Cell Growth Rate Regulates Expression of Group B *Streptococcus* Type III Capsular Polysaccharide

LAWRENCE C. PAOLETTI,* ROBIN A. ROSS, AND KENNETH D. JOHNSON

Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

Received 7 November 1995/Returned for modification 15 December 1995/Accepted 9 January 1996

The capsular polysaccharide (CPS) of group B streptococci (GBS) is an important virulence factor that also serves to protect cells from nonspecific host defense mechanisms. Expression of CPS by GBS, as with other encapsulated bacterial pathogens, is not constitutive but varies during growth in vitro and in primary cultures isolated from different sites of infection. Despite this understanding, little is known about regulation of this surface-expressed carbohydrate antigen in GBS. Here we report that expression of type III CPS by GBS strain M781 grown in continuous culture with a modified chemically defined medium is regulated by growth rate. Cells in steady state at mass doubling times (t_d s) of 0.8, 1.4, and 1.6 h expressed an average of sixfold more cell-associated CPS than did cells held at t_d s of 2.3 and 11 h. Strain M781 grown at a t_d of 1.4 h repeatedly produced more type III CPS than those held at a t_d of 11.0 h, even when limited for glucose, pyridoxamine, or thiamine. In our studies, $\geq 93\%$ of the total CPS expressed by strain M781 was cell associated. Strain M781 grown at a t_d of 11.0 h (i.e., lowered CPS expression) was susceptible to in vitro complement-mediated opsonophagocytosis and killing by human peripheral blood leukocytes, whereas cells grown at a t_d of 1.4 h (i.e., higher CPS expression) were not killed unless type III CPS-specific antibody was present. Factors that allow GBS to asymptotically colonize women yet cause invasive infection to both mother and infant are poorly understood. Our results shed new light on parameters that regulate the pathogenic potential of GBS and may also serve as a way to discern more fully the genetics and biochemistry of GBS capsule synthesis.

Lancefield's group B *Streptococcus* (GBS), also known as *S. agalactiae*, is the leading cause of bacterial sepsis and meningitis among neonates in the United States, with a prevalence in the 1990s of 1.8 per 1,000 live births and a fatality rate of 6% (36). The capsular polysaccharide (CPS) antigens of GBS are important virulence determinants and are the targets of protective antibody. These surface-expressed antigens are composed of repeating-unit polymers of galactose, glucose, *N*-acetylglucosamine, and *N*-acetylneuraminic (sialic) acid arranged differently for each of the five serotypes (Ia, Ib, II, III, and V) most often associated with GBS disease (18, 21, 43, 46). The presence on the capsule of side chains that terminate with sialic acid makes cells of GBS poor activators of the alternative pathway of complement and thus resistant to phagocytosis in the absence of specific antibody (12, 13, 25). The degree of encapsulation can also affect activation of the alternative pathway of complement, with increased deposition of C3 molecules on strains expressing small amounts of CPS (25).

Variability in the degree of encapsulation of human isolates of several GBS serotypes has been well documented (11, 22, 25, 29, 49). Primary isolates from the blood of a newborn with GBS type III septicemia contained a greater portion of GBS cells with larger amounts of CPS, as measured by cell density in a hypotonic Percoll gradient, than did cells isolated from the cerebrospinal fluid of the same child (16). Differences in the degree of encapsulation by GBS cells isolated from different sites in the host were corroborated by Gray and Pritchard (15), who found that GBS type Ib isolated from the cerebrospinal fluid of an infant with meningitis had large amounts of capsule, whereas isolates of the same serotype from the cervix of the infant's asymptomatic mother had relatively small amounts of

capsule. These studies suggest that the in vivo environment plays an important role in capsule expression by GBS. Several studies have described a correlation between CPS expression and virulence in bacteria (11, 22, 29, 49). Conclusive evidence for this association in GBS was obtained with isogenic mutants that lacked CPS generated by Tn916 transposon mutagenesis of fully encapsulated GBS type III strains (22, 35). Cells of the nonencapsulated mutant strain COH1-13 were relatively avirulent in a neonatal rat model of GBS disease compared with the fully encapsulated parent strain COH-1 (35). Unlike COH-1, COH1-13 cells were susceptible to in vitro opsonophagocytic killing in the absence of specific antibody (33). Edwards et al. (14) have recently demonstrated that interactions of GBS type III with neutrophil complement receptor 3 were attenuated by the degree of encapsulation and the presence of sialic acid on the CPS.

Few studies have examined growth rate-dependent regulation of bacterial virulence factors. Differences in the amount of cell-associated K1 capsule of *Escherichia coli* at various stages of growth in batch culture profoundly affected susceptibility of these organisms to serum killing in vitro (41). Cells of *E. coli* O18:K1:H7 Bort produced the greatest amounts of K1 capsule in early log phase of growth and substantially smaller amounts during early stationary phase of growth (41). In chemostat growth under glucose limitation, enterotoxigenic *E. coli* maximally produced the fimbrial adhesins K99 and F41 at mass doubling times (t_d s) of 1.75 and 2.0 h, respectively (40). These investigators speculated that *E. coli* cells growing in vivo at a t_d of >14 h possess few fimbriae and thus adhere poorly to intestinal epithelium, but under more favorable growth conditions cells proliferate, fimbrial subunits assemble, and colonization ensues. Collectively, these studies indicate that the regulation of *E. coli* surface components such as the capsule and fimbriae is affected by the phase or rate of cell growth.

Although the CPS is a critical cell surface virulence factor for GBS, mechanisms of its regulation remain unknown. Using

* Corresponding author. Mailing address: Channing Laboratory, 180 Longwood Ave., Boston, MA 02115. Phone: (617) 432-2678. Fax: (617) 731-1541. Electronic mail address: paolettc@warren.med.harvard.edu.

TABLE 1. Components of parts I to IV of the CDM for the cultivation of GBS

Part	Chemical	Amt (g)/liter
I	K ₂ HPO ₄	0.30
	KH ₂ PO ₄	0.44
	Glucose	20.00
	Sodium acetate · 3H ₂ O	6.00
	Na ₂ HPO ₄	3.15
	NaH ₂ PO ₄ · H ₂ O	2.05
	(NH ₄) ₂ SO ₄	0.60
	Glycine	0.20
	MgSO ₄ · 7H ₂ O	0.20
	MnSO ₄ · H ₂ O	0.01
II	p-Aminobenzoic acid	0.0002
	Biotin	0.0002
	Folic acid	0.0008
	Niacinamide	0.0010
	β-NAD	0.0025
	Pantothenate	0.0020
	Riboflavin	0.0020
	Thiamine	0.0010
	Pyridoxamine	0.0008
III	Adenine	0.0020
	Guanine	0.0020
	Uracil	0.0020
IV	DL-Alanine	0.10
	L-Aspartic acid	0.10
	L-Cystine	0.05
	L-Glutamic acid	0.10
	L-Glutamine	0.20
	L-Histidine	0.10
	L-Isoleucine	0.10
	L-Lysine	0.10
	L-Methionine	0.10
	L-Phenylalanine	0.10
	L-Proline	0.10
	L-Hydroxyproline	0.10
	L-Serine	0.10
	L-Threonine	0.20
	L-Tryptophan	0.10
	L-Tyrosine	0.10
	L-Valine	0.10

continuous culture, we endeavored to examine growth parameters that regulate expression of CPS by GBS type III. To our knowledge, all studies with GBS have been performed with cells grown in batch culture, which is characterized by changing growth rate, nutrient concentrations, and pH. Growth parameter shifts experienced by cells in batch culture lead to metabolic changes that affect the composition of the cell. Conversely, continuous culture allows for continuous exponential growth in an environment of stable substrate, product, and biomass concentrations and at a rate controlled by the researcher. If growth conditions are maintained, a steady metabolic state will be achieved. We describe the regulation of GBS type III CPS expression by growth rate, with strain M781 cells grown in a modified chemically defined medium (mCDM) in continuous culture. Our studies show that strain M781 cells maintained at faster t_d s expressed 6- to 30-fold more CPS than those maintained at slower t_d s, a finding that may shed new light on the role of the environment in cell growth rate, regulation of CPS expression, and the pathogenic potential of GBS.

MATERIALS AND METHODS

Bacterial strain, strain maintenance, and culture medium. *S. agalactiae* type III strain M781, originally isolated from a newborn with GBS meningitis, was provided by Carol J. Baker, Baylor College of Medicine, Houston, Tex. Strain M781 cells were grown in a modified version of a CDM initially developed for growth of group A streptococci (39). The composition of the CDM used in the batch culture study is listed in Table 1. CDM parts II to IV are prepared as stock solutions and added to part I before sterilization by filtration through a 0.22-μm-pore-size membrane filter. For continuous culture studies with unknown growth limitation, the following modifications were made to the CDM: 5 g of Casamino Acids (acid-hydrolyzed casein; Difco, Detroit, Mich.) per liter was substituted for the amino acid mix, β-NAD was omitted, and 0.1 g of tryptophan per liter and 0.02 ml of Antifoam 240 (Sigma Chemical Co., St. Louis, Mo.) per liter were added. For experiments involving pyridoxamine limitation, the amount of pyridoxamine was adjusted to a growth-limiting concentration of 1 μg/liter, the concentration of thiamine was 100 μg/liter, and acid-hydrolyzed casein was replaced with 5 g of vitamin-assay Casamino Acids (Difco) per liter. For experiments involving thiamine limitation, thiamine was added at the growth-limiting concentration of 1 μg/liter and pyridoxamine was added at 10 μg/liter. In glucose limitation experiments, the concentration of glucose was reduced from 20 to 2.5 g/liter, and thiamine and pyridoxamine were included at 6 and 10 μg/liter, respectively. Growth-limiting substrate concentrations were calculated by determining the yield of cell biomass in batch culture on each substrate. A series of screw-cap glass tubes containing 5 ml of modified CDM (mCDM) without the nutrient to be limited were prepared. The nutrient was then added in incremental concentrations to the tubes containing mCDM. The seed culture was prepared by growing cells overnight in mCDM. Cells were pelleted by centrifugation, resuspended in an equal volume of phosphate-buffered saline (PBS), and used to seed tubes at 0.1%. Tubes were incubated at 37°C, statically, for 48 to 72 h until cell growth ceased. Final cell densities were determined by culture *A*₆₅₀. Cell densities were plotted against the concentration of the limiting nutrient, and a concentration from the linear portion of this curve was used in limitation experiments.

Growth systems. (i) Batch culture. Preliminary experiments on the expression of CPS by strain M781 were performed in batch culture. A tissue culture spinner flask was retrofitted to accommodate a pH probe, a sample port, and a glass inlet tube to deliver 10 N NaOH. Strain M781 cells were grown overnight at 37°C on blood agar plates, and several colonies were used to seed two 10-ml tubes of CDM. These cultures were incubated at 37°C for approximately 3 h, and cells were pelleted by centrifugation and washed twice with PBS, pH 7.2. Washed cells were suspended in 2 ml of CDM and used as the seed culture. Cells were added to the culture flask containing 150 ml of CDM and grown with mixing at 37°C. The culture pH was maintained between 7.0 and 7.2 by the addition of 10 N NaOH with a pH titrator. Culture purity was confirmed by characteristic colony morphology and beta-hemolysis of cells grown overnight at 37°C on blood agar plates. At various times during growth, 5-ml samples were removed and placed on ice. Cells were pelleted by centrifugation, washed three times with 40 mM sodium phosphate buffer, and suspended in 250 μl of sodium phosphate buffer. For these studies, total cellular protein was used to estimate total cell dry weight. We have determined that under all growth conditions tested, 50% of GBS cell dry weight is protein. Cells (5 ml) were washed twice with 40 mM sodium phosphate buffer, pH 7.0, pelleted by centrifugation, and suspended in 0.30 ml of buffer. Cells were solubilized by boiling for 5 min in an equal volume of 1 N NaOH (3). Total cell protein concentrations were determined with 0.1 ml of alkaline cell extract by the method of Lowry et al. (24), with bovine serum albumin as the standard. Cell-associated CPS was also determined with these samples by using methods described below. The t_d during the exponential phase was determined by regression analysis of the linear portion of the growth curve. The slope of this line corresponds to the maximum specific growth rate, μ_{max} , and t_d according to the formula $t_d = \ln 2/\mu$.

(ii) Continuous culture. A 100-ml batch culture of strain M781 was grown overnight at 37°C, statically, in mCDM. Culture aliquots (1 ml in 10% glycerol) were stored at -80°C. Starter cultures were prepared from thawed aliquots by seeding 50 ml of mCDM and incubating the culture statically overnight at 37°C. Studies of cells growing in continuous culture were initiated by the addition of the 50-ml starter culture to a 1-liter reaction vessel (Applikon, Inc., Foster City, Calif.) containing a final volume of 500 ml of mCDM. All experiments were conducted at 37°C with agitation at 400 rpm. Culture pH was monitored and maintained at 7.0 to 7.2 by the addition of 1 N NaOH. Sterile medium inflow rates and growth vessel volumes were adjusted to generate the dilution rate (D) for the desired cell growth rate (mass doubling time, t_d). D indicates the volume of medium replaced in the fermentor per hour and, if kept constant, corresponds to a t_d determined by the formula $t_d = \ln 2/D$. The t_d s used were 0.8 h ($D = 0.862 \text{ h}^{-1}$), 1.4 h ($D = 0.493 \text{ h}^{-1}$), 1.6 h ($D = 0.431 \text{ h}^{-1}$), 2.3 h ($D = 0.300 \text{ h}^{-1}$), and 11 h ($D = 0.063 \text{ h}^{-1}$). To insure a steady state, cells were maintained at each growth rate for at least five generations before removal of samples for analysis. In continuous culture experiments, a steady-state sample (10 ml) of cells was removed from the reaction vessel and separated into two 5-ml fractions, one of which was used to determine total cell biomass and the other to determine total cellular protein and cell-associated CPS.

Growth measurements. Optical densities of culture samples were determined

FIG. 1. Expression of GBS type III CPS by strain M781 grown in pH-controlled batch culture. Growth (closed circles) of strain M781 and amount of specific type III CPS measured (open circles) are shown. The dashed line was generated from regression analysis performed on the linear portion of the growth curve whose slope is equal to the maximal specific growth rate reached under these conditions. t_d was determined according to the formula $t_d = \ln 2/\mu$.

at A_{650} . Bacterial cell biomass was calculated from cell dry weight as described previously (5), except that the method of O'Toole (31) was used to compensate for moisture absorbed during weighing. Briefly, a 5-ml sample collected from the reaction vessel was fixed with a final concentration of 4% (vol/vol) formaldehyde. Cells were pelleted by centrifugation and washed twice with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. After the final centrifugation, the pellet was adjusted to a final volume of 0.5 ml with 4% formaldehyde in 0.1 M phosphate, pH 7.4. Dry weights were determined by placing 400 μ l of cell suspension on a predried, preweighed 0.22- μ m-pore-size polycarbonate filter. The filters were dried at 60°C for at least 24 h and reweighed. The cell biomass reported is the difference in the weight of the filter before and after addition of sample and is expressed as milligrams per milliliter.

Determination of cell-associated CPS. Cells (5 ml) were washed twice with 40 mM sodium phosphate buffer, pH 7.0, pelleted by centrifugation, and suspended in 0.30 ml of buffer. Washed cells (0.20 ml) were adjusted to 0.75 ml with 40% (wt/vol) sucrose in 50 mM phosphate, pH 7.0. Mutanolysin (Sigma Chemical Co.) was added (0.25 ml of a 0.1-mg/ml solution), and cell wall digestion proceeded at 37°C with end-over-end mixing for 1 h (4, 8). Protoplasts and debris were removed by centrifugation at $13,000 \times g$ for 5 min, and the supernatant fluid was collected and stored at -20°C. The type III CPS concentration in the mutanolysin cell wall extract was determined by an inhibition enzyme-linked immunosorbent assay (ELISA) with type III CPS-poly-L-lysine-coated microtiter plates, rabbit antisera raised to type III CPS coupled to tetanus toxoid (III-TT) (44), and purified type III CPS as the standard. III-TT rabbit antiserum reacts specifically with type III CPS and fails to recognize other GBS cell wall carbohydrates. Serial doubling dilutions of mutanolysin cell wall extracts were tested in duplicate in a microtiter plate with a final volume of 100 μ l. An equal volume of antiserum at a 1:100,000 dilution was then added to each well and mixed. Extract-antiserum mixtures were transferred to type III CPS-coated microtiter plate wells, and the ELISA was developed as described previously (32). The concentration of purified type III CPS required to inhibit 50% of antiserum binding (IC_{50}) was determined from the linear portion of the standard curve. The dilution of mutanolysin cell wall extracts that resulted in an IC_{50} was determined, and the concentration of type III CPS was calculated by multiplying the reciprocal of the dilution by the amount of purified type III CPS required to achieve an IC_{50} . Duplicate cell samples were processed at each growth rate, and results are reported as specific type III CPS (micrograms of CPS per milligram of cell dry weight).

In vitro opsonophagocytosis of cells held at steady state at two different rates of growth. The relative sensitivities of strain M781 cells grown at two different rates to in vitro opsonization for killing by peripheral blood leukocytes (PBLs) were tested. The assay was performed with and without the addition of type III CPS-specific antibody, complement, or both. Rabbit serum raised to III-TT vaccine was diluted 1:100 and used as the source of type III-specific antibody. Serum enriched for type III CPS-specific immunoglobulin G (IgG) was made by passage over a protein A column of whole III-TT rabbit serum as described previously (32). In some tests, type III CPS-specific IgG was used in place of whole antiserum at a concentration that approximated the amount present in whole antiserum. Complement was prepared by the adsorption of normal human serum with GBS strain M781 cells as described previously (45). For these studies, two side-by-side chemostats were used, with either pyridoxamine or glucose as the growth-limiting nutrient. One chemostat held cells at a fast t_d of 1.4 h ($D = 0.493 \text{ h}^{-1}$), while the other held them at a slower t_d of 11.0 h ($D = 0.063 \text{ h}^{-1}$). After at least five generations at the particular growth rate, cell samples were removed from the chemostat and used immediately in the opsonophagocytic

assay (1) and for determination of cell surface polysaccharide as described above. Differences in CFU were determined by quantitative plating of duplicate aliquots before and after adding GBS to the mixture and after incubation for 1 h at 37°C with end-over-end mixing.

Analysis of GBS type III capsular polysaccharides. (i) **Compositional.** The composition of type III CPS purified from strain M781 cells maintained in a steady state under glucose limitation at t_d s of 1.4 and 11 h was determined by high-performance anion-exchange chromatography. For comparison, type III CPS lot Nc isolated from strain M781 cells grown in a 20-liter pH-controlled batch culture with Columbia broth was used. GBS type III CPS was purified using mutanolysin extraction by methods described previously (44). For compositional analysis type III CPS (5 μ g) and 2.5 μ g of arabinose (internal standard) were dissolved in 1.0 ml of 0.5 M trifluoroacetic acid and incubated at 100°C for 18 h. The hydrolyzed mixture was dried with a flash evaporator at 53°C. The residue was dissolved in 1.0 ml of deionized water (dH_2O), redried, and dissolved in 500 μ l of dH_2O . For hydrolysis of *N*-acetylneuraminic acid, 5 μ g of purified type III CPS and 2.5 μ g of galacturonic acid (internal standard) were dissolved in 1.0 ml of 3% glacial acetic acid and incubated at 80°C for 1 h. The hydrolyzed material was dried, dissolved in 1.0 ml of dH_2O , redried, and dissolved in 250 μ l of dH_2O .

The high-performance anion-exchange chromatography system (Dionex, Sunnyvale, Calif.) was composed of a gradient pump module and a pulsed amperometric detector with a gold working electrode. For detection, the pulse potentials and durations were as follows: $E_1 = 0.05 \text{ V}$ ($t_1 = 480 \text{ ms}$), $E_2 = 0.60 \text{ V}$ ($t_2 = 120 \text{ ms}$), and $E_3 = -0.60 \text{ V}$ ($t_3 = 60 \text{ ms}$). Saccharides were separated on a CarboPac PA-1 column (4 by 250 mm; Dionex) connected to a CarboPac PA guard column (3 by 25 mm) at a flow rate of 1.0 ml/min. The eluant used for analysis of neutral and amino sugars was 20 mM NaOH prepared from a 50% NaOH solution (Fisher Chemical Co.), while the eluants used for analysis of acidic sugars were 100 mM NaOH and 150 mM sodium acetate. The pulsed amperometric detector response at 300 nA full scale was determined for a hydrolyzed reference type III CPS whose composition and structure (46) were confirmed by nuclear magnetic resonance spectrometry in the laboratory of H. J. Jennings, National Research Council of Canada, Ottawa.

(ii) **Relative molecular weight.** The relative molecular weight of type III CPS purified from 2 liters of cells held at t_d s of 1.4 and 11 h was determined by use of a Superose 6 HR gel filtration column (10 mm by 30 cm; Pharmacia Fine Chemicals, Piscataway, N.J.) and a fast protein liquid chromatography system as described previously (44). The Superose 6 gel filtration column was calibrated with 10 different dextrans with molecular weights that ranged between 2×10^6 and 6×10^3 . Polysaccharides were detected with a differential refractometer, model 401 (Waters, Division of Millipore, Marlborough, Mass.) with PBS as the eluate.

(iii) **Antigenicity.** Antigenic similarity of type III CPSs isolated from strain M781 cells cultured at t_d s of 1.4 and 11 h in continuous culture was determined by type III inhibition ELISA (32). Rabbit serum to III-TT was used at a dilution

FIG. 2. Effect of growth rate on GBS type III CPS expressed by strain M781 cells grown in an mCDM in continuous culture. The average of duplicate measurements is shown in the bar graph with each datum point shown (closed circles). CPS expressed at a t_d of 11 h compared with that expressed at 0.8, 1.4, or 1.6 h, $P < 0.01$; at 2.3 h compared with 0.8 or 1.4 h, $P < 0.01$; at 2.3 h compared with 1.6 h, $P < 0.05$; all other combinations were not significant at $P > 0.05$.

FIG. 3. Comparison of GBS type III CPS expression by strain M781 maintained at a steady state of growth in continuous culture at a t_d of 1.4 h (gray bars) or 11.0 h (white bars) under the indicated growth limitation. Values are means and standard deviations of at least three measurements.

of 1:100,000, and inhibition of specific antibody binding to type III poly-L-lysine-coated wells was calculated as described previously (32). For comparative purposes, GBS type III CPS lot Nc and *Streptococcus pneumoniae* type 14 CPS (lot 79756; American Type Culture Collection) were used. *S. pneumoniae* type 14 CPS is identical in composition and structure to desialylated GBS type III CPS (20).

Statistics. Comparison of the amounts of CPS expressed at each of the five growth rates was determined by one-way analysis of variance with the Student-Newman-Keuls multiple comparisons test using InStat version 2.0 software (Graphpad Software, Inc., San Diego, Calif.).

RESULTS

Production of GBS type III CPS in pH-controlled batch culture. Strain M781 cells grown in a CDM with the pH maintained between 7.0 and 7.2 repeatedly produced the greatest amount (13 µg/mg of cell dry weight) of cell-associated CPS during mid-exponential growth (Fig. 1). The t_d during this phase of growth was 0.8 h. When cells entered post-exponential growth, the amount of type III CPS produced was reduced by ~70% and remained at this low level as cells entered stationary phase (Fig. 1). These results suggested an association between maximum expression of type III CPS by strain M781 and a fast t_d ; however, since this study was performed with batch culture, other factors such as depletion of a specific nutrient could account for the decreased CPS expression.

Effect of cell growth rate on GBS type III CPS expression. Continuous culture was used to more accurately study the influences of growth rate and nutrient concentrations on GBS type III CPS expression. Examination of both cells and cell-free supernatant fluids showed that the majority ($\geq 93\%$) of type III CPS detected from strain M781 was cell associated. This pattern of CPS association was observed for GBS grown under all continuous culture conditions (i.e., growth rates and nutrient limitation) tested. Strain M781 cells expressed significantly ($P < 0.01$) more CPS (> 160 µg/mg of cell dry weight) when maintained at t_d s of 0.8, 1.4, and 1.6 h compared with those maintained at a t_d of 11.0 h (≤ 12 µg/mg of cell dry weight) (Fig. 2). The amount of type III CPS produced when the growth rate slowed from a t_d of 1.6 h to a t_d of 2.3 h decreased by 69% ($P < 0.05$; Fig. 2). In these studies the growth-limiting nutrient was not determined experimentally, as opposed to the chemostat studies detailed below, in which the growth-limiting nutrient was selected.

Effects of growth limitation on CPS production. Cells were maintained at a t_d of either 1.4 or 11.0 h under three different growth-limiting conditions: pyridoxamine (vitamin B₆), a coenzyme in transamination reactions involving α -amino acids (23); thiamine (vitamin B₁) an essential component of a coenzyme involved in energy metabolism (7); or glucose, a carbon/

energy source. Strain M781 cells at a t_d of 1.4 h under each of the three limitations produced a greater amount of cell-associated type III CPS than did cells held at the slower t_d of 11.0 h (Fig. 3). The difference in specific CPS produced at these two growth rates ranged from 5- and 6-fold for cells grown under thiamine and glucose limitation, respectively, to 24-fold for those under pyridoxamine-limited growth (Fig. 3). These data suggest that the limitation of these metabolically important nutrients alone cannot account for the variation in type III CPS expression by strain M781 but that under each of these three conditions cell growth rate was the principal factor that regulated CPS expression.

Effect of GBS type III CPS expression and in vitro opsonophagocytic killing by human PBLs. In addition to comparing the amount of surface CPS expressed at t_d s of 1.4 h (i.e., higher CPS expression) and 11.0 h (i.e., lower CPS expression), we tested the relative sensitivity of these cells to in vitro opsonophagocytic killing by PBLs under four different assay conditions. In the presence of PBLs, III-TT antiserum, and complement, the numbers of cells cultured under pyridoxamine limitation at t_d s of 1.4 and 11.0 h were reduced by 2.8 and 1.1 log₁₀ CFU/ml, respectively (Fig. 4). This result was expected since both groups of cells contain type III CPS and the antisera used is opsonic for GBS type III (44). In the presence of PBLs and type III CPS-specific IgG, but in the absence of complement, the degrees of reduction in CFU per milliliter were similar for cells maintained at both growth rates (Fig. 4). Thus, the difference in killing between cells at the two rates of growth under the initial condition (PBLs, III-TT antiserum, and complement) could not be explained by the opsonic activity of antibody alone. The difference in the degree of killing of cells cultured at the two growth rates was most striking in the presence of PBLs and complement. The numbers of cells cultured at a t_d of 11.0 h were reduced by 2.6 log₁₀ CFU/ml, whereas

FIG. 4. In vitro opsonophagocytosis and killing by human PBLs of GBS strain M781 maintained in a steady state under pyridoxamine limitation in continuous culture at a t_d of 1.4 h (gray bars) or 11.0 h (white bars). Reduction of GBS CFU/ml was determined after a 1-h incubation of cells in the presence of PBLs, type III CPS-specific antibody, and complement (PBL + Ab + C); PBLs and type III CPS-specific IgG (PBL + IgG); PBLs and complement (PBL + C); and PBLs in the absence of added antibody or complement (PBL). Values are means and standard deviations of triplicate measurements.

FIG. 5. Inhibition of binding of GBS type III-TT antiserum to microtiter plates coated with GBS type III CPS by type III CPS purified from cells grown in continuous culture at a t_d of 1.4 h (closed circles) or 11.0 h (open squares) or from cells grown in batch culture (open triangles). *S. pneumoniae* type 14 CPS (open circles) was used as a negative control. Datum points represent means and standard deviations of triplicate determinations in the presence of inhibitor compared with binding in control wells lacking inhibitor.

cells cultured at a t_d of 1.4 h showed no change in CFU during the 1-h incubation (Fig. 4). Thus, the physiologically regulated expression of CPS influenced greatly the susceptibility to killing of GBS in vitro by nonspecific host defense mechanisms. With PBLs in the absence of both specific antibody and complement, the numbers of cells cultured at a t_d of 11.0 h were reduced by 0.6 $\log_{10}$ CFU/ml while cells cultured at a t_d of 1.4 h grew (Fig. 4). As expected, cells used in this assay cultured at the faster t_d expressed 13-fold more CPS than cells cultured at the slower t_d .

Opsonophagocytosis studies were also performed with strain M781 cells held at these two rates of growth but under glucose limitation. No differences were seen in susceptibility to killing of cells held at the two growth rates with type III antibody in the presence or absence of complement. However, glucose-limited cells at a t_d of 1.4 h were resistant to killing (cells actually grew) in the presence of complement alone, whereas in this condition the numbers of cells held at a t_d of 11.0 h were reduced by $>1.5 \log_{10}$ CFU/ml. This pattern of susceptibility to nonspecific killing was identical to that observed with cells held at a t_d of 1.4 h under pyridoxamine limitation. Glucose-limited cells used in this study at a t_d of 1.4 h expressed 16-fold more CPS than those cultured at a t_d of 11.0 h.

Chemical characteristics of GBS type III polysaccharide.

Size, compositional, and antigenic analyses were performed on type III CPS isolated from strain M781 cells maintained at t_d s of 1.4 and 11.0 h. Type III CPS isolated from cells held at t_d s of 1.4 and 11.0 h had relative molecular weight peaks of 150,000 and 130,000, respectively. The ratios of galactose, glucose, *N*-acetylglucosamine, and *N*-acetylneuraminic acid were 2.0:0.9:1.2:0.6 and 2.0:1.1:1.0:0.4 for CPS purified from cells grown at t_d s of 1.4 and 11.0 h, respectively. The ratio of these component saccharides for type III CPS lot Nc purified from cells grown in batch culture with Columbia broth was 2.0:1.0:0.9:0.9. The IC_{50} s of purified type III CPS determined by an inhibition ELISA with cells held at t_d s of 1.4 and 11.0 h were 9.1 and 10.5 ng/ml, respectively (Fig. 5). The IC_{50} of GBS type

III CPS lot Nc was 8.1 ng/ml. In this assay, $<30\%$ of binding inhibition was obtained with *S. pneumoniae* type 14 CPS even at a concentration of 1 μ g/ml.

DISCUSSION

Several bacterial pathogens, especially those that cause disease in newborns and young children, are encapsulated with polysaccharides. Like other encapsulated pathogens, including *Haemophilus influenzae* (37) and *Neisseria meningitidis* (6), GBS express various amounts of CPS when grown in vitro and when isolated from various sites of infection (e.g., cervix, blood, and CSF). Despite studies that have identified this surface antigen as critical for virulence and pathogenicity in GBS, little is known of the mechanisms that regulate CPS expression. Cell growth rate has been shown to modulate CPS expression in bacteria (26, 27, 38), but how this physiologically controlled regulation influences susceptibility of the organism to the host defense system has not been addressed. Here we examined the physiology of CPS expression by GBS and the consequences of the physiologically mediated regulation of CPS for the susceptibility of GBS to killing in vitro by specific and nonspecific immune mechanisms.

Strain M781 cells grown in pH-controlled batch culture in a CDM produced maximal amounts of cell-associated CPS during exponential growth; however, the amount of CPS decreased by 70% as cells entered a late log-early stationary phase of growth. This pattern of cell-associated CPS expression during growth in batch culture was also observed for GBS type III strain M732, type Ia strain A909, and type Ib strain H36B (data not shown). These results are at variance with those of von Hunolstein et al. (42), who found that levels of cell-associated sialic acid (used as a marker of CPS) on cells of GBS type IV did not decrease during batch growth in Todd-Hewitt broth. Differences between our results and those of von Hunolstein et al. (42) may reflect culture media used, serotype and strain tested, and method of CPS quantification.

A correlation among the amount of capsule produced, the growth medium used, and cell doubling time was noted in the study of *E. coli* K1 capsule production (41). When grown in enriched (brain heart infusion broth), nonenriched (Casamino Acids), and glucose-minimal salts (deficient) media, cell doubling times (amount of K1 capsule produced per 10^{10} CFU) were 18 min (800 μ g), 30 min (450 μ g), and 60 min (300 μ g), respectively. These studies, performed in batch culture, as was the initial GBS study (Fig. 1), are suggestive of growth rate-dependent K1 production by *E. coli* (41).

The rate of growth that resulted in maximal CPS production of GBS type III strain M781 cells in batch culture (t_d of 0.8 h) was also shown to be in a range of rates optimal for expression of CPS in continuous culture. The transition in continuous culture of cells expressing large to small amounts of type III CPS between t_d s of 1.6 and 2.3 h suggests that mechanisms critical for the regulation of this surface antigen were affected within this narrow range of growth rates.

GBS CPS can be isolated from both cells and spent culture fluids (10, 19). In batch culture, the greatest amount of GBS CPS recovered from supernatant fluids occurred when cells entered stationary phase of growth (10). In continuous culture, $\geq 93\%$ of the total type III CPS expressed by strain M781, under all conditions tested, was cell associated. This result was predicted from studies performed in batch culture and consistent with that expected for cells maintained at exponential rates of growth (Fig. 1).

Although a growing culture requires the metabolism of many different nutrients, at any given instant growth will be

limited by the slowest step of nutrient metabolism. Changing the growth-limiting nutrient can result in alterations in the metabolic pathways of the cell (9). Continuous culture of strain M781 cells was initially performed in an mCDM without a defined nutrient limitation. Increases in cell-associated CPS at t_d s of 1.4 and 11.0 h suggested, but did not prove, a role for growth rate in CPS regulation. That strain M781 showed a growth-rate-dependent shift in CPS expression independent of the particular growth-limiting nutrient (glucose, thiamine, or pyridoxamine) strongly suggests that growth rate, as opposed to nutrient limitation, is responsible for this event.

In vitro opsonic-mediated killing of encapsulated GBS relies on the presence of specific antibody and is augmented by the addition of active complement components. The presence of a CPS protects not only GBS but other encapsulated bacteria, such as *E. coli* K1 (2, 41), from killing by nonspecific host defense mechanisms. GBS strain M781 cells grown in continuous culture at the slower t_d of 11.0 h were susceptible to complement-mediated opsonophagocytosis and killing by PBLs, a finding identical to that reported previously for the nonencapsulated mutant GBS strain COH1-13 (33). Therefore, susceptibility to in vitro opsonophagocytic killing by human PBLs in the presence of complement was observed whether cells of GBS type III (i) contained CPS that lacked sialic acid (12, 47), (ii) possessed CPS with chemically reduced sialic acid residues (12), (iii) lacked CPS as a result of genetic manipulation (33), or (iv) expressed substantially lower amounts of CPS via a physiologically regulated mechanism.

Purified CPS from strain M781 cells maintained at t_d s of 1.4 and 11.0 h were essentially identical in composition, size, and antigenicity. Therefore, decreases in the amount of specific CPS from cells held at a t_d of 11 h cannot be attributed to expression of an altered capsule. Moreover, the composition of CPS from cells grown in continuous culture in an mCDM was similar to that of CPS purified from cells grown in batch culture with Columbia broth. GBS grown in continuous culture as described here can be used in purification of CPS for vaccine production and in studies designed to elucidate more fully genes essential to (33) and the enzymes required for capsule biosynthesis.

We propose that cell growth rate is a mechanism by which CPS expression by type III GBS is regulated. The variability of invasion of respiratory epithelial cells by GBS harvested at different stages of growth in batch culture prompted Rubens and coworkers (34) to suggest that growth rate may have a role in invasion. On the basis of our results and those of studies cited throughout this paper, we hypothesize that GBS may be able to attach and adhere to host tissue when cells are growing slowly and expressing small amounts of CPS, thus exposing other cell wall components necessary for attachment (17, 28, 30, 48). However, under conditions (e.g., in blood or amniotic fluid) that may increase GBS growth rate, CPS expression is up-regulated and cells become resistant to nonspecific defense mechanisms and thus more virulent. Cell growth rate regulation of surface CPS may further our understanding of the pathogenesis of GBS and perhaps of other encapsulated bacterial pathogens in humans.

ACKNOWLEDGMENTS

We thank Dennis L. Kasper and William Chesbro for helpful advice and critical review of this work and Julieanne Pinel and Barbara Reinap for polysaccharide purification and analysis.

This work was supported by NIH contract AI-25125.

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Editor: V. A. Fischetti