NUTRITION OF *VIBRIO FETUS*

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ABSTRACT

SMIBERT, ROBERT M. (Virginia Polytechnic Institute, Blacksburg, Va.). Nutrition of *Vibrio fetus*. J. Bacteriol. **85**:394–398. 1963.—A chemically defined medium has been developed for the cultivation of both catalase-positive H_2S-negative and catalase-positive H_2S-positive strains of *Vibrio fetus*. A total of 87 strains of *V. fetus*, representing bovine, ovine, porcine, human, and chicken isolates, were grown in both semisolid and liquid defined media. The medium was also used to isolate *V. fetus* from the stomach contents of aborted bovine fetuses. The strains investigated did not require purines or pyrimidines. They required nicotinic acid, and were stimulated by other B vitamins. There was considerable variation in the amino acid requirements of *V. fetus*. Some strains required only one amino acid, and others required many more. The final complete medium contained 18 amino acids and supported growth of all 87 strains. There was no significant correlation between amino acid requirements and source of isolation or biochemical characteristics of *V. fetus*.

Vibrio fetus is a gram-negative, motile, microaerophilic, curved rod which has been associated with abortion and infertility in cattle and sheep. The organism has also been associated with infection in man (King, 1957). *V. fetus* was first described by McFadyean and Stockman (1913) as being associated with abortion in sheep. Smith and Taylor (1919), after extensive work, established the organism as a cause of infectious abortion in cattle and named it *V. fetus*.

Alexander (1957) studied the energy sources of two ovine strains of *V. fetus* by the addition of various substrates to a peptone medium, which was diluted so that it permitted only minimal growth. He found that lactate, pyruvate, acetate, α-ketoglutarate, succinate, fumarate, malate, aspartate, asparagine, glutamate, and proline all served as energy sources. Serine was metabolized by one of the strains. Kuzdas and Morse (1956) reported that nicotinic acid, thiamine, calcium pantothenate, pyridoxal-HCl, and biotin were needed for growth of *V. fetus*. Leece (1958), using the Thunberg technique, studied the oxidative activity of 27 strains of *V. fetus* on 30 substrates. He reported that lactate, formate, pyruvate, α-ketoglutarate, and succinate were used as electron donors. Glutamate was only slightly active with 4 of 27 strains of *V. fetus*. Kiggins and Plastridge (1958), using the Warburg technique, studied the activity of two strains of *V. fetus* on various substrates. Carbohydrates and glycolytic intermediates were not oxidized, and pyruvate and all tricarboxylic cycle intermediates were oxidized by either resting cells or cell-free extracts of *V. fetus*. Acetate was the only fatty acid oxidized. Glutamate, aspartate, asparagine, proline, and cysteine were the only amino acids oxidized. Zemjanis and Hoyt (1960), by adding various substrates to a peptone semisolid medium, observed that cysteine, lactate, α-ketoglutarate, glutamate, glutamine, uracil, thymine, *p*-aminobenzoic acid, and 17-β-estradiol enhanced growth of *V. fetus*. They also stated that the addition of magnesium, manganese, and iron stimulated growth of 22 strains of *V. fetus*.

Batlin and Wilson (1950) reported the cultivation of *V. fetus* in a semisolid medium composed of 16 amino acids, salts, growth factors, and 1% Methocel as a gelling agent.

Hoff (1956) also reported the growth of two ovine strains of *V. fetus* in a semisolid medium containing amino acids, purines, pyrimidines, vitamins, salts, and agar.

The purpose of this work was to study the nutritional requirements of selected strains of *V. fetus*, and to develop a chemically defined liquid medium which would support growth of a large number of strains.

MATERIALS AND METHODS

A total of 87 strains of *V. fetus*, representing bovine, ovine, human, porcine, and chicken iso-

lates, were employed in this study. They were obtained from Virginia, Kentucky, Maryland, Montana, Agricultural Research Service (U.S. Department of Agriculture), the American Type Culture Collection, and the Communicable Disease Center. The strains of *V. fetus* represented both catalase-positive H_2S-negative and catalase-positive H_2S-positive varieties. They have been named *V. fetus* var. *venerialis* and *V. fetus* var. *intestinalis*, respectively, by Florent (1959). Some were old laboratory strains, and others were recent isolates that had been lyophilized after isolation.

All stock cultures were grown in Brucella broth (Albimi) containing 0.15% agar and were incubated at 37 C. Cells used to inoculate experimental media were grown in Brucella broth in an atmosphere containing 85% N_2, 10% CO_2, and 5% O_2 (Kiggins and Plastridge, 1956). After 72 hr of incubation of the broth cultures, the cells were centrifuged, washed once in sterile 0.15 M phosphate buffer (pH 7.0), and the cell suspension was adjusted to an optical density of 0.30 at 420 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer. Cell suspensions of *V. fetus* (0.1 ml) were inoculated into 8 ml of the experimental media contained in 16-mm screw-cap culture tubes. Liquid cultures were grown in the gaseous atmosphere described above, and cultures in semisolid media were grown aerobically. All cultures were incubated at 37 C. Growth of vibrios in broth cultures was determined turbidimetrically at 420 mμ in a Spectronic 20 spectrophotometer. Semisolid experimental media containing 0.15% agar were inoculated with cell suspensions of *V. fetus* (0.1 ml) and growth was compared visibly with that obtained in Brucella semisolid medium. Cultures grown in experimental media were subcultured in the same medium for at least three passages to eliminate any carry-over of nutrients. Cultures were examined with a phase-contrast microscope to check cellular morphology and purity.

Amino acids, purines, pyrimidines, and vitamins were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. Amino acids were tested for purity by paper chromatography, using *n*-butanol-acetic acid-water (12:3:5) as the developing solvent (Smith, 1960). Ammonia was determined by the microdiffusion technique (Conway, 1950), and estimated colorimetrically by nesslerization.

RESULTS

A medium containing Trypticase (BBL) and yeast extract (BBL) or yeast extract alone supported the growth of *V. fetus*, although Trypticase alone did not yield as heavy a growth. Vitamin-free acid hydrolysate of casein (Nutritional Biochemicals Corp., Cleveland, Ohio), supplemented with tryptophan and cystine or cysteine, did not support the growth of *V. fetus*. The addition of B vitamins, however, did permit growth of a few strains. Most strains of *V. fetus* would not grow in a medium containing 21 amino acids, purines, pyrimidines, vitamins, minerals, acetate, and citrate. A few strains, however, did produce poor and irregular growth in this medium.

A liquid or semisolid medium containing 18 amino acids, B vitamins, and minerals supported the growth of all 87 strains of *V. fetus* (Table 1). The chemically defined semisolid medium was used successfully to isolate *V. fetus* from the stomach contents of four aborted bovine fetuses. Growth of most strains in the chemically defined medium compared favorably with growth in Brucella broth. However, the growth of different strains in either medium was not always comparable. Growth of strain Kohler, grown in either Brucella broth or the defined medium, reached an average optical density of 1.8, and the optical density of strain UM was only 0.06. Some strains, however, grew better in semisolid than in liquid media. After 5 days of growth, the average total number of cells per ml of the defined medium (Petroff-Hausser counting chamber) for strain Kohler was 1.9×10^9; for strain 277, 1.1×10^9; for 085, 10^9; and for strain 3530, 5×10^8. The maximal turbidity was usually reached by the first three of the above strains after 72 hr of growth. A few strains, (e.g., 3530 and 86565) had a long lag time (24 to 36 hr) when grown in the defined liquid medium. Spermine, cadavarine, putrescine, betaine, and reducing agents such as thioglycolate, thiosulfate, cystine, and ascorbic acid neither reduced the lag time nor enhanced the growth of slower growing strains in the liquid defined medium. The average total number of cells per ml of Brucella broth for strain Kohler was 8×10^9; for strain 277, 5×10^9; and for 085, 4.7×10^9.

The optimal pH for growth of *V. fetus* in the chemically defined liquid medium was between 6.8 and 7.2. After 5 days of growth, the pH of the medium remained between 7.1 and 7.3.

TABLE 1. *Composition of chemically defined medium for Vibrio fetus*

Amino acids	Amount	Salts	Amount	Vitamins	Amount
	mg		*mg*		*μg*
L-Glutamic acid	200	$(NH_4)_2SO_4$	300	Nicotinic acid	1 mg
L-Proline	120	K_2HPO_4	400	Pyridoxal-HCl	200
L-Aspartic acid	70	NaCl	5	Pyridoxine-HCl	200
L-Leucine	70	Salt solution*	3 ml	Pyridoxamine·2HCl	100
L-Methionine	50	Sodium thiosulfate	80	*p*-Aminobenzoic acid	400
L-Arginine	40	Sodium carbonate	40	Biotin	10
DL-α-Alanine	30			Folic acid	100
DL-Serine	30			Calcium pantothenate	500
L-Lysine-HCl	20			Thiamine-HCl	400
Hydroxy-L-proline	20			Riboflavine	100
DL-Valine	10			B_{12}	10
Glycine	30			*i*-Inositol	200
L-Threonine	30			Choline chloride	400
L-Tryptophan	30			Ascorbic acid	500
L-Phenylalanine	20			Water (double-distilled)	100 ml
L-Tyrosine	30				
DL-Isoleucine	50				
L-Cystine	40				

* Salt solution: 800 mg of $MgSO_4$ and 40 mg of $FeSO_4 \cdot 7H_2O$ in 100 ml of distilled water, pH adjusted to 7.0 to 7.1, medium filtered and sterilized by autoclaving. Semisolid medium contained 0.15% agar.

Sodium carbonate, although not required, slightly enhanced the growth of a few strains, and sodium bicarbonate did not affect growth. Sodium thiosulfate enhanced the growth of a few strains and also served as a sulfur source for some vibrios as well as a substrate for H_2S production by some catalase-positive H_2S-positive strains of *V. fetus* Sodium chloride was not required for growth. Only the additions of magnesium and ferrous salts were needed to support growth of *V. fetus*. The addition of other inorganic salts ($MnSO_4$, $CaCl_2$, $ZnSO_4$, and $CuSO_4$) did not seem to affect growth. Ammonium sulfate or chloride was not needed in the medium to support growth of *V. fetus*. Ammonia could be detected after 2 to 3 days of incubation in the chemically defined broth which did not contain $(NH_4)_2 SO_4$.

Nicotinic acid (or nicotinic acid amide) was the only vitamin required by most strains of *V. fetus*. The addition of the other B vitamins to the medium, although not required, yielded larger cell crops, a faster growth rate, and more reliable growth of most of the strains. Purines or pyrimidines neither enhanced growth of *V. fetus* nor decreased the lag time of slower growing strains.

There was only one observable difference between colonies of *V. fetus* grown on Brucella agar and colonies grown on the chemically defined agar medium. Colonies of some strains grown on the defined agar were smaller than colonies on Brucella agar. Cells of *V. fetus* grown in Brucella broth or semisolid medium were similar to cells grown in the chemically defined broth or semisolid medium when observed under a phase-contrast microscope.

The amino acid requirements of *V. fetus* were quite heterogeneous. Growth of selected strains of *V. fetus* in media containing various amino acid mixtures was investigated (Table 2). Two strains (Kohler and 102) required only glutamate, which could be partially replaced by proline. Aspartate and leucine were stimulatory. Another strain (1430) required glutamate, proline, leucine, aspartate, and methionine. Alanine was stimulatory. Strain 12 and 86565 required glutamate, proline, aspartate, leucine, methionine, arginine, and alanine. However, alanine could be partially replaced by serine or lysine. *V. fetus* strain 86565 grew sporadically in this medium, even on repeated subculture, but gave consistent growth in the complete medium.

Other strains (UM, B-24, B-22, 105, ATCC 11311, 78, 21085, and 12351) required glutamate, proline, aspartate, leucine, methionine, arginine, alanine, tryptophan, and tyrosine, and the other amino acids were highly stimulatory. In addition,

TABLE 2. *Growth response of selected strains of Vibrio fetus to media containing various amino acid mixtures**

Amino acid mixtures	Strain				
	Kohler	102	12	1430	86565
Control†	1.9	1.5	1.5	1.0	0.98
Glutamic acid	1.2	1.0	0.50	0.05	0.04
Proline	1.1	0.66	0.30	0.07	0.26
Glutamic, proline	1.4	1.2	0.05	0.06	0.17
Glutamic, proline, leucine	1.0	1.0	0.12	0.08	0.39
Glutamic, proline, aspartic	1.6	0.98	0.01	0.06	0.18
Glutamic, proline, aspartic, leucine	1.6	1.6	0.04	0.10	0.31
Glutamic, proline, methionine	1.0	0.60	0.02	0.04	0.22
Glutamic, proline, aspartic, methionine, arginine	1.0	1.0	0.64	0.66	0.41
Glutamic, proline, leucine, aspartic, methionine	1.8	1.1	0.48	0.92	0.45
Glutamic, proline, leucine, aspartic, methionine, alanine	1.7	1.6	0.70	1.2	0.59
Glutamic, proline, leucine, aspartic, methionine, arginine, alanine	1.7	1.3	1.5	1.2	1.0
Glutamic, proline, leucine, aspartic, methionine, arginine, lysine	1.7	1.5	1.3	1.1	0.50
Glutamic, proline, leucine, aspartic, arginine, alanine, lysine	1.7	1.3	0.27	0.31	0.63
Albimi broth‡	0.69	0.74	0.60	0.09	0.21

* Results expressed as optical density at 420 mμ. All media contained vitamins and salts listed in Table 1.

† Medium in Table 1.

‡ Albimi broth cultures diluted 1:6 with sterile 0.85% salt solution and optical density read in a Spectronic 20 spectrophotometer.

TABLE 3. *Amino acid composition of media which supported growth of 87 strains of Vibrio fetus**

Group A (26/87)†	Group B (9/87)	Group C (15/87)	Group D (34/87)	Group E (3/87)
L-Glutamic acid L-Proline L-Aspartic acid L-Leucine	L-Glutamic acid L-Proline L-Aspartic acid L-Leucine L-Methionine	L-Glutamic acid L-Proline L-Aspartic acid L-Leucine L-Methionine L-Arginine DL-α-Alanine	Amino acids listed in Table 1 minus L-lysine-HCl, L-phenylalanine, and DL-isoleucine	Amino acids listed in Table 1

* All media contained salts and vitamins listed in Table 1.

† Number of strains that required the amino acids in each group over total number of strains tested.

strains 21085, ATCC 11311, and 12351 required isoleucine. The additional amino acids in the medium listed in Table 1 were greatly stimulatory, yielding higher cell crops and more regular growth. Histidine was neither required nor stimulatory for any of the strains tested.

Media containing the amino acids listed in Table 3 were tested for their ability to support growth of *V. fetus*. Of 87 strains, 26 grew in medium A, 9 grew in medium B but not in A, 15 grew in medium C, but not in A or B, and 34 grew in medium D, but not in A, B, or C. Of, 87 strains, 3 grew in medium E, but not in A, B, C, or D. All 87 strains, however, grew in medium E. Vibrios grown in medium A used thiosulfate as the source of sulfur, and strains growing in the other media had both organic and inorganic sources of sulfur.

Catalase-positive H_2S-negative and catalase-positive H_2S-positive strains of *V. fetus*, and strains isolated from different sources (bovine, ovine, and human), could not be readily separated on the basis of their amino acid requirements.

DISCUSSION

The main purpose of this investigation has been accomplished, namely, the development of a chemically defined medium for *V. fetus*. The present data indicated that the requirements of different strains of *V. fetus* for amino acids were not similar, and that a chemically defined medium could be formulated. Thus, the difficulty involved in isolating and culturing *V. fetus* may be mainly in the atmospheric requirements of the organism and in the physicochemical conditions of the growth medium, but not in requirements for unidentified growth factors.

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