Use of a dynamic *in vitro* attachment and invasion system (DIVAS) to determine influence of growth rate on invasion of respiratory epithelial cells by group B *Streptococcus*

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Expression of capsular polysaccharide (CPS) and some surface proteins by group B *Streptococcus* (GBS) is regulated by growth rate. We hypothesized that precise control of GBS growth, and thus surface-expressed components, could modulate the ability of GBS to invade eukaryotic cells. To test this hypothesis, a dynamic *in vitro* attachment and invasion system (DIVAS) was developed that combines the advantages of bacterial growth in continuous culture with tissue culture. Tissue culture flasks were modified with inlet and outlet ports to permit perfusion of GBS. Encapsulated type III GBS strains M781 and COH1 and strains COH1-11 and COH1-13 (transposon mutants of COH1 that express an asialo CPS or are acapsular, respectively) were grown in continuous culture in a chemically defined medium at fast mass doubling time (t_d = 1.8 h) and slow (t_d = 11 h) growth rates, conditions previously shown to induce and repress, respectively, type III CPS expression. Encapsulated GBS strains invaded A549 respiratory epithelial cells 20- to 700-fold better at the fast than at the slow growth rate, suggesting a role for CPS. However, unencapsulated GBS were also invasive but only when cultured at the fast growth rate, which indicates that GBS invasion is independent of CPS expression and can be regulated by growth rate. Growth rate-dependent invasion occurred when GBS was grown in continuous culture under glucose-defined, thiamine-defined, and nondefined nutrient limitations. These results suggest a growth rate-dependent regulation of GBS pathogenesis and demonstrate the usefulness of DIVAS as a tool in studies of host–microbe interactions.

Bacterial attachment to, colonization of, and invasion into host tissue are mediated through a complex series of events that involves changes of surface constituents. Most protocols developed to study attachment and invasion involve growing bacteria in batch culture to a certain phase, placing them onto a monolayer of eukaryotic cells for a specific amount of time, and treating the monolayer to study either attached cells or cells that have invaded, or both.

Bacterial growth is a critical, but often overlooked, variable in many types of experiments, including those of attachment and invasion. Although in some reports the effect of the bacterial growth phase on invasiveness *in vitro* was unequivocally established (1–4), the conclusiveness of the results is curbed by the limitations of the methodology, as growth cannot be controlled by batch culture methods where steady rates of bacterial multiplication occur briefly during the exponential phase and in an ever-changing nutritional environment (5). Moreover, when host–microbe interactions are studied, bacteria placed in a eukaryotic cell culture medium experience a new nutritional environment that may effect expression of certain cell components (6, 7). Indeed, differences in the ability of bacteria to adhere to and invade host tissue may be controlled by surface component expression that is, in turn, regulated by bacterial growth rate and metabolic status.

Although harmless as a member of the normal human gut microflora, group B *Streptococcus* (GBS) can be life threatening when vertically transmitted from a colonized mother to her newborn during birth (8). Human isolates of GBS are surrounded by capsular polysaccharide antigens (CPS), major virulence factors that serve to protect GBS from host defense mechanisms (9). Of the nine known GBS serotypes, type III is the best studied because of its prevalence in cases of early-onset neonatal GBS disease.

Expression of several surface antigens of GBS, including type III CPS and some important proteins, has been shown to be regulated by the rate of cell growth (10, 11). Different rates of GBS growth in chemically defined medium were achieved with use of a continuous culture system. In continuous culture, bacteria achieve a steady state of growth by precise control of nutrients, temperature, pH, and oxygen. Here, we test whether precise control of GBS metabolism, specifically growth rate and thus surface-expressed CPS, can modulate the ability of GBS to invade permissive eukaryotic cells.

To test this hypothesis, we developed the dynamic *in vitro* attachment and invasion system (DIVAS), a system that combines the advantages of controlling bacterial growth by continuous culture methods with perfusion tissue culture. The tissue culture flasks used with DIVAS were modified to allow bacteria from the chemostat to perfuse over an established monolayer of respiratory epithelial cells. Serotype III GBS strains M781 and COH1 and transposon mutants of COH1 (9) that either lack CPS (strain COH1-13) or have a CPS that lacks the negatively charged side-chain terminal sialic acid (strain COH1-11) were grown in continuous culture in a chemically defined medium at a fast mass doubling time (t_d = 1.8 h) and at a slow mass doubling time (t_d = 11 h), conditions previously shown to influence type III CPS expression (10, 11). These strains of COH1, grown in batch culture, have been shown to attach to and invade A549 respiratory epithelial cells by using conventional attachment and invasion protocols (1, 12), thus making them useful agents to validate DIVAS and to evaluate its applicability as a methodological approach in the study of bacteria–host interactions.

Materials and Methods

Bacterial Strains. Strain COH1 is a highly encapsulated type III isolate from an infected infant with early-onset GBS disease

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Abbreviations: GBS, group B *Streptococcus*; CPS, capsular polysaccharide; DIVAS, dynamic *in vitro* attachment and invasion system; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; CDM, chemically defined medium; t_d, mass doubling time; MOI, multiplicity of infection.

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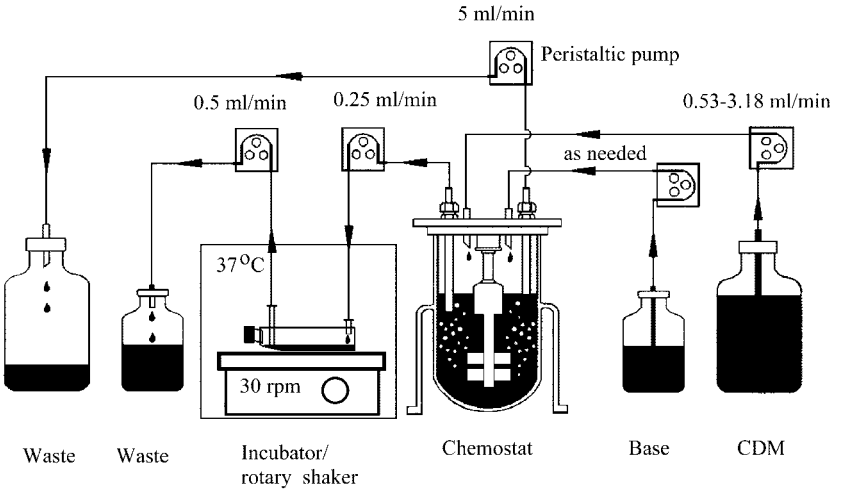

Fig. 1. Schematic representation of the DIVAS, a continuous culture system combined with tissue culture. CDM flows into the continuous culture growth vessel (chemostat) where GBS are grown under specific growth conditions. Base (NaOH) is added to maintain the culture pH at 7.3. GBS and medium are pumped over a confluent monolayer of tissue culture cells grown in a culture flask modified with inlet and outlet ports to allow perfusion of bacteria. The tissue culture flask on a rotary shaker is perfused with GBS in a 37°C incubator. GBS cells that do not attach to the tissue culture cells, along with media, are pumped to waste. Shown are the flow rates used at each part of the system. Not shown are the pH and dissolved oxygen probes, a chemostat heating unit, and the motor drive used to mix the chemostat contents.

(13). Strain COH1-13 is devoid of CPS, whereas strain COH1-11 produces a CPS that lacks sialic acid; both strains are isogenic mutants of COH1 derived by transposon-insertional mutagenesis (13). Type III strain M781 was isolated from a newborn with GBS meningitis (14).

Epithelial Cell Culture. A549 cells (ATCC CCL-185), a human type II alveolar epithelial carcinoma cell line, were maintained in RPMI 1640 tissue culture medium with 1% L-glutamine/50 units/ml penicillin G/50 μg streptomycin/10% FBS (GIBCO/BRL). Confluent monolayers for the invasion assays were grown without antibiotics, and the medium was replaced a day before the experiment.

Cell Viability Assay. Cell viability was measured with a (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. In this assay, dissolved MTT is converted to an insoluble purple formasan by cleavage of the tetrazolium ring solely by active mitochondrial dehydrogenase (15); 5 mg of MTT/ml was dissolved in PBS (pH 7.5) and filtered through a 0.22-μm membrane; aliquots were kept at −20°C until used. After incubation of A549 cells as described above, 10 μl of the MTT stock solution was added to 100 μl of medium in each microtiter well, and the microtiter plates were incubated at 37°C for 2.5 h. After aspiration of the medium, 100 μl of acid isopropanol (0.04 M HCl in propan-2-ol) was added and mixed, and absorbances were measured with a microELISA reader by using a test wavelength of 570 nm and reference wavelength of 630 nm (16).

DIVAS. The DIVAS comprises two main components (Fig. 1). The chemostat was used to maintain GBS at steady states of growth, and tissue culture flasks were modified to allow perfusion of chemostat-derived bacteria.

Chemostat. A 1-liter reaction vessel (Applikon, Foster City, CA) containing 500 ml of modified chemically defined medium (CDM) was used to support growth of GBS at a t_d of 1.8 h (fast growth) and 11.0 h (slow growth) with glucose-defined, thiamine-defined, or nondefined limitation as detailed (10). Culture pH was monitored and maintained at 7.3 by the addition of NaOH. The agitation rate was maintained at 400 rpm, and the dissolved oxygen levels were maintained at 2.6–2.8 mg/liter. Real time monitoring of absorbance at 650 nm was achieved by pumping bacterial culture from the outflow of the reaction vessel through a UV/VIS monitor (model 1790, Bio-Rad) to ensure that cells had reached a steady growth state. Bacteria that had achieved steady growth state were pumped from the reaction vessel through a single line of tubing (Masterflex 6404–14 Norprene, Cole–Parmer) that was split to allow perfusion of four tissue culture flasks (described below) with use of an eight-channeled peristaltic pump (Dynamax RP-1, Rainin Instruments).

Perfusion Tissue Culture Flasks. Twenty-five-milliliter (12.5 cm^2) tissue culture flasks with vented caps (Falcon, Becton Dickinson) were modified to accept flow of steady-state cells from the chemostat by insertion of a sterile 21-G infusion set needle into the upper right section of the flask, and with use of a male-luer coupler connecting each inlet tubing to the infusion set needle (Fig. 2). The inlet insertion hole was made by puncturing the flask with a hot 21-G needle. The inlet needle was placed ≈5 mm above the monolayer. Outlet ports were created diagonally opposite the input line (lower left section of the flask) to allow for even distribution of GBS over the established monolayer. For this port, a sterile 16-G needle attached to a female-luer connector was inserted into the flask after the flask was punctured with a hot 16-G needle. The outlet needle was inserted such that the bevel of the needle could be positioned to determine the level of the medium above the tissue culture monolayer, allowing for the maintenance of a desirable and constant volume over the monolayer. The inlet flow rate was 0.25 ml/min, which corresponds to a contact time (time required to traverse the flask) of 15–20 min. By using tubing with an internal diameter of 1.6 mm, the flow rate from the outlet of the tissue culture flasks exceeds the inlet flow rate (an internal diameter of 0.8 mm) to assure a constant fluid level. Fluids from the outlet ports were collected, and the volume was measured to check the amount of bacterial culture that passed through the flasks. Typically, four culture flasks were used per experiment; thus, four inlet lines and four outlet lines were placed in the eight-channel head of the peristaltic pump to complete the DIVAS.

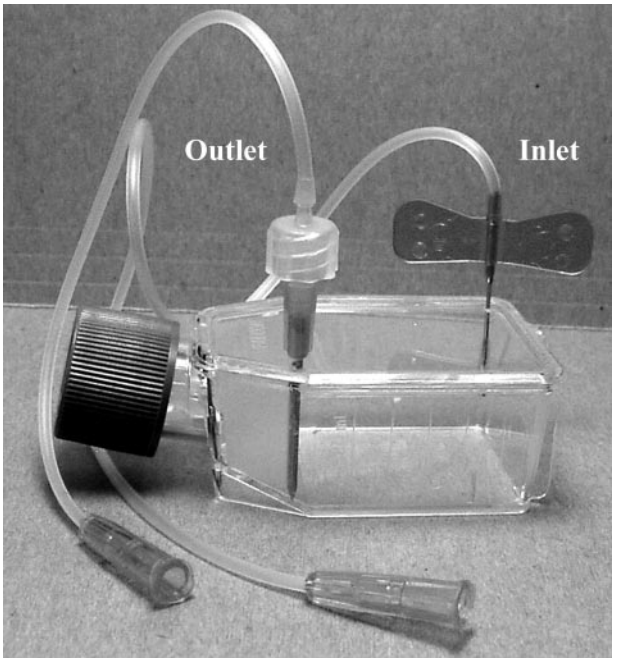

Fig. 2. Details of the perfusion tissue culture flask. The outlet port is a sterile 16- or 18-G needle attached to a female-luer connector inserted after the tissue culture flask is punctured with a hot 16-G needle. The bevel of the needle can be positioned to determine the level of the medium above the tissue culture monolayer, which allows for the maintenance of a desirable and constant volume over the monolayer. The inlet port is a sterile 21-G infusion set inserted after the flask is punctured with a hot sterile 21-G needle. The bevel of the needle is inserted 5–6 mm above the tissue culture monolayer and is in contact with the side wall of the flask to minimize turbulence of the flow. Bacteria enter the flask through this port.

Epithelial Cell Invasion Assays. Two cell invasion assays, based on the inability of gentamycin and penicillin G to penetrate epithelial cells and kill internalized GBS, were used in this study. The conventional, static invasion assay was done essentially as described by Rubens *et al.* (1), with modifications indicated below and omission of the centrifugation step after the inoculation of bacteria. For the dynamic invasion assay, inlet and outlet ports were inserted shortly before the assay into the cell culture flasks with monolayers of A549 cells (as described above); the perfusion flasks were placed on a rotary shaker (20 rpm) in a 37°C incubator and connected to the chemostat. After the cell culture medium was replaced with the bacterial culture, flasks were incubated for 2 h with continuous flow of the bacteria from the chemostat. [The multiplicity of infection (MOI) is always in flux because of the continuous perfusion of bacteria over the cell monolayer. On the basis of the number of A549 cells present in a confluent monolayer in a 12.5-cm^2 flask and the density of GBS culture (colony-forming unit/ml) in the chemostat, the moi for all experiments was estimated to be 1:1]. The perfusion flasks were disconnected from the chemostat, the needles were removed, and the inlet and outlet port holes were sealed with paraffin wax. After washing of infected monolayers three times with PBS, pH 7.3, fresh RPMI 1640 medium with 10% FBS/5 μg of penicillin G/100 μg of gentamycin was added, and flasks were incubated in a CO_2 incubator for 2 h. After four washes with PBS, monolayers were detached from the plastic by 0.2 ml of trypsin, lysed by 0.8 ml of 0.025% Triton X-100, transferred quantitatively to microfuge tubes, and vortexed vigorously for 1 min; appropriately diluted aliquots were plated on tryptic soy/5% sheep blood agar plates. Plates were incubated at 37°C overnight, and GBS colony-forming units were enumerated.

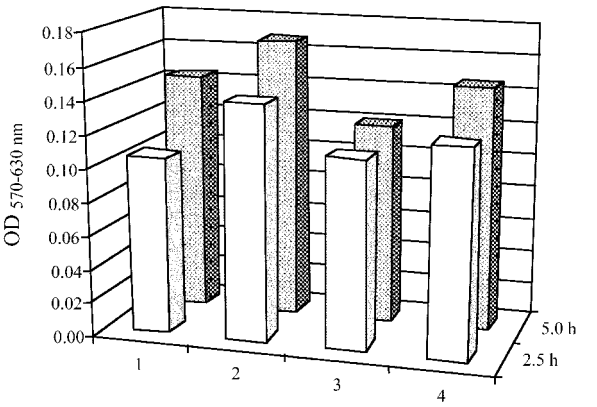

Fig. 3. Metabolic activity of A549 respiratory epithelial cells as measured with the MTT assay. Cells were cultured for 2.5 or 5 h with RPMI 1640 medium containing 25 mM Hepes and 10% FBS (lane 1), RPMI 1640 medium with 25 mM Hepes (lane 2), CD medium with 10% FBS (lane 3), and CDM without FBS (lane 4).

Statistical Analysis. Significance of the invasiveness of GBS at each growth rate was determined with a nonparametric two-tailed Mann–Whitney test (INSTAT, version 2.0, GraphPad, San Diego).

Results

Validation of DIVAS. The flow of a stable bacterial culture over the surface of the attached eukaryotic cells presents conditions typically not encountered by the eukaryotic cell culture in the conventional *in vitro* bacterial invasion assay. Imposed for 2 h during a typical dynamic invasion assay, these conditions include: (*i*) fluid movement over the cell monolayer, (*ii*) absence of CO_2 in the gas phase, and (*iii*) substitution of the cell culture medium for the bacterial CDM. Although the latter two conditions are possible to overcome by simple technical modifications, they would present GBS with nutrients that differ dramatically from those of the steady-state growth conditions established in the chemostat. Therefore, we chose to maintain the nutritional conditions of GBS (10) and evaluate the impact of perfusion on the stability of the eukaryotic cell culture.

To evaluate the effect of sheer force on the integrity and health of confluent monolayer, RPMI 1640 containing 10% FBS or CDM without FBS was perfused at a flow rate of 0.25 ml/min over the A549 cells in the rotary shaker at 37°C in the absence of CO_2. No significant changes were visible microscopically after 5 h of medium perfusion, a length of time that is well above the length of the typical dynamic invasion assay, although a notable number of detached cells were seen after 30 h of the medium perfusion. Moreover, A549 cells did not show any significant changes in viability, as measured by their ability to reduce MTT, after a 2.5-h or 5-h incubation period in the RPMI 1640 or CDM with or without FBS (Fig. 3).

Invasion of A549 by GBS Strain M781 in Conventional, Static Conditions. Because physiological parameters that affect expression of important surface antigens of GBS type III strain M781 have been described (10), we sought to establish the basic parameters of invasion for this strain in a conventional static invasion assay in a multiwell plate with A549 cells as described by Rubens *et al.* (1) for GBS type III strains COH1 and COH31r/s. The well encapsulated type III strain M781 invaded A549 cells in a time-dependent manner, whereas the control *Escherichia coli*

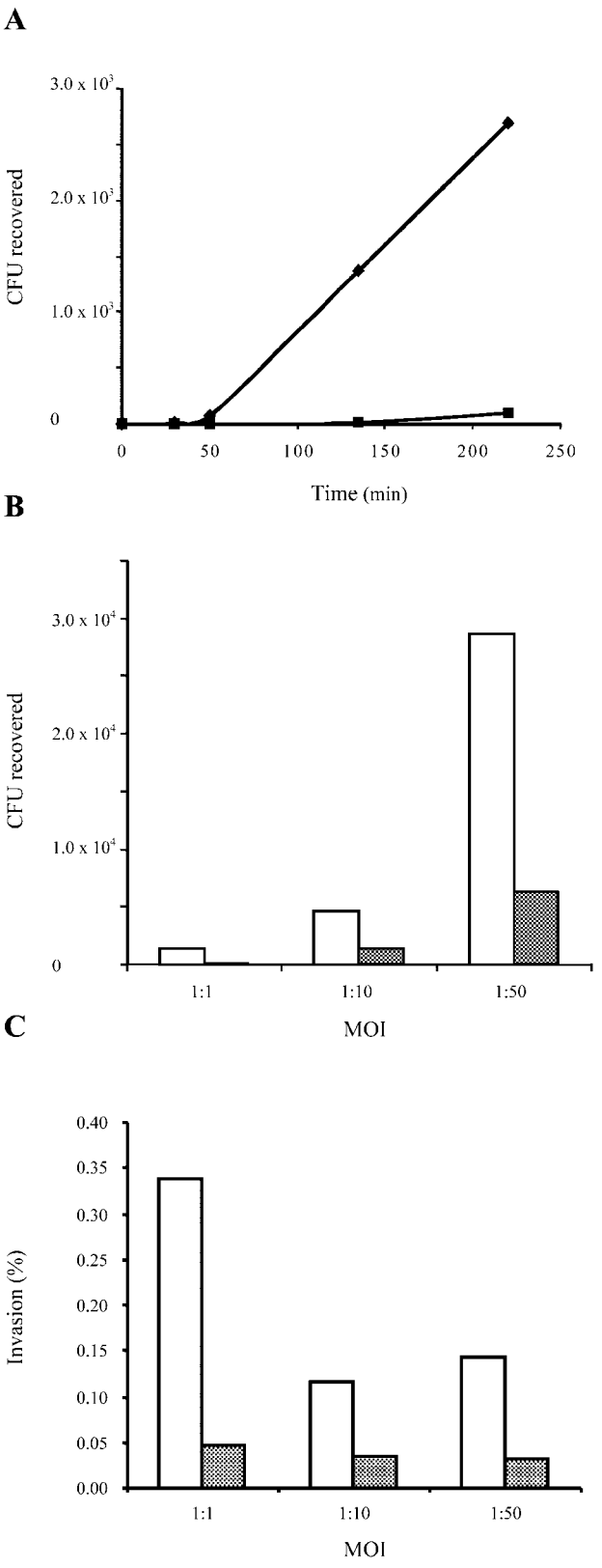

Fig. 4. Characteristics of invasion of A549 respiratory epithelial cells by GBS strain M781 using static, conventional invasion methods. Kinetics of invasion (*A*) of A549 cells by GBS strain M781 (◆) and by *E. coli* strain DH5 (■). Assay was performed in a 24-well plate in RPMI with 10% FBS and moi (eukaryotic cell/bacteria) was 1:1 for GBS and 1:10 for *E. coli* DH5. Invasion (*B*) and efficiency of invasion (*C*) of A549 respiratory epithelial cells by GBS strain M781 at different MOI levels with (white bars) and without (gray bars) addition of 10% FBS.

strain DH5 was poorly invasive (Fig. 4*A*). In addition to exposure time, the MOI or the ratio of the eukaryotic cells to infecting GBS had a pronounced effect on the number of bacteria internalized. The MOI ratios of 1:10 and 1:50 resulted in a greater number of internalized GBS (Fig. 4*B*); however, the increase of the number of internalized bacteria expressed as a percentage of the inoculum was not directly proportional to the increase of the inoculum size (Fig. 4*C*). Thus, GBS strain M781 invaded A549 respiratory epithelial cells with characteristics (MOI and kinetics of invasion) similar to those measured with other type III strains of GBS in a conventional, static invasion assay (1).

Invasion of A549 by GBS Strains in DIVAS. Because conventional static invasion assays are conducted in a CO_2 incubator, we compared the effect on the ability of GBS to invade A549 cells by placing one culture on a rotary shaker at 37°C without CO_2 (Fig. 1) for 2 h and another in a CO_2 incubator under static conditions for the same duration. The numbers of invading GBS in these two systems were 8.0×10^3 and 8.5×10^3 colony-forming unit/well (average of three determinations), respectively, suggesting that the CO_2 atmosphere was not essential for, nor the agitation deleterious to, invasion of these cells by GBS.

In the static invasion assay, omission of FBS from the RPMI medium during the infection led to 3-fold fewer internalized GBS. Hence, apart from the question of whether the FBS can be omitted temporarily as a cell culture growth factor, we sought to determine whether FBS can effect the invasion of A549 cells by GBS in DIVAS the way it did in the static invasion assay. To test this possibility, an additional line to deliver FBS was inserted into the bacterial culture delivery line proximal to its entry into the invasion perfusion flasks. This procedure allowed FBS to mix with the bacterial culture at the required concentration (10%), but kept the bacteria exposed to FBS for only 15 min, which was the time required for bacteria to traverse the tissue culture flask. No significant differences in the number of internalized GBS strain COH1-13 were seen in the presence or absence of FBS at the fast or slow growth rate. This observation suggests that growth of GBS in the presence of FBS during the 2-h assay, rather than the effect of FBS on the invasion itself, was responsible for the higher number of internalized bacteria measured with the conventional static invasion assay. It also suggests that FBS can be omitted from studies using DIVAS without compromising the health of the eukaryotic cells (Fig. 3) or invasiveness of GBS.

The now-validated DIVAS was used to compare the invasion of heavily encapsulated GBS strain M781 maintained in the chemostat at a fast and at a slow rate of growth in a CDM with glucose-defined, thiamine-defined, and nondefined limitation. As shown in Fig. 5, glucose-limited GBS strain M781 cells held at a fast growth rate (t_d = 1.8 h) efficiently invaded A549 cells, whereas strain M781 cells held at a slow growth rate (t_d = 11.0 h) invaded significantly less ($P = 0.002$). The fast rate of growth that resulted in efficient invasion was similar to that shown previously to cause high-level expression in type III CPS by strain M781 (10). To examine further the role, if any, of this important virulence factor, we used DIVAS to test the ability of glucose-limited GBS strains COH1, COH1-11, and COH1-13 to invade A549 cells. GBS strain COH1 showed a pattern of invasion similar to that of M781, with significantly ($P < 0.0001$) greater invasion when cells were maintained at the fast than at the slow rate of growth (Fig. 5). GBS strain COH1-11 cells bearing an asialylated type III CPS also showed characteristics of growth rate-dependent invasion. When cultured at a fast rate of growth, cells of the acapsular strain COH1-13 invaded A549 cells as efficiently ($P = 0.13$) as did its well encapsulated parent strain (Fig. 5). However, when held at the slow rate of growth, these unencapsulated GBS were unable to invade A549 cells ($P <$

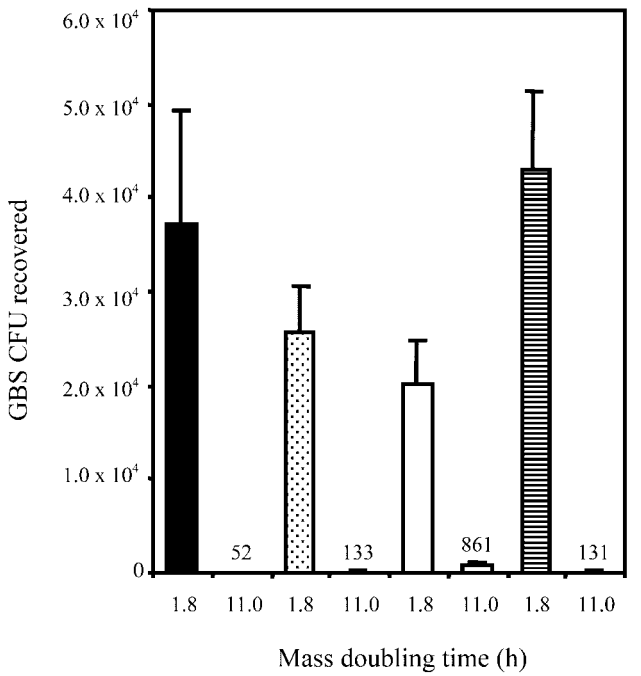

Fig. 5. Invasion of A549 respiratory epithelial cells using DIVAS and GBS strain M781 (black bars), COH1 (stippled bars), COH1-11 (white bars), and COH1-13 (striped bars) grown in the chemostat at a slow (t_d = 11 h) and at a fast (t_d = 1.8 h) cell mass doubling time. The mean and standard errors of 8–16 determinations are shown.

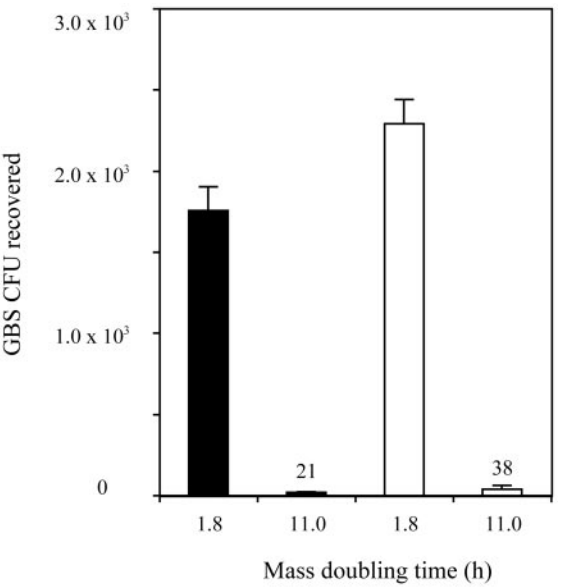

Fig. 6. Invasion of A549 respiratory epithelial cells using DIVAS and GBS strain M781 grown in the chemostat with thiamine limitation (black bars) or nondefined limitation (white bars) at the fast (t_d = 1.8 h) and slow (t_d = 11 h) cell mass doubling times. The mean and standard errors of 19–32 determinations are shown.

0.0001 compared with invasion by COH1-13 held at the fast growth rate). These results suggest a growth rate-dependent regulation of the factor(s) involved with GBS invasion and an apparent lack of influence on the invasion process by the type III CPS antigen.

To test whether differences in invasion were the result of growth rate changes as opposed to nutrient limitation *per se*, DIVAS experiments were performed with GBS strain M781 cells held at the fast and slow growth rates with CDM limited for thiamine, or with a nondefined nutrient limitation. At both limitations, GBS invaded A549 respiratory epithelial cells in numbers significantly ($P < 0.0001$) greater when GBS was grown at t_d of 1.8 h compared with the relatively slow t_d of 11.0 h (Fig. 6). These results strongly suggest that growth rate, as opposed to the limiting nutrient used to maintain steady-state growth, is responsible for the regulation of GBS invasion.

Discussion

In the 1990s, Rubens and coworkers published a series of papers describing the invasiveness of highly and poorly encapsulated, as well as transposon-derived, mutants of type III GBS that were devoid of or produced an asialylated CPS (1, 17, 18). Collectively, these studies revealed that invasion by GBS of A549 respiratory epithelial cells required actin microfilament elements, actively metabolizing bacteria, and was strain dependent, with greater invasion occurring with a highly than with a poorly encapsulated wild-type GBS (1). They also observed that lag- and stationary-phase GBS invaded in greater numbers than did cells harvested during log-phase growth, which suggested a growth-rate or growth-phase regulation of components involved with invasion. That GBS factors other than CPS were required for invasion of this cell line was demonstrated with use of acapsular mutants and blocking studies using CPS-specific antibody (17). Additional studies using human umbilical vein endothelial (HUVE) cells concluded that GBS type III CPS "attenuates but does not prevent type III entry into HUVE cells" (18) because the acapsular and asialo mutants invaded more readily than did the fully encapsulated parent strain. Corroborating these results are those of Tamura and Nittayajarn (19), who showed, using GBS strains COH1 and the unencapsulated COH1-13, that attachment to cytokeratin 8 on A549 epithelial cells was not mediated by the CPS.

Because expression of GBS surface CPS and some protein antigens are regulated by growth rate (10, 11), we hypothesized that invasion by GBS also may be regulated in a growth rate-dependent manner. DIVAS was developed to test this hypothesis, which is predicated on controlling bacterial growth, and thus surface-expressed components, during the invasion process.

Validation of DIVAS included testing the viability and the metabolic activity of the A549 epithelial cells during perfusion and upon incubation in the absence of FBS or CO_2 with use of an MTT assay. Maintenance of metabolic activity of A549 cells under the conditions deemed necessary for use with DIVAS, as well as the comparable invasion results obtained with the static or the rotating platform, provided assurance that the system was not inducing variables new to the process other than the dynamic flow of steady-state bacteria over the monolayer.

That the highly encapsulated GBS type III strains M781 and COH1, as well as the asialo mutant strain COH1-11, invaded A549 cells when maintained under glucose limitation at the fast but not slow growth rate suggested a role for CPS in this process, as all strains express more CPS under that growth condition. However, if CPS contributes predominately to the invasion of A549 by GBS, the invasiveness of the COH1-13 acapsular mutant should differ from that of its parent strain and should not be influenced by growth rate changes. Indeed, the ability of strain COH1-13 cells to invade A549 cells at levels comparable to those of the encapsulated wild type suggests that the CPS is not required for invasion by GBS. Moreover, that invasion by all GBS strains tested was significantly abrogated when cells were held at a slow rate of growth implies that the factor(s) responsible for this event is/are under growth rate regulation. Prelim-

inary results suggest that GBS cultured at a t_d of 1.8 h (invasive condition) expressed membrane proteins that were completely absent from membrane preparations of GBS grown at a t_d of 11 h (unpublished results). Whether these proteins are involved with attachment to and/or invasion of GBS remains to be determined, but they are in a size range similar to that of hydrophobic surface proteins involved with adherence of type III GBS to buccal epithelial cells (20) and a 21-kDa GBS protein involved with binding to human macrophages (21).

To ensure that growth rate, and not factors involved with glucose limitation, was responsible for the observed differences in invasiveness, GBS was cultured to steady state in the CDM with defined (thiamine) and nondefined nutrient limitations then used in DIVAS. Three growth-limiting conditions (glucose, thiamine, and nondefined) were used previously with GBS strain M781 to demonstrate growth rate-dependent expression of CPS (10). The fact that invasion of A549 cells by GBS was growth rate-dependent even when the bacteria were grown at each of three nutrient limitations unequivocally signals the importance of growth rate in regulating this event.

Studies of the interactions between host and microbe may benefit from the many advantages of DIVAS. Advantages range from using various tissue cell lines to the ability to alter a single bacterial nutritional requirement such as carbon or nitrogen by adjusting the composition of the CDM and studying the impact of this singular change on pathogenesis. By controlling bacterial growth and perfusion parameters, DIVAS may be particularly useful in reproducibly generating microarrays to study gene expression by both mammalian and bacterial cells during the attachment and invasion process (22). It may also help to resolve discrepancies in published results, especially where influences by bacterial growth phase and/or nutritional requirements were observed. For example, separate studies showed that *Salmonella* spp. failed to readily invade several tissue cell lines when harvested in the stationary phase of oxygen-limited, batch culture growth (2, 4). However, others showed that the moi, not the growth phase, was responsible for these results (3). Study of the rate, instead of the phase, of growth as well as the influences of moi on the invasiveness of *Salmonella*, in the presence or absence of nutrients such as oxygen, can be readily achieved with DIVAS.

Properties of bacterial growth are not new but rather have been on the forefront of the microbiologists' way of thinking for decades (23). Despite this understanding, *in vitro* studies of bacterial pathogenesis have not routinely involved the control of bacterial growth. Results presented herein demonstrate the importance of defining and controlling growth during studies of the infection process and illuminate the possibilities for revealing the underlying mechanisms responsible for intimate host–pathogen interactions.

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