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Identification of a High-Virulence Clone of Serotype III *Streptococcus agalactiae* by Growth Characteristics at 40°C

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A high-virulence clone of serotype III *Streptococcus agalactiae* causing invasive neonatal disease was previously identified by multilocus enzyme electrophoresis. A simple procedure involving growth at 40°C distinguished all isolates classified in this high-virulence clone from other serotype III isolates, which are more frequently associated with asymptomatically colonized infants, as well as the other serotypes of group B streptococci. The high-virulence clone failed to grow at 40°C in FMC, a chemically defined medium, in contrast to the other organisms, which grew readily.

A recent study examined the chromosomal genotypes of five serotypes of *Streptococcus agalactiae* (group B streptococci) by characterizing electrophoretically the allelic profiles at 11 metabolic enzyme loci (4). Nineteen distinctive electrophoretic types were identified, and cluster analysis indicated two primary phylogenetic divisions. A single clone (electrophoretic type) comprising division I was composed of 40 serotype III isolates from human neonates, 34 of which were isolated from infected infants and 6 from asymptomatically colonized infants. The 18 remaining electrophoretic types comprised division II, which included serotypes Ia, Ib, Ic, II, and III. All were isolated from infected infants, with the exception of serotype III, the majority of which were isolated from asymptomatically colonized infants. In addition to the presence of the type III polysaccharide antigen, characteristics associated with the high-virulence clone include the elaboration of elevated levels of extracellular neuraminidase (2) and lipoteichoic acids (5) and the inability to grow in culture medium containing 200 mM sodium phosphate (1, 4). However, some of these characteristics can also be found in division II isolates. This study reports that serotype III (division I) isolates can rapidly be distinguished from the other 18 electrophoretic types comprising division II by their inability to grow at 40°C.

S. agalactiae (group B streptococci) strains (118 isolates) used in this study have been described in detail elsewhere (4). Routinely, isolates were cultured on 5% sheep blood agar plates (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 16 to 24 h before each experiment. A chemically defined medium (FMC) formulated for the oral streptococci by Teleckyj et al. (6) and modified for the group B streptococci (3) by increasing the sodium phosphate to 65 mM was used for growth of the cultures. For some experiments, whole Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) was used and prepared according to the directions of the manufacturer. Growth was monitored by measuring the A_{675} in a spectrophotometer (Junior model 35; The Perkin-Elmer Corp., Norwalk, Conn.). The optical density was multiplied by 1,000 and converted to adjusted optical density units (AOD) so that readings would agree with Beer's law and be proportional to bacterial mass (7). It was determined that 1 AOD is the equivalent to 0.43 μg of cellular dry weight per ml or 8.0×10^5 CFU/ml. The cell density of each culture inoculum was adjusted to 30 to 40 AOD. The pH was routinely monitored and maintained between 6.5 to 7.0 by addition of 2 N NaOH. When the cultures reached 400 to 500 AOD (mid-exponential phase; 30 to 35 min doubling time), two 10-ml tubes of FMC were inoculated at 37 and 40°C, respectively, at a starting inoculum of 30 to 40 AOD. Growth at both temperatures was monitored as previously described, and temperature shift experiments were routinely conducted for 3 h. Inability of cultures to grow at 40°C was defined as a less than twofold increase in cellular mass during this time period.

In addition to the previous demonstration of the production of elevated levels of extracellular neuraminidase (2) and the inability to grow in 200 mM phosphate (1, 4), the present study indicated that a high-virulence clone of serotype III *S. agalactiae* (GBS 110, isolated from an infant with meningitis [5]) could also be characterized by marked inhibition of growth at 40°C in a chemically defined medium (FMC) (Fig. 1). In contrast, a type III isolate obtained from an asymptomatically colonized infant (GBS 181) was unaffected by the increase in growth temperature from 37 to 40°C (Fig. 1). The latter isolate was shown previously to represent an electrophoretic type quite distinct from that of the isolates composing the high-virulence clone (4). Examination of 118 isolates of *S. agalactiae* representing five serotypes previously examined (4) indicated that inhibition of growth at 40°C was associated only with the serotype III high-virulence clone (Table 1). A comparison of the chemically defined medium with Todd-Hewitt broth indicated that the ability to distinguish the high-virulence clone at 40°C was lost when the organisms were grown in Todd-Hewitt broth (Fig. 1). Similar results were obtained with brain heart infusion and tryptic soy broth (data not shown), suggesting that the complex media may supply a factor(s) required for growth of the high-virulence clone at 40°C.

In addition to the requirement of the chemically defined medium to demonstrate the inhibitory effect of increased temperature on growth, we found that only exponential-phase cultures respond to the increased temperature and require about 60 to 90 min of growth of an isolate in the appropriate medium (FMC) before being tested for inhibition of growth at 40°C. Since an increase in cell mass of the high-virulence clone is not immediately inhibited upon temperature shift, a low starting inoculum (30 to 40 AOD; 2×10^7 to 4×10^7 CFU/ml) is required to obtain an accurate

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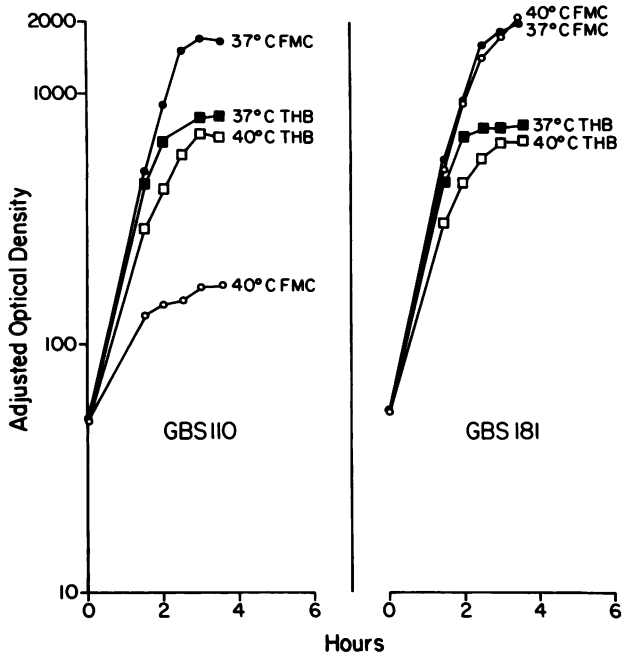

FIG. 1. Effect of temperature (37 and 40°C) on the growth of GBS 110 (division I [4], infected neonate) and GBS 181 (division II, asymptomatic neonatal carrier) in FMC and Todd-Hewitt broth.

measure of the increase in cell mass (usually less than two cell doublings). Starting inocula greater than 2×10^8 CFU/ml decrease the sensitivity of the assay and make it difficult to distinguish between the two classes of organisms. Positive (serotype III high-virulence clone, division I [4]) and negative (serotype III, asymptomatic or any other serotype, division II) controls must always be included. The inhibitory effect of the increased temperature is apparent within 2 to 3 h, so the entire procedure can be completed in less than 5 h.

Efforts are under way to develop gene probes based on the unique temperature effect, and transconjugants of the high-virulence clone which grow at 40°C have been isolated. Once the temperature-sensitive gene product(s) is identified, the gene(s) involved can be cloned and used as probes to further decrease the time required for identification.

TABLE 1. Correlation of growth of division I and division II isolates of *S. agalactiae* at 40°C[a]

Division and serotype	Growth response at 40°C (% inhibited)	
	Invasive isolates	Carrier isolates
I: III	100 (34)[b]	100 (6)
II		
III	0 (8)	0 (15)
Ia	0 (8)	
Ib	0 (16)	
Ic	0 (14)	
Ic/II	0 (6)	
II	0 (11)	

[a] Divisions I and II are based on electrophoretic types as determined by Musser et al. (4).
[b] Numbers in parentheses are numbers of culture isolates.

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