gradients. The specific gravity of the banded bacteria was determined by weighing replicate 100-μl aliquots.
[b] No density determination was performed; the tube broke.

equivalent numbers of colonies on the three media, but the colonies were considerably smaller on Todd-Hewitt agar. Variant 1.1 did not grow on Todd-Hewitt agar. Transparent variants grew well in Todd-Hewitt broth and maintained their phenotype, but there was little, if any, growth of either opaque variant in this medium overnight. However, growth was obtained by diluting this overnight culture 1:10 in fresh Todd-Hewitt broth and incubating overnight again. When the phenotypes of surviving GBS were examined, 1.5 remained opaque whereas 1.1 had converted to the transparent form. Todd-Hewitt medium was incapable of supporting the growth of the 1.1 variant in its opaque form, and growth of 1.5 was markedly slower than that of the transparent GBS. Thus, the standard growth medium used for GBS selects against opaque variants. This likely accounts for others not having observed opaque GBS variants.

Our initial preparation of GBS strain Fu gave virtually all transparent colonies identical to those of variants 1.2, 1.3, and 1.4. Because strain Fu had been propagated in Todd-Hewitt broth, which doesn't support the growth of opaque variants, the opacity state of the original isolates is unknown. Many studies of GBS virulence used this medium (3, 10, 16), and any contribution of opaque GBS to disease phenomena has not been explored. We have also isolated stable opaque variants from type Ia and II GBS, although these are not characterized in this report.

Morphology of GBS opacity variants. The architecture of GBS variants was examined by both light and electron microscopy (Fig. 2 and 3). The opaque colonies (1.1 and 1.5) appeared white under the dissecting microscope with oblique lighting, and variant 1.5 had rough colony edges (Fig. 2). In Gram stains (Fig. 2) variants 1.2, 1.3, and 1.4 had very short chains and appeared smaller in diameter than the opaque GBS. Opaque variant 1.1 formed chains of intermediate length, and 1.5 had very long chains that gave a rodlike appearance. All variants were gram positive.

SEM of colonies in situ (Fig. 2) demonstrated that the transparent variants formed short chains and had smooth, homogeneous surfaces. In contrast, the surfaces of opaque colonies were rougher because of irregular projections and longer chains. Occasionally small regions of the transparent colonies had longer chains and rougher surfaces (as in the low-power scanning electron micrograph of variant 1.3). This was most likely due to areas within the colony having a somewhat higher density of bacteria, resulting in packing effects. At higher magnification, small strands were seen on the cell surface of opaque but not transparent variants. We have been unable to visualize these strands on intact GBS strains 1.1 and 1.5 by negative staining (not shown) and thin sectioning followed by TEM (Fig. 3) or in pellets of a bacterial supernatant. If they are artifacts of fixation or staining, these strands are unique to opaque variants and likely reflect their underlying differences from nonopaque forms.

Several features were observed in thin sections of opacity variants examined by TEM (Fig. 3). Variant 1.2 had smaller-diameter cells than either opaque variant, and only cells that were completely separated had secondary septa. Variant 1.1 exhibited secondary septa even in cells whose primary septa were incomplete. Incomplete separation was most extreme in 1.5, for which numerous complete, incomplete, and "aberrant" septa were seen in a single chain of cells. Such incomplete separation of multiple cells in variant 1.5 accounts for their rodlike appearance in Gram stains. The intracellular distances between dense cell wall laminae of adjacent cells were measured (Table 1). The distances between variant 1.5 cells was significantly less than those between either 1.1 or 1.2 cells ($P < 0.001$ by Student's t test), suggesting that 1.5 has thinner cell walls and/or less extracellular matrix.

Buoyant density of variant GBS. Variation in expression of the GBS capsule is reflected in the buoyant density of the GBS (8, 9). In hypotonic medium, the capsule swells and decreases the buoyant density. High-density GBS have been shown to produce less type-specific capsular polysaccharide. Variant 1.1 had a greater density than 1.2, 1.3, or 1.5, all of which had densities which were approximately equal, suggesting that there may be less capsular polysaccharide in variant 1.1 (Table 2). It is unlikely that chain length alters buoyant density, since both the GBS forming the longest chains (1.5) and those with the shortest chains (1.2 and 1.3) had the same buoyant density.

In opaque colonies of streptococci, chain length was related to the number of septa that formed without cell separation. This likely reflects uncoupling of division, septum formation, and cell separation. The relationship among these elements in streptococci has been extensively studied by Higgins and coworkers, who used *Enterococcus hirae* (group D streptococci) grown in high-viscosity medium to induce the formation of long chains. Buoyant density measurements of group D streptococci harvested during log-phase growth indicate that the buoyant density increases during the initiation of septum formation and that this is not due to changes in the density of the cell wall or the relative ratios of the cell wall to cytoplasm (6, 7). Nutritional shift-up leads to buoyant density increases in the terminal stages of the cell cycle when the surface grows slower than the cytoplasm (11). Thus, septum formation may be initiated by signals indicating that the volume of the cell is increasing more rapidly than the surface area. Because of other differences among our GBS opacity variants, we cannot subject our data regarding the buoyant density of the variants to this type of analysis. Nevertheless, alterations in the signal

FIG. 2. Morphologic characteristics of GBS opacity variants. Different microscopic views of the five GBS opacity variants are shown. The left column shows the appearance of whole colonies as viewed under a dissecting microscope with oblique light. Colonies of 1.1 and 1.5 were opaque; colonies of 1.2, 1.3, and 1.4, were transparent. The next column shows Gram stains of the variants. Bacterial colonies were dispersed in PBS, spread on microscope slides, and Gram stained. The two right columns show scanning electron micrographs at two different magnifications (×750 and ×10,000, respectively); the opaque bacteria form irregular surfaces and longer chains.

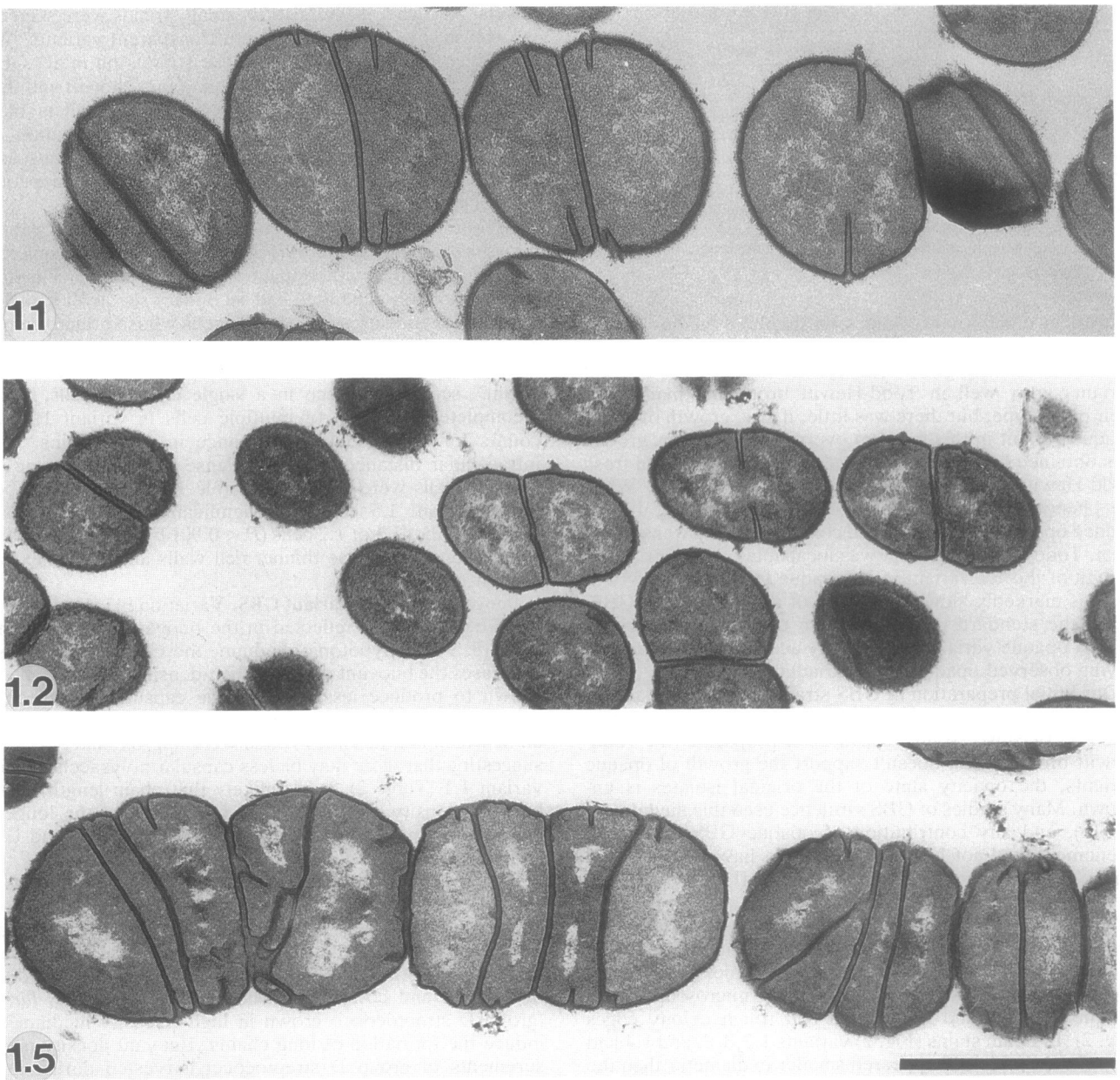

FIG. 3. Ultrastructural analysis of variants. Thin sections of opacity variants 1.1, 1.2, and 1.5 were examined by TEM. Representative photographs are shown. All samples are shown at the same magnification. Bar, 1 μm.

initiating septation could account for the increased chain length noted in the opaque GBS.

EPM of variant GBS. The EPM of variant 1.1 cells was consistently less negative than those of the other four variants, all of which were very similar (Table 3). This implies a relative paucity of negatively charged, surface-exposed residues on variant 1.1. Any contribution of GBS chain length to EPM measurements appears slight, given the equivalent values exhibited by variants 1.2 to 1.4 and 1.5, since 1.5 forms such long chains.

Opacity phase variation. The ability of the variants to switch opacity phenotype was studied both in vitro and in vivo. Switching of transparent variants 1.2, 1.3, and 1.4 to opaque in vitro was estimated to occur at a frequency of between 1:50 and 1:500. Switching involved only partial colonies, as shown in Fig. 1, because each streptococcal CFU contains multiple bacteria and switching of individual cells only affects that part of the colony containing its descendants. We estimated the frequency of 1.1 switching (opaque to transparent) in vitro by the selective growth of transparent variants on Todd-Hewitt medium. The frequency of phase variation of 1.1 was between 10^{-4} and 10^{-5}. In this case, entire colonies were transparent. On GC typing medium, these transparent colony variants were stable, spawning opaque forms at frequencies like those for transparent variants 1.2, 1.3, and 1.4. We have yet to demonstrate any phase variation of 1.5 to nonopaque.

We have also encountered a phenomenon that may repre-

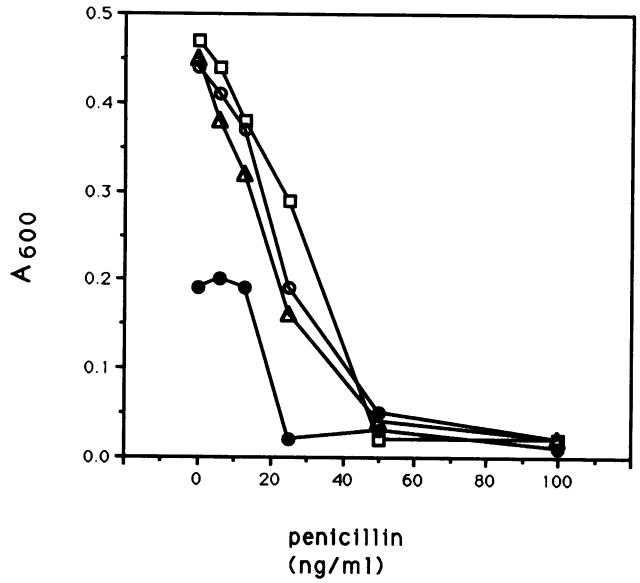

FIG. 4. Sensitivity of opacity variants to penicillin. GBS variants were grown in GC broth in the presence of various concentrations of penicillin. The bacteria were grown for 7 h at 37°C with shaking. Bacterial growth was determined by measuring the A_{600} at the end of the growth period. In the absence of penicillin, variant 1.5 grows to lower densities than the others. Symbols: □, 1.1; ○, 1.2; △, 1.3; ●, 1.5.

sent high-frequency phase variation in vitro. Several opaque variants derived from nonopaque parents never became stable. Offspring of the colony in the culture with the greatest degree of opacity (often the parent colony appeared completely opaque) were never more than 25 to 50% opaque, with most opaque colonies having a variegated appearance. This seemingly metastable situation could result from high-frequency reversion to transparent forms. An alternative explanation that could not be ruled out is that this apparent metastability may be a function of chain length and that we were unable to obtain a chain or fragment (i.e., CFU) that was entirely opaque.

We studied phase variation in vivo by inoculating neonatal RML mice with GBS intraperitoneally. One and two days later, the animals were sacrificed and cultures were obtained at the site of infection (peritoneal wash) or at a distant site (brain). Identical results were obtained for each site. Transparent variants remained transparent when passaged in vivo. Although opaque variant 1.5 retained its phenotype, variant 1.1 switched completely to the transparent phenotype in vivo.

TABLE 3. EPMs of GBS variants

Expt and variant	EPM (μM-cm/V-s)[a]
1	
1.1	−0.432 ± 0.025
1.2	−0.522 ± 0.037
1.3	−0.498 ± 0.010
1.4	−0.510 ± 0.015
1.5	−0.502 ± 0.032
2	
1.1	−0.328 ± 0.018
1.2	−0.492 ± 0.016
1.3	−0.512 ± 0.011
1.5	−0.497 ± 0.007

[a] A negative sign indicates that the bacteria moved toward the anode.

Penicillin and colony opacity. Penicillin interferes with the synthesis of cell wall peptidoglycan. In pneumococci, mutations in penicillin-binding proteins can cause altered septation (20). Therefore, we explored the relationship between penicillin and colony opacity in GBS. The sensitivity of the opacity variants to penicillin was measured in broth cultures grown in the presence of various concentrations of penicillin or no penicillin (Fig. 4). Transparent variants and opaque variant 1.1 gave similar curves. Variant 1.5 may have been slightly more sensitive to penicillin. It was completely inhibited at 25 ng/ml, a dose that only partially inhibited (30 to 60%) the other variants. However, 1.5 was uninhibited at 12.5 ng/ml, whereas the others were somewhat inhibited (15 to 30%) at this dose. Thus, there probably was no major difference in the penicillin sensitivity of the variants.

Similar results were obtained with penicillin disks (data not shown). Transparent variants exhibited a ring of opacity immediately surrounding the clear zone around the disks (Fig. 5). Closest to the penicillin disk were very small opaque colonies. Adjoining this were larger opaque colonies which merged into the transparent parental GBS. This concentric pattern indicated that the colony type was a function of penicillin concentration. SEM of opaque colonies revealed the formation of long chains. Colonies closest to the penicillin disk also formed a dense mat of the strands we had observed in SEM of variants 1.1 and 1.5 (Fig. 2). The increased chain length of GBS in these opaque colonies was clearly induced by penicillin. Opaque colonies from near the penicillin disk reverted to transparent on subsequent passage in the absence of penicillin, indicating that the phenotypic opacity changes induced by penicillin did not result from an induced genetic switch or selection for opaque variants.

Penicillin inhibits bacterial cell wall synthesis, primarily by inhibiting transpeptidation reactions. Mutations in penicillin-binding proteins have been associated with altered septum formation in other gram-positive cocci (20). The relationship between penicillin and GBS opacity is complex. Penicillin can induce opacity in transparent GBS in a dose-dependent fashion (Fig. 5). But there is no difference in penicillin sensitivity between opaque and transparent variants (Fig. 4). These data suggest that there are several routes to opacity in GBS, some influenced by penicillin and others not.

Antigenic structure of opacity variants. The binding of monoclonal antibodies to intact GBS variants is shown in Fig. 6. Antibody S9 binds a neuraminidase-sensitive determinant on the type III capsular polysaccharide (16); all variants expressed the type III antigen on the bacterial surface. Antibodies S3 and S7 recognize two different epitopes on the group B carbohydrate; variant 1.5 was markedly deficient in the binding of these antibodies when compared with the other variants. None of the variants bound monoclonal antibodies S10 and S11, which are specific for type I and II GBS. The deficiency of group B antigen on 1.5 variants was confirmed in an agglutination assay with polyclonal anti-group B antibodies on latex beads (data not shown). We then assayed the antigenic content of mutanolysin extracts from the variant GBS. The extracts were used to competitively inhibit the binding of antibodies to purified group B or type III antigen (Fig. 7). As expected, there was a marked decrease in the group B antigen present in the extract of variant 1.5 (Fig. 7, top). The inhibition seen at high

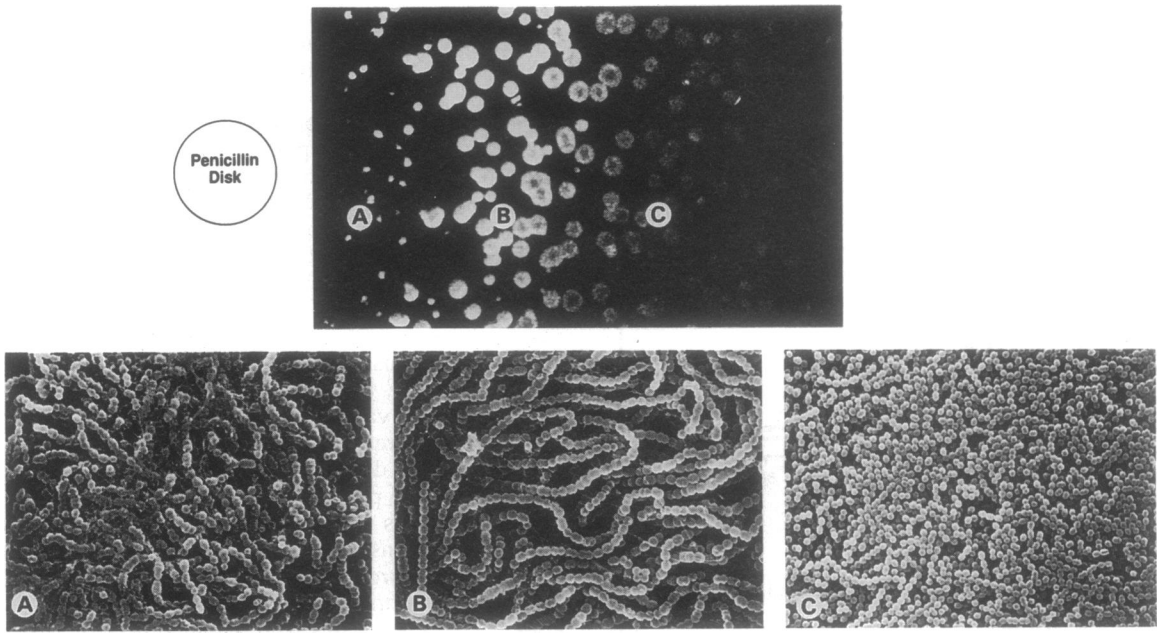

FIG. 5. Effects of penicillin on transparent GBS. GBS variant 1.2 was plated on a GC agar plate at sufficient density to form a bacterial lawn. A filter disk soaked in 100 ng of penicillin per ml was placed on the plate, and the bacteria were incubated overnight at 37°C. Inspection the following day revealed the expected clear zone around the penicillin disk. The photograph at the top shows a low-magnification view of the bacterial colonies adjacent to the clear zone. The colonies were viewed through a dissecting microscope with oblique light. The direction of the penicillin disk is indicated, although the distance is not to scale. Three zones of colonies were identified: (A) very small opaque colonies, (B) opaque colonies of approximately normal size, and (C) transparent colonies with the same morphology as that of 1.2. The three bottom photographs are scanning electron micrographs of colonies in the indicated zones at a magnification of ×3,000.

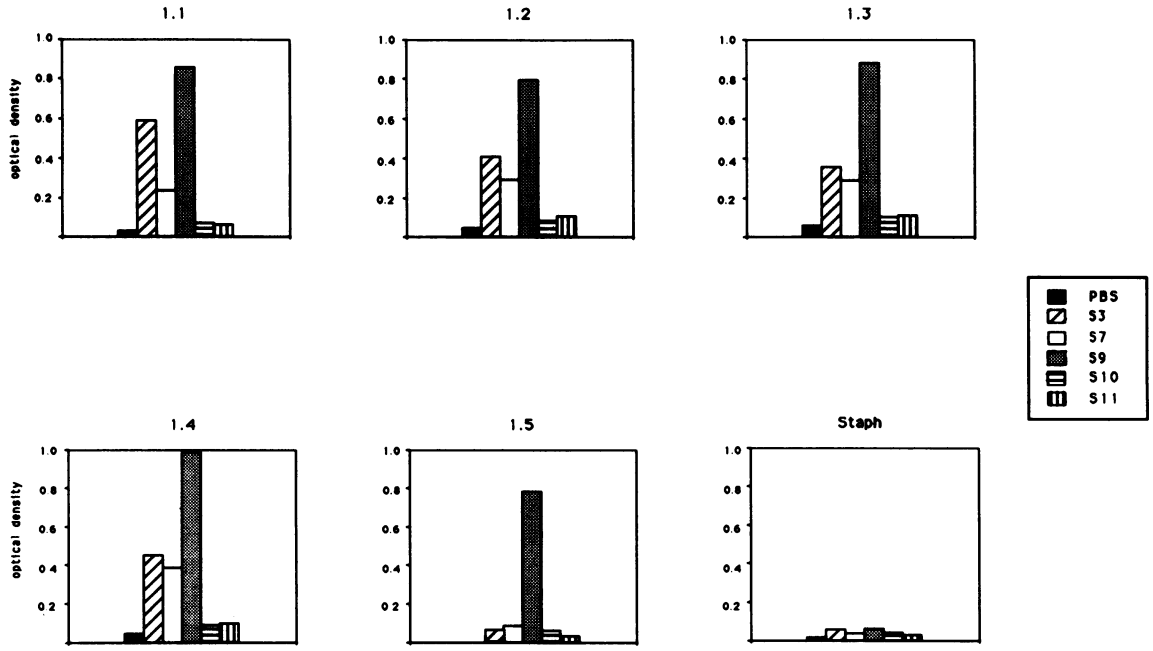

FIG. 6. Binding of monoclonal antibodies to intact GBS. The GBS variants, or *S. aureus* as a control, were incubated with monoclonal antibodies against group B (antibodies S3 and S7), type III (S9), and type I and II (S10 and S11) antigens. Antibody binding to the intact bacteria was detected with alkaline phosphatase conjugated anti-mouse immunoglobulin and *p*-nitrophenyl phosphate. The data are presented as optical density.

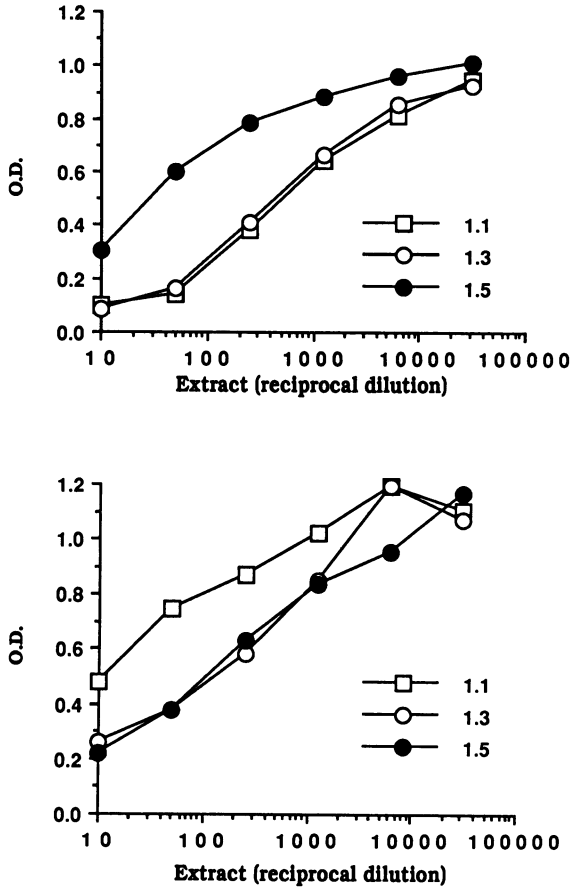

FIG. 7. Presence of group B and type III antigens in bacterial extracts. A competitive inhibition assay was used to measure the amounts of antigens in the GBS variants. Serial dilutions of mutanolysin extracts of bacteria were incubated with antisera specific for either group B (top) or type III (bottom) antigens. The serum-extract mixture was then plated onto microtiter plates coated with purified group B (top) or type III (bottom) polysaccharide. Antibody binding was measured as optical density in this ELISA.

extract concentrations may either indicate the presence of a low level of group B antigen or nonspecific inhibition caused by high concentrations of the carbohydrate extract. Although the monoclonal antibody studies indicated the presence of type III polysaccharide on all the variants (Fig. 6), the amount of type III polysaccharide detected in mutanolysin extracts of variant 1.1 was reduced more than 10-fold compared with that in extracts from the other variants (Fig. 7, bottom). One explanation for these data is that the capsule of variant 1.1 is present and expressed at the bacterial surface but the total quantity of capsule is diminished. This interpretation is also consistent with the increased buoyant density of variant 1.1 and with the diminished net surface charge.

Protein analyses. SDS-PAGE of bacteria digested with mutanolysin revealed two bands either unique or overexpressed in the 1.1 variants, at ~75 and ~46 kDa (Fig. 8). The 75-kDa band in variant 1.2 was absent in 10-fold-concentrated supernatants. Because there are a number of protein bands in all variants that run at about 46 kDa, it is impossible to say whether the 46-kDa protein expressed in large amounts by variant 1.1 was absent or just diminished in the other variants.

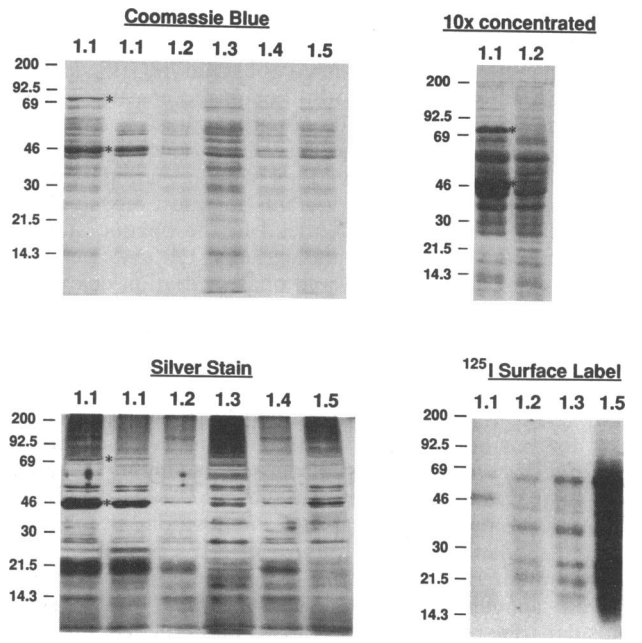

FIG. 8. SDS-PAGE analysis of proteins of the GBS variants. The variants were digested with mutanolysin, and the supernatant was loaded onto a 12.5% acrylamide gel. The top left panel shows Coomassie blue-stained gels of all the variants, including two different preparations of 1.1. The 46- and 75-kDa proteins are indicated with asterisks. The top right panel shows a Coomassie blue stain of 10-fold concentrates of variants 1.1 and 1.2. A silver stain is shown on the bottom left. An autoradiogram of ^{125}I surface-labelled GBS is shown on the bottom right; equal quantities of total protein were loaded into each lane as measured by Coomassie blue staining of the gel (not shown).

When intact GBS were labeled with ^{125}I, variant 1.5 was labelled the most and 1.1 was labelled the least. This was a true difference in labelling efficiency, since Coomassie blue staining of the same gel indicated that equal amounts of protein were loaded in each lane. There was a 46-kDa band unique to 1.1. As best as could be evaluated, all the other bands were present in all variants.

Because overexpression of the 46- and 75-kDa proteins in variant 1.1 correlated with its opacity phenotype, they were subjected to amino-terminal sequencing. The sequence for the 46-kDa protein was (NH_2)-ASKTTINLFVPTDDLAFR KIQNVPG. No significant homologies were detected in the NBRF protein data bank or in the translated bacterial sequences in GenBank. The amino terminus of the 75-kDa protein was blocked, and no sequence was obtained.

The appearance of two unique proteins at or near the surface of variant 1.1 suggests that the expression of these proteins may be associated with opacity in this variant, much as expression of the Opa protein is associated with opacity in gonococci (25, 26). However, alternative explanations must be considered. One possible alternative is differential mutanolysin digestion of the cell wall and selective release of proteins into the digestion supernatant. Perhaps the 46- and 75-kDa proteins are expressed in all the GBS but more easily released upon digestion of 1.1. In this case, the appearance of the proteins in the digestion supernatant, as well as the opacity phenotype of the GBS, might be related to the structure of the cell wall rather than the production of the protein. In other bacterial systems opacity has been associated with expression of specific proteins (22,

24), alterations in lipopolysaccharides (27), and changes in the carbohydrate capsule (19, 30). Thus, the molecular basis for the opacity variation we have observed in GBS remains very much an open question, although the 46- and 75-kDa proteins in variant 1.1 provide potential clues.

Conclusions. We have described GBS opacity variation. On the basis of our observations with penicillin and the distinctions between opaque variants 1.1 and 1.5, we believe that multiple molecular routes lead to the opaque state in GBS. The two stable opaque variants 1.1 and 1.5 have different physical, antigenic, and phase variation characteristics and probably represent two different routes to increased chain length and opacity. Variant 1.1 has capsule alteration, as evidenced by decreased type III antigen production, increased buoyant density, and decreased negative EPM. The EPM change is consistent with a reduced amount of or compositional change in the capsule (2). Capsulated *Escherichia coli* organisms display more highly negative EPMs than do noncapsulated variants. In *E. coli*, the size of the difference depends on capsule composition but is generally greater than that found for variant 1.1 versus other GBS we examined. Diminished capsule expression alone is unlikely to account for the change in colony opacity since acapsular transposon mutants of two different type III GBS strains do not differ from the wild type in colony opacity (32). In addition to alterations in capsule, other characteristics that distinguish variant 1.1 are that it can switch to the transparent phenotype, cannot grow in Todd-Hewitt medium, and has abundant expression of 46- and 75-kDa proteins. Variant 1.5, which forms rough-edged opaque colonies, cannot revert to the transparent phenotype, grows to a lower density than do the other GBS, and has a thinner extracellular matrix. Variant 1.5 also lacks the group B antigenic structure, as defined by both monoclonal and polyclonal antibodies. Although these two opaque variants have different characteristics, both form elongated chains.

The role of opacity phase variation in the life cycle of GBS and in the pathogenesis of GBS infections remains to be established. There are selective growth conditions for the opacity variants, both in vitro and in vivo. We have studied the interaction of the GBS opacity variants with the host defense system (15a). Opaque bacteria differ from transparent GBS in several important ways, including virulence, immunogenicity, and interactions with neutrophils. It is possible that the ability of GBS to phase vary their opacity type may play a role in their ability to survive in selective environments and to cause infection.

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