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Growth and Amino Acid Requirements of Various Strains of Group B Streptococci

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A chemically defined medium (FMC; B. Terleckyj, N. P. Willett, and G. D. Shockman, Infect. Immun. **11**:649-655, 1975) was used to compare the growth and amino acid requirements of 16 strains of group B streptococci, consisting of both laboratory-passaged organisms and fresh clinical isolates from adult and neonatal infections. The 5 standard Lancefield immunizing strains of group B streptococci, 090 (Ia), H36B (Ib), A909 (Ic), 18RS21 (II), and D136C (III), had doubling times in FMC (28 to 36 min) similar to those observed in Todd-Hewitt glucose broth (24 to 30 min). Similar doubling times were obtained with 11 clinical isolates growing in Todd-Hewitt glucose broth and FMC. The optimum buffering capacity of FMC was provided by 0.06 M sodium phosphate, and 1% glucose gave maximum cell yield. The group B streptococci, with minor exceptions, were very homogeneous in their amino acid requirements under both aerobic and anaerobic growth conditions. Phenylalanine, tyrosine, tryptophan, glutamate, arginine, valine, leucine, lysine, methionine, isoleucine, cystine, and histidine were required by all 16 strains under both aerobic and anaerobic growth conditions. In addition, threonine was required by all strains under aerobic growth conditions, whereas only 9 strains required threonine under anaerobic conditions. Serine was required by only 3 type III fresh clinical isolates aerobically, but not anaerobically. A requirement for glycine varied from strain to strain, apparently influenced by the oxidation-reduction potential of the growth medium.

The recognition of group B streptococci as significant etiological agents of serious neonatal infections has stimulated renewed interest in the study of these microbes. Although several aspects of human infection with the group B streptococci have been studied, such as the host immunological response (2, 4) and the epidemiology of neonatal infections by these organisms (1, 5), few reports have appeared concerning the in vitro growth characteristics and nutritional requirements of these agents. Niven (8) examined the nutritional requirements of 18 human and bovine strains of the group B streptococci in a chemically defined medium and found that all 18 strains were homogeneous in their vitamin requirements, with the exception of riboflavin, which was not required by 16 strains. However, serotyping of the individual strains was not reported. Unfortunately, 44% of the strains tested did not show repeated transfer in the complete chemically defined medium, possibly indicating that an unknown growth factor(s) was missing from the medium. The amino acid requirements of 2 of the strains were examined, and valine, leucine, isoleucine, phenylalanine, glutamate, arginine, lysine, histidine, and tryptophan were found to be essential for growth. Willett et al. (12, 13) formulated a completely defined medium for the growth of a bovine strain of *Streptococcus agalactiae* (Cornell strain C-48) that yielded growth comparable to that seen in brain heart infusion broth. These authors found that arginine, cysteine, glutamate or glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine, tryptophan, and valine were essential for growth after initial transfer. In another study, Mickelson (6) examined the cystine requirement of *S. agalactiae* in detail and found that five cultures had an absolute requirement for L-cystine that could be replaced with cysteine, glutathione, or the disulfide form of glutathione. The requirement for cystine was clearly shown to be nutritional, since dithiothreitol could not replace the sulfur-containing amino acids or glutathione. Finally, Baker and Kasper (3) examined the effects of increasing glucose and phosphate concentrations on the growth response of the group B streptococci in Todd-Hewitt broth. They observed that by in-

creasing the buffering capacity of the broth medium, acid accumulation was prevented, and the number of viable cells obtained was increased at the stationary phase of growth.

None of the above studies, however, has examined the growth characteristics and nutritional requirements of group B streptococcal strains of defined serotypes from both laboratory-passaged strains and fresh clinical isolates to determine if differences might exist among the various serotypes. The purpose of the present study, therefore, was to examine the growth characteristics and amino acid requirements of 16 strains of defined serotypes, using a chemically defined medium that supported growth rates comparable to those obtained in Todd-Hewitt broth. In addition, the influence of glucose and phosphate concentrations on final cell yield was examined.

MATERIALS AND METHODS

Bacterial strains. Sixteen strains of group B streptococci, representing all five known serotypes, were used in this study. Table 1 lists the various strains, along with the source of each strain and the specific serotype. Strains 090, H36B, A909, 18RS21, and D136C were kindly supplied by Hazel Wilkinson, Center for Disease Control, Atlanta, Ga. Strains 110, 111, 112, 113, and 114 were obtained from Carol J. Baker, Baylor College of Medicine, Houston, Tex. Strains 100, 101, 102, 108, 115, and 117 were obtained from the Microbial Pathology Section of Bexar County Hospital, San Antonio, Tex., through the generosity of James H. Jorgenson. These strains were typed by the capillary precipitin reaction, using antisera kindly supplied by Rebecca C. Lancefield. Multiple aliquots of each strain were lyophilized in 1.0% skim milk and/or frozen at −70°C in Todd-Hewitt broth (Difco) for long-term storage. To obtain cells for experiments, samples from the frozen or lyophilized cultures were streaked onto 5% sheep blood agar plates and, after overnight incubation at 37°C, were stored at 4°C for up to 3 weeks.

Liquid media and growth conditions. Todd-Hewitt broth (Difco) was prepared according to the manufacturer's directions and modified by the addition of a filter-sterilized solution of glucose after the remainder of the medium had been autoclaved at 121°C for 15 min. Additionally, the final pH of the Todd-Hewitt broth was aseptically adjusted to 7.0 after autoclaving. The chemically defined medium (FMC) formulated for the growth of oral streptococci was prepared as described by Terleckyj et al. (10), with a final pH of 7.0 and a sodium carbonate concentration of 0.019 M. To vary the sodium phosphate (Na_2HPO_4/NaH_2PO_4 [10]) or glucose concentration, a 2× solution of the medium was prepared that lacked sodium phosphate (pH 7.0) or glucose. These two solutions were then added aseptically to give various required concentrations.

All components of FMC were obtained from Sigma Chemical Co. Cells were grown in optically calibrated culture tubes (18 by 150 mm) containing 6 ml of medium, either aerobically, in a 37°C water bath, or anaerobically, using the GasPak (Baltimore Biological Laboratory) anaerobic system, in a 37°C incubator.

TABLE 1. *Strains of group B streptococci*

Strain no.	Serotype	Source of strain and clinical disease
090	Ia	Standard Lancefield immunizing strain
114	Ia	Blood, neonatal early-onset sepsis
101	Ia	Blood, adult female sepsis
102	Ia	Blood, adult female sepsis
H36B	Ib	Standard Lancefield immunizing strain
A909	Ic	Standard Lancefield immunizing strain
18RS21	II	Standard Lancefield immunizing strain
113	II	Blood, neonatal early-onset sepsis
D136C	III	Standard Lancefield immunizing strain
110	III	Cerebrospinal fluid, neonatal late-onset meningitis
111	III	Cerebrospinal fluid, neonatal early-onset sepsis with meningitis
112	III	Cerebrospinal fluid, neonatal late-onset meningitis
115	III	Cerebrospinal fluid, neonatal late-onset meningitis, isolated before initiation of ampicillin and gentamicin chemotherapy
117	III	Cerebrospinal fluid, neonatal late-onset meningitis, isolated 13 h after initiation of ampicillin and gentamicin chemotherapy
108	III	Blood, adult female sepsis
100	Untyped	Blood, adult female sepsis

Growth measurements. Growth was monitored by measuring the absorbance in a Coleman Junior II spectrophotometer at 675 nm. The observed optical density was multiplied by 1,000 and converted to adjusted optical density (AOD) units so that readings would agree with Beer's law and be proportional to bacterial mass (11). Turbidities were quantitated by dry-weight determinations of 10-ml samples of washed stationary-phase cell suspensions. Samples were dried at 84°C for 48 h and desiccated over $CaSO_4$ before weighing. It was ascertained that 1 AOD unit corre-

sponded to 0.43 μg of cellular dry weight per ml. One AOD unit also was equivalent to 5.0×10^5 colony-forming units per ml, determined on 5% sheep blood agar plates. Doubling times (T_D) were estimated graphically, by plotting AOD versus time.

Amino acid studies. Amino acid requirements were determined basically as described by Terleckyj and Shockman (9). Briefly, cells were initially grown in "complete" FMC, containing 1.0% glucose and 0.060 M sodium phosphate. Cells were grown to mid- to late-exponential phase (AOD = 600 to 1,000, 0.23 to 0.39 mg of cellular dry weight per ml). The cultures were then chilled on ice, centrifuged, washed twice in FMC minus the amino acid under study, and then resuspended in 2 to 3 ml of the same medium. An aliquot of this cell suspension was then inoculated into duplicate tubes of FMC deficient in the amino acid to be tested so that the intial AOD of the culture would be between 10 and 40. One tube was then incubated aerobically, the other was placed in a GasPak anaerobic jar, and both were incubated for 24 h, after which culture turbidities were determined.

If growth did not occur within 24 h, cultures were reincubated for up to 48 h. Lack of growth after 48 h indicated that the missing amino acid was required for the growth of that particular strain. If growth occurred at 24 h, a subculture was performed and growth was measured after 24 h. The growth of each strain in amino acid-deficient media was compared with a control culture containing FMC with all amino acids present. An amino acid was considered essential if growth in the original culture or subculture was 40% or less than that of the control culture after 24 h of incubation. This figure was found to be convenient, because additional subcultures in amino acid-deficient media further decreased the amount of growth obtained, so that by three or four subcultures the amount of growth was usually less than 5% of that of the control culture. If growth was more than 40% of the control, the particular amino acid was not considered essential for growth, and in no case did additional subculture further decrease the level of growth to below 40% of that of the control culture.

RESULTS

Growth of standard immunizing strains of group B streptococci in THGB and FMC. The standard immunizing strains of group B streptococci, representing each of the five serotypes, were grown in Todd-Hewitt glucose broth (THGB) and FMC for comparison of T_D values and final AODs after 24 h (Table 2). Both media contained 0.5% glucose and were adjusted to a pH of 7.0. In addition, FMC contained 0.045 M phosphate and 0.019 M Na_2CO_3. Little difference was seen in the T_D values of the five strains grown in THGB (24 to 30 min) compared with those grown in FMC (28 to 36 min). The clinical isolates of group B streptococci listed in Table 1 had similar T_D values in THGB and FMC (data not shown).

In three of the strains examined (090, H36B, and A909), the final AODs after 24 h were 1.6 to 2.1 times higher in FMC than in THGB. Since this difference possibly could be attributed to the increased buffering capacity of FMC (0.045 M phosphate) compared with THGB (0.003 M phosphate), the effect of phosphate concentration on T_D and maximum cell yield was further examined in one type III (D136C) strain.

TABLE 2. *Growth of standard immunizing strains of group B streptococci in FMC[a] and THGB[b]*

Strain	Serotype	T_D[c] (min)		Final AOD (24 h)	
		FMC	THGB	FMC	THGB
090	Ia	36	24	790	505
H36B	Ib	30	28	885	465
A909	Ic	28	28	775	365
18RS21	II	35	30	ND[d]	380
D136C	III	30	26	ND	505

[a] Chemically defined medium of Terleckyj et al. (10), containing 0.5% glucose. The phosphate concentration was 0.045 M and Na_2CO_3 was 0.019 M.

[b] Glucose, 0.5%.

[c] Determined graphically from a semilogarithmic plot of AOD versus time.

[d] ND, Not done.

Effect of sodium phosphate concentration on T_D and cell yield of group B streptococcus D136C type III. The T_D, maximum turbidity, and final pH after 24 h of incubation of strain D136C in FMC containing 1% glucose (see Table 4) were examined at phosphate concentrations ranging from 0.015 to 0.240 M (Table 3). The final AOD, or cultural turbidity, increased to a maximum at a phosphate concentration of 0.06 M, with little change when the phosphate concentration was doubled to 0.12 M. However, there was a decline in culture turbidity above 0.12 M, possibly indicating a toxic effect of sodium phosphate. The final pH of the cultures increased with phosphate concentration, indicating the expected buffering capacity of phosphate.

The T_D values in varying phosphate concentrations increased only slightly, from 27 min at 0.015 M phosphate to 33 min at 0.06 M. Above 0.06 M phosphate, the T_D values increased significantly, indicating a major effect on cell growth. Thus, the optimum phosphate concentration producing maximum cell yield with minimum disturbance in T_D appeared to be 0.06 M, and this concentration was used in all subsequent experiments.

Effect of glucose concentration on cell yield of group B streptococcus D136C type III. Maximum culture turbidity occurred at a 1% glucose concentration in FMC containing 0.06 M phosphate (Table 4). Glucose concentra-

TABLE 3. *Effect of sodium phosphate concentration on final AOD, pH, and T_D[a] of group B streptococcus strain D136C type III grown in FMC containing 1% glucose*

Sodium phosphate concn (M)	Maximum tubidity[b] (AOD)	Final pH[b]	T_D (min)
0.015	1,205	4.13	27
0.030	1,255	4.15	29
0.045	1,500	4.30	32
0.060	1,725	4.37	33
0.120	1,670	4.89	45
0.180	1,150	5.75	>50
0.240	710	6.16	>50

[a] Determined graphically from a semilogarithmic plot of AOD versus time.

[b] Values recorded after 24 h of growth at 37°C aerobically as described in the text.

TABLE 4. *Effect of increasing glucose concentration on the cell yield of group B streptococcus strain D136C type III in FMC containing 0.060 M phosphate*

Glucose (%)	Maximum turbidity[a] (AOD)	Final pH[a]
0.5	1,170	6.02
0.6	1,270	5.68
0.7	1,470	5.15
0.8	1,840	4.74
0.9	1,750	4.54
1.0	1,950	4.39
1.5	1,780	4.29
2.0	1,730	4.29

[a] Values recorded after 24 h of growth at 37°C aerobically as described in the text.

tions below 1% were growth limiting, as evidenced by increasing culture turbidity with increasing glucose concentrations to 1%. Above 1% glucose, the turbidity values at 24 h decreased slightly, although it is not clear what caused the reduction. Thus, maximum cell yields (approximately 0.85 mg of cell dry weight per ml) of group B streptococci can be obtained in FMC containing 1% glucose and 0.06 M sodium phosphate. This yield was about four to six times the maximum cell dry weight obtained in unmodified Todd-Hewitt broth (Table 2).

Amino acid requirements of group B streptococci determined under aerobic and anaerobic growth conditions. The amino acid requirements of the 16 strains of group B streptococci listed in Table 1 are summarized in Tables 5 and 6. With the exception of glycine, serine, and threonine (Table 6), the 16 strains of group B streptococci were very homogeneous in their requirements for the 17 other amino acids under both aerobic and anaerobic growth conditions (Table 5). The growth ranges observed for the 16 strains, especially after subculture, tended to be narrow for some amino acids (tyrosine, tryptophan, glutamate, arginine, leucine, and lysine) and somewhat wider for others (phenylalanine, valine, methionine, isoleucine, cystine, and histidine). In the latter case, this may reflect quantitative differences in pool sizes of these amino acids in the various strains. Amino acids that were found to be nonessential (glutamine, hydroxyproline, proline, alanine, and aspartate) always had turbidity values greater than 40% of control cultures, even after three or four subcultures in amino acid-deficient media.

In contrast to the consistent requirements by all 16 strains for 12 of the amino acids presented in Table 5, the requirements for glycine, serine, and threonine were somewhat more strain dependent. In addition, the oxidation-reduction potential influenced the amino acid requirements. For example, threonine was required by all strains growing aerobically, but only 9 strains (56%) required threonine under anaerobic conditions. Of the 16 strains of group B streptococci, only 3 type III clinical isolates (108, 110, and 111) required serine aerobically, however, there was no requirement for serine under anaerobic conditions. The requirement for glycine also varied from strain to strain, with some strains (H36B, A909, and D136C) exhibiting a requirement under both aerobic and anaerobic conditions, whereas other strains required glycine either aerobically (100, 108, and 112) or anaerobically (110).

DISCUSSION

A chemically defined medium (FMC) originally formulated for oral streptococci (10) was used in these studies and supported rapid, luxuriant, and transferable growth of 16 strains of group B streptococci representing the five known serotypes. The T_D values of group B streptococci in either THGB or FMC were comparable, although final cell yields were increased severalfold in unmodified FMC. Maximum growth yields were obtained in FMC containing 0.06 M phosphate and 1% glucose. To define further the minimum nutritional requirements of the group B streptococci, the amino acids essential for growth were determined in both laboratory-passaged strains and fresh clinical isolates. In most instances, the group B streptococci were homogeneous in their amino acid requirements. Thirteen amino acids were required under aerobic conditions by all 16 strains examined. These results were identical to those obtained by Willett et al. (13) for one bovine strain of *S. agalactiae*. In addition to the requirement for phenylalanine, tryptophan, glu-

TABLE 5. *Amino acid requirements of 16 strains of group B streptococci*

Amino acid	Aerobic conditions			Anaerobic conditions		
	Required[a]	Percentage of control growth[b]		Required[a]	Percentage of control growth[b]	
		24-h culture	24-h subculture		24-h culture	24-h subculture
Phenylalanine	+	8–24	5–33	+	6–33	3–26
Tyrosine	+	3–9	ND[c]	+	2–7	ND
Tryptophan	+	7–57	3–12	+	8–62	1–8
Glutamate	+	7–18	4–10	+	8–19	4–9
Glutamine	−	47–116	46–124	−	59–106	56–120
Proline	−	85–123	66–132	−	39–103	63–114
Hydroxyproline	−	85–121	70–113	−	73–126	86–128
Arginine	+	4–22	1–13	+	3–25	1–9
Alanine	−	68–117	72–119	−	75–142	57–123
Valine	+	3–16	2–23	+	2–15	2–27
Leucine	+	3–10	2–5	+	2–15	2–4
Aspartate	−	86–125	84–129	−	88–111	73–126
Lysine	+	2–14	1–4	+	1–5	1–3
Methionine	+	4–90	2–27	+	3–20	1–34
Isoleucine	+	3–113	5–40	+	2–25	5–37
Cystine	+	33–111	2–34	+	22–115	2–32
Histidine	+	6–27	2–24	+	4–49	2–8

[a] +, In the absence of the amino acid, the 16 strains grew to 40% or less of control growth either on initial culture or on subculture; −, growth was greater than 40% of the control cultures. Note that the ranges given are for 16 strains.

[b] Control values for the 16 strains ranged from 1,500 to 2,100 AOD units when grown in complete FMC.

[c] ND, Not done; 48-h incubation of the primary culture did not result in a turbidity increase above the 24-h level.

TABLE 6. *Strain-dependent requirements for glycine, serine, and threonine by 16 strains of group B streptococci*

Amino acid	Aerobic conditions			Anaerobic conditions		
	Percentage of control growth[a]		Strains requiring amino acid (%)[b]	Percentage of control growth[a]		Strains requiring amino acid (%)[b]
	24-h culture	24-h subculture		24-h culture	24-h subculture	
Glycine	15–70	2–95	38[c]	11–93	5–117	25[d]
Serine	7–114	6–115	19[e]	61–131	69–148	0
Threonine	4–60	1–11	100	3–65	3–145	56[f]

[a] Control values for the 16 strains ranged from 1,500 to 2,100 AOD units when grown in complete FMC.

[b] Determined after 24-h subculture.

[c] Strains 100, 090, H36B, A909, 108, and 112.

[d] Strains H36B, A909, D136C, and 110.

[e] Strains 108, 110, and 111.

[f] Strains 101, 102, A909, D136C, 108, 110, 111, 112, and 114.

tamate, arginine, valine, isoleucine, leucine, lysine, and histidine, Niven (8) reported that cystine, methionine, threonine, and tyrosine were stimulatory but not required for the growth of two strains of group B streptococci. In the present study, tyrosine was shown to be a requirement on primary culture, whereas cystine, threonine, and methionine were not required by all strains on primary culture but were required on subculture. Thus, in three separate studies, the amino acid requirements of the group B streptococci under aerobic conditions were remarkably similar for 13 amino acids.

Anaerobic growth conditions eliminated the requirement for threonine in seven strains, but had no effect on the requirements for the other amino acids listed in Table 5. Three type III clinical isolates that required serine aerobically had no requirement for serine under anaerobic growth conditions (Table 6). These results are difficult to interpret, since aerobic environments are generally associated with the operation of the tricarboxylic acid cycle and the resulting synthesis of C_4 and C_5 precursors for a number of amino acids. Terleckyj and Shockman (9), however, have reported similar observations in various oral streptococci in which anaerobic incubation reduced the amino acid requirements

in some cases to only one amino acid, cystine.

In addition to an increased buffering capacity, FMC also allowed rapid doubling of all strains of group B streptococci. In view of the fulminant nature of group B streptococcal infections, this medium may afford an opportunity to study the in vitro elaboration of potential virulence factors under conditions of rapid cell proliferation similar to those expected in group B infections. Moreover, FMC may greatly facilitate the isolation and purification of any such virulence factors. In this regard, Milligan et al. (7) demonstrated that rapidly dividing cells of group B streptococci growing in FMC supplemented with human or bovine serum albumin elaborated an extracellular neuraminidase during the exponential phase of growth, but destruction of the enzyme by extracellular protease occurred within 1 h after the onset of the stationary phase. These results indicate that several possible virulence factors elaborated by the group B streptococci that would be difficult to isolate and purify from complex media can be readily examined in FMC and correlated with the physiological status of the organism.

Since the elaboration of antigenic cell surface components is also well known to be a reflection of the nutritional and physiological status of the bacterial cell, this medium will also provide the opportunity to modify the environment so as to allow maximum expression of protection-inducing antigens. Baker and Kasper (3) reported that they could double the yield of type III capsular polysaccharide by increasing the phosphate and glucose concentrations. Although quantitation of cellular yield was not reported, they did observe an average of a two-log increase in the number of viable organisms after 12 h of growth in the buffered medium. Preliminary experiments in our laboratory, using FMC (described above), indicate that the yield of sialic acid-containing, cell-associated polysaccharide (with type III specificity) can be increased seven- to eightfold (unpublished data) over that reported by Baker and Kasper. Whether this yield is due to more ideal physiological conditions for antigen production provided by FMC or, perhaps, due to an increase in the cellular yield is currently under investigation in our laboratory.

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