FORMULATION OF IMPROVED MEDIA FOR ISOLATION

AND CULTIVATION OF *CAMPYLOBACTER FETUS*

by

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APPROVED:

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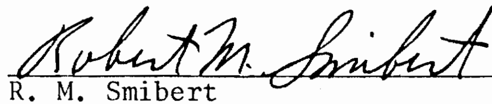

R. M. Smibert T. D. Wilkins

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INTRODUCTION

Campylobacter fetus, a small, microaerophilic, Gram negative rod, is a well known cause of contagious abortion in cattle and sheep. In recent years, it is evident that the organism can also cause human disease, usually affecting individuals who have been debilitated in some manner, such as cancer patients, alcoholics, and elderly persons.

It is generally held that the incidence of human infection is higher than the number of confirmed cases would indicate. Being a microaerophile, normal levels of atmospheric oxygen are toxic to *C. fetus*. To successfully culture the organism, oxygen tensions must be adjusted to microaerobic conditions. In a clinical setting, necessary equipment to establish a microaerobic environment, such as special gas mixtures, are not routinely used. Thus, the extent to which human infection occurs is difficult to assess because the microaerophilic nature of the organism imposes restrictions upon the methods used for its optimum recovery.

Work begun by Bowdre *et al.* (3) indicated that it is possible to enhance the aerotolerance of another microaerophile, *Spirillum volutans*, by the addition of dihydroxypehnyl compounds to its culture medium. Further study showed that these same compounds, as well as high levels of iron salts, could allow three strains of *C. fetus* subsp. *jejuni* to grow at higher oxygen tensions. Clearly, the formulation of a medium which would enhance the growth and aerotolerance of many strains of

C. fetus would be a valuable tool for the recovery of the organism in any clinical situation.

The purpose of the present investigation was, therefore, to (i) examine the growth response of *C. fetus* to other stimulatory compounds, (ii) find the optimum levels for these compounds for three reference strains (one from each subspecies), (iii) evaluate the growth response of many strains of *C. fetus* to the new formulation, and (iv) gather any preliminary evidence as to the mechanisms of exygen toxicity in microaerophilic organisms.

LITERATURE REVIEW

This literature review is divided into the following sections: (i) taxonomy of the genus *Campylobacter*, (ii) pathogenicity of *C. fetus*, (iii) general physiology and culture techniques, and (iv) aerotolerance and oxygen toxicity.

Taxonomy of the genus Campylobacter

Members of the genus *Campylobacter* Sebald and Veron 1963 are small, Gram negative, helically-curved rods which are motile by means of a single polar flagellum at one or both ends of the cell. The genus has a mol% guanine + cytosine (G+C) content of 30-35. They possess a strictly respiratory metabolism and are microaerophilic to anaerobic, differentiating them from the genus *Vibrio* whose members are facultative anaerobes with a fermentative metabolism. The current classification by Smibert (57) indicates three species within *Campylobacter*: *C. fetus* (the type species), *C. sputorum*, and *C. fecalis*. *C. fetus* is further subdivided into three subspecies on the basis of physiological characters and host specificity (see Table 1).

Pathogenicity of C. fetus

C. fetus subsp. *fetus* is a well known cause of contagious abortion and infertility in cattle. The mode of transmission is venereal and the organism can be recovered from the preputial cavity and semen of bulls and the lumen of the vagina, cervix, and uterus of infected cows.

Table 1. Differential characteristics of species of the genus *Campylobacter* (57).

Species	Catalase	Nitrite reduction	H_2S on TSI	H_2S lead acetate strips	1% glycine	3.5% NaCl	25 C
C. fetus subsp. *fetus*	+[a]	-	-	-	-	-	+
C. fetus subsp. *intestinalis*	+	-	-	+	+	-	+
C. fetus subsp. *jejuni*	+	-	-	+	+	-	-
C. sputorum subsp. *sputorum*	-	+	+	+	+	-	+
C. sputorum subsp. *bubulus*	-	+	+	+	+	+	d
C. fecalis	+	?	+	+	+	d	-

[a]+ = most (90% or more) strains positive for this characteristic; - = most (90% or more) strains negative; d = some (less than 90%) strains positive, some negative; ? = reaction not known.

This subspecies will not multiply in the gastrointestinal tract of man or animals.

C. fetus subsp. *intestinalis* is one cause of abortion in sheep and sporadic abortion in cattle. In ovine vibriosis, the mode of transmission is oral and the organism invades the bloodstream, finally residing in the reproductive tract (24, 25, 38). Unlike subspecies *fetus*, this organism will grow in the gastorintestinal tract of man and animals, and it can be isolated from placentas and stomach contents of aborted fetuses from cattle and sheep.

Subspecies *jejuni* can also cause abortion in sheep; however, it can be recovered from the gastrointestinal tract of clinically normal swine, cattle, sheep, goats, chickens, turkeys, and wild birds. The mode of transmission is also believed to be oral.

As this classification suggests, isolates of *C. fetus* from animal sources have long been known to differ in biologic activity. Experimental infections have confirmed that isolates exhibit characteristic disease syndromes in different hosts. For example, bovine venereal strains (subspecies *fetus*) cannot establish infection in the digestive tract of cattle, sheep, rabbits, guinea pigs, or mice; however, strains of subspecies *intestinalis* can consistently infect the gall bladder and intestines of many test animals (6). In addition, the intravenous inoculation of bovine or ovine strains (subsp. *fetus* and subsp. *intestinalis*) will not readily cause a bacteremia in cattle, but in sheep this may occur even by the oral route (6). Strains of subspecies *intestinalis* will cause abortion in

pregnant cows (49), but only a transient infection in young heifers (63). Thus, the physiological parameters now used to distinguish the three subspecies of C. fetus in vitro also reflect differences in biologic activity as well.

Of recent research interest, attention has been drawn to the isolation of an antiphagocytic component of the outer envelope of virulent strains of C. fetus (36). Mutants lacking this component are immobilized, i.e., exhibit a loss of motility, by the addition of antiserum to the flagellar hooks and filaments, reactions unlike that of the parent strain (35). Furthermore, several bovine venereal isolates have been shown to undergo antigenic variation, and this is considered to be a possible mechanism for the maintenance of the asymptomatic cervicovaginal carrier state (9).

As in other Gram negative organisms, C. fetus has been shown to produce an endotoxin. Osborne and Smibert (48) demonstrated that the C. fetus toxin is abortifacient in bovine, ovine, porcine, and caprine species, a reaction which appeared to be of an allergic nature. Anaphylactoid hypersensitive reactions were also demonstrated in many species. The histopathology of C. fetus endotoxic shock shares many characteristics with endotoxins from other sources, such as edema, disseminated intravascular coagulation, stasis, and embolism (47). Highly purified lipopolysaccharide fractions obtained after phenol extraction have been shown to induce a biphasic febrile response in rabbits (45), as well as a hypothermic reaction in mice (11).

In addition to the well-documented role of C. fetus in bovine and ovine disease, the organism is now recognized as an opportunistic

human pathogen. Two subspecies, intestinalis and jejuni, have been implicated, usually affecting individuals with some predisposing condition, such as alcoholics, diabetics, cancer patients, infants, pregnant women, and elderly persons, especially males (R. M. Smibert, personal communication; 1, 60, 64). Human infection has been associated with such varied disorders as enteritis (10), abortion (22), peritonitis (60), endocarditis (33), septicemia with meningitis (8), phlebitis (62), localized abcess (32), and salpingitis (5).

It is generally held that the incidence of human infection is higher than the number of confirmed cases would indicate. Although the epidemiology of campylobacteriosis is obscure, it is assumed that most human cases are acquired by ingestion or handling of contaminated food or water. For example, M. V. Smith has obtained three isolations of C. fetus subsp. jejuni from examination of 165 poultry meat samples purchased from local stores in Blacksburg, Virginia (58). Wild bank voles have been suggested as a possible natural resevoir for campylobacters in the United Kingdom (12).

With regard to human infection with C. fetus, Krieg (31) has noted the apparent similarities between this organism and Spirillum minus, one of the causitive agents of rat-bite fever in humans. Both have a characteristic corkscrew-like motility and usually possess single flagella at one or both poles. One report of spirillum endocarditis indicates that the organism recovered was oxidase positive, catalase positive, and failed to ferment carbohydrates (37).

The difficulty in growing S. minus in vitro might be accounted for by assuming an unusual oxygen requirement as in the microaerophilic campylobacters.

General Physiology and Culture Techniques

C. fetus possesses a strictly respiratory metabolism using oxygen (or nitrate under anaerobic conditions) as a terminal electron acceptor. Amino acids such as glutamate and aspartate, or tricarboxylic acid cycle intermediates, can be used as carbon and energy sources (29, 54). Carbohydrates are neither oxidized or fermented, and no detectable oxygen uptake occurs for several glycolytic intermediates with whole cells or cell-free extracts (29).

Many complex media can satisfy the basic growth requirements of C. fetus. Nutrient agar with blood (28, "homemade" yeast extract-polypeptone medium with thioglycollate (40), thiol agar (13), and Brucella medium (2) have been satisfactory to differing degrees. Simon (53) has recommended double strength cystine heart agar with a thioglycollate broth (no agar) overlay for production of large numbers of cells for use in serological studies. Catalase (30) and hematin (2) have been reported to be stimulatory for growth when added to a complex media base. A defined medium for C. fetus has been formulated by Smibert (54) consisting of 18 amino acids, B vitamins, and minerals. Selective media, often containing bacitracin, novobiocin, and polymyxin, have been used for isolation of C. fetus from bull preputial fluid (52), bull semen (50), animal feces (55, 56), and commercially processed poultry (58).

C. fetus appears to have a wide range of pH tolerance. Cultures started at an initial pH of 6.6 may reach pH 8.0 after stationary phase (40). The optimum pH for growth depends upon the medium and culture techniques used. For example, in a survey of the growth characteristics of 10 bovine strains, Miyamae (40) demonstrated that two strains had an optimum pH in broth of 6.6; however, with the addition of 0.1% thioglycollate, two optima (pH 6.4-6.6 and at 7.0-7.4) were observed in semisolid medium. Smibert's defined medium is adjusted to a pH value of 6.8-7.2, and most media used are adjusted to pH near 7.0.

The microaerophilic nature of C. fetus has undoubtedly complicated its recovery from animal and human sources. For growth on agar plates, optimum concentrations for oxygen and carbon dioxide have been found to be 5% and 10%, respectively (28). Semisolid media (ca. 0.16% agar) is highly desirable for maintenance of stock cultures. Under these conditions, oxygen diffusion is retarded and the organism can find its own tolerable environment within the oxygen gradient established. The Anaerobe Laboratory Manual of the Virginia Polytechnic Institute and State University Anaerobe Laboratory (21) recommends the use of Brucella medium in semisolid form. It is interesting that in one of the first dexcriptions of the organism, attempts to recover C. fetus from cattle on beef-peptone agar slants were unsuccessful unless an overlay of broth was used (59). As summarized by Smibert (54), "the difficulty involved in isolating and culturing C. 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."

Aerotolerance and Oxygen Toxicity

While oxygen is highly desirable as a terminal electron acceptor due to its high oxidation-reduction potential, it is evident that oxygen can also exert toxic effects on living organisms. Mechanisms possibly responsible for oxygen toxicity include oxidation of critical sulfhydryl groups, elevation of cultural E_h, draining of the cell's reducing power due to the great oxidizing ability of molecular oxygen, inhibition of a key redox couple, or the production of toxic derivatives of oxygen such as the superoxide radical, the hydroxyl radical, singlet oxygen, and peroxides (41). Radical-mediated oxygen damage to biomolecules is well documented. It has been proposed that singlet oxygen can participate in the destruction of histidine residues, inactiviation of DNA, and lipid hydroperoxidations (14). The superoxide radical has been implicated in the degradation of tryptophane residues and lipid peroxidation (14). Hydroxyl radicals have been shown to react with nucleic acids and pentoses (43).

In addtion to their interactions with specific molecules, oxygen-related radicals have also been studied with regard to effects on whole cells. For example, the post-phagocytic killing of bacteria in polymorphonuclear leukocytes is believed to involve the preferential production of oxygen radicals by the enzyme, myleloperoxidase. As noted by Morris (41), the bactericidal events associated with the myeloperoxidase-H_2O_2-halide ion system has been variously attributed to

a direct action of the H_2O_2, iodination of the cell envelope, or the production of superoxide, singlet oxygen, or hydroxyl radicals. Similarly, the lethal effects of X-irradiation on bacterial cells is more pronounced under aerobic rather than anaerobic conditions. The ratio of the rate of killing under aerobic versus anaerobic conditions is said to be the oxygen enhancement ratio (OER). This oxygen-enhanced effect can be at least partially reversed with superoxide dismutase and catalase, as well as with other compounds believed to degrade radicals (39).

Survival and growth under aerobic conditions is thus viewed as a balance between the beneficial and detrimental effects of oxygen. Organisms which can tolerate atmospheric oxygen tensions are still susceptible to oxygen toxicity, since hyperbaric oxygen can be lethal for some aerobes (19). The inhibitory effect of oxygen for organisms having a respiratory type of metabolism is most easily demonstrated with obligate microaerophilic organisms, which cannot grow with the levels of oxygen present in air. In such cases, the mechanism of the oxygen toxicity has not been fully resolved; however, work begun in our laboratory with another microaerophile, the large fresh-water bacterium Spirillum volutans, has provided important clues (3). S. volutans is incapable of initiating growth from a small inoculum in a defined medium unless microaerobic conditions (ca. 6% oxygen) are employed. However, the addition of tyrosine prepared in autoclaved, alkaline stock solution would allow growth under aerobic conditions (static incubation in an air atmosphere). Unheated tyrosine stock solutions were ineffective. The growth-stimulating effect of autoclaved alkaline tyrosine had previously been noted by Gorini and Lord in work with an

aerobic coccus (16). In this study, a number of dihydroxyphenyl compounds such as nor-epinephrine at low concentrations (10^{-6}M) could substitute for the tyrosine. Bowdre et al. (3) found that nor-epinephrine, epinephrine, and 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA) would all allow aerobic growth of S. volutans from a small inoculum. With the addition of these compounds, tyrosine was no longer required.

Wishing to test the effectiveness of these compounds on another microaerophile, Bowdre et al. (3) obtained strain H840 of C. fetus subsp. jejuni from the collection at the Anaerobe Laboratory, Virginia Polytechnic Institute and State University. Using a Brucella agar base, streaked plates of H840 incubated at 6% oxygen grew well. However, with incubation at 21% oxygen, no growth occurred. The addition of 2×10^{-4}M nor-epinephrine was shown to allow good growth at 17% oxygen (approximating candle jar conditions) and moderate growth at 21% oxygen. Growth was seen to occur in areas of heaviest inoculation, while no colonies appeared on the areas of the plate that had received only a few cells.

A report by Hutner (23) suggested that the stimulatory effect of dihydroxyphenyl compounds found by Gorini and Lord was probably due to their ferric-iron binding properties. Bowdre et al. (3) showed that Brucella agar supplemented with 0.02-0.05% $FeSO_4 \cdot 7H_2O$ was indeed effective in enhancing the aerotolerance of strain H840.

Sodium bisulfite is a component of Brucella medium and is present at a level of 0.01%. Subsequent work by Hoffman (20 has indicated that the addition of 0.05% sodium bisulfite (giving a total of 0.06%)

greatly enhanced the aerotolerance of three strains of *C. fetus* subsp. *jejuni*. Hoffman tested a number of other compounds for their ability to stimulate growth at 19.5% O_2 in the presence of 0.005% $FeSO_4 \cdot 7H_2O$ (in itself not stimulatory). Effective compounds were: sodium phosphite, sodium pyruvate, sodium sulfite, sodium hydrosulfite, and sodium sulfide. Other sulfur compounds such as glutathione, cystine, thioglycollate, and sodium or potassium sulfate were ineffective. Therefore, it appeared that the stimulation seen with sodium bisulfite was not due to a requirement for sulfur. In addition, it was doubtful that it was acting as a reducing agent since thioglycollate and other sulfhydryl reagents, as well as ascorbic acid, were not effective. Of a number of tricarboxylic acid cycle intermediates, only pyruvic acid gave strong stimulation. Thus, an effective medium for enhancing aerotolerance of *C. fetus* strain H840 was found to be a *Brucella* agar base supplemented with $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid. The optimal concentration of these additives were found to be 0.025%, 0.050%, and 0.025%, respectively.

MATERIALS AND METHODS

Reagents and Media Preparation

The basic media used throughout this investigation were *Brucella* agar and broth (Pfizer Diagnostics, Clifton, NJ). The composition of this medium is given in Table 2. Sodium bisulfite and pyruvic acid were "Sigma Grade I" quality (Sigma Chemical Co., St. Louis, MO). $FeSO_4 \cdot 7H_2O$ was Fisher reagent grade quality (Fisher Scientific, Fair Lawn, NJ).

Bovine erythrocyte superoxide dismutase (10,500 U/mg protein) was obtained from the Research Division of Becton-Dickinson Co., Rockville, MD. Bovine liver catalase (13,000 U/mg protein) and bovine serum albumin were purchased from Sigma.

Crude enterochelin and ferrichrome preparations were kindly donated by J. B. Neilands.

All glassware used in media preparation was rinsed in 1N HCl followed by distilled water prior to use. $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid were added to the *Brucella* media prior to autoclaving. Enterochelin, ferrichrome, superoxide dimutase, catalase, and albumin were prepared as stock solutions in distilled water and sterilized by filtration before use. These stock solutions were added to sterile *Brucella* agar at 45 C. Heat-inactivated enzyme preparations were made by placing the stock solutions in a boiling water bath for 20 minutes.

Agar plates (20 ml/plate) and broth were prepared the day before use and stored overnight at room temperature in a closed, dark cabinet.

Table 2. Composition of Brucella agar.

Ingredient	Approximate grams/liter
Pancreatic digest of Casein	15.0
Enzymatic digest of Animal tissue	5.0
Yeast extract	2.0
Sodium chloride	5.0
Dextrose	1.0
Sodium citrate	1.0
Sodium bisulfite	0.1
Agar	15.0

Source of Strains and Maintenance of Stock Cultures

Stock cultures were maintained in semisolid *Brucella* medium supplemented with 0.02% $FeSO_4 \cdot 7H_2O$, pH 7.4. Cultures were transferred weekly. All cultures used in this investigation were incubated at 37 C.

A total of 64 strains of *C. fetus* from animal and human sources were obtained from the collection of R. M. Smibert at the Anaerobe Laboratory, Virginia Polytechnic Institute and State University. The origins of strains of *C. fetus* subsp. *intestinalis* is indicated in Table 3. The origins of strains of subsp. *jejuni* and subsp. *fetus* are indicated in Table 4.

Direct Microscopic Counts

To insure that turbidity in Klett units was a reliable measure of cell numbers in *Brucella* broth and *Brucella* broth supplemented with $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid (where the medium tends to become more amber in color), direct microscopic counts (DMC) were done using a Hausser Improved Neubauer Counting Chamber. Cells of strain H840 were grown in *Brucella* broth and also *Brucella* broth supplemented with $FeSO_4 \cdot 7H_2O$ (0.2%), sodium bisulfite (0.025%), and pyruvic acid (0.050%). 50 ml volumes of broth (per 250 ml flask) were incubated at 37 C with shaking under 6% O_2, 2.5% CO_2. Turbid cell suspensions were diluted to 55 Klett units (red filter, 16 mm cuvettes). The DMC for each broth was determined by counting the total number of bacteria for 25 squares of the counting grid. The average number of bacteria per square (1/20 millioneth ml) was then calculated. Serial diluation of the 55 Klett unit suspensions were made with sterile

Table 3. Sources of strains of *C. fetus* subsp. *intestinalis* used in this study.

Strain	Source	Strain	Source
2221	ovine (fetus)	11529	human (blood)
Nelson 1	human	11639-B-76	human (blood)
10951	human (blood)	Grant	ovine
18151-B-76	unknown	Langford	ovine
Keith 76-1435	bovine	DT-74	bovine
319-A-76	human (abdominal fluid)	702	bovine
10451	unknown	436	bovine (fetus)
10148	human (blood)	PB 1/35	human
Pacemaker	human (pacemaker fluid)	HCB	bovine
Pedro	human (abdominal cyst fluid)	B6286	human
11669	human (blood)	HF1	human (blood)
13161	ovine (fetus)	6339	ovine
1510 MB	human (blood)	PB 1/1	human
10296	unknown	4440	ovine (fetus)
11164	human (blood)	EMB 1545	bovine
PB 1/76	human	273	bovine (fetus)
11324-A-76	unknown	1925	human
8391-B-76	human (hip infection)	Barr	human
W5-184	unknown	10583	human (blood)

Table 4. Sources of strains of C. fetus subsp. jejuni and subsp. fetus used in this study.

Subsp. jejuni Strain	Source	Subsp. fetus Strain	Source
Wang III	feline (feces)	998	bovine (vaginal)
C14	chicken	21085	bovine
Otis P.	human	14701	bovine
8945-A-76	unknown	14093	bovine
6963	human	7721	bovine
8916	ovine (bile)	75-183	bovine
H641	human (feces)	13841	bovine
B7619	human (blood)	14664	bovine
H840	human (feces)		
29A	chicken		
13136	ovine (fetus)		
Smith	human (blood)		
4849	ovine		
H550	human (feces)		
11642-B-76	human (blood)		
H325	human (blood)		
Holy Cross	human (blood)		

broth and for each dilution, the turbidity measured. Cell numbers/ml were calculated on the basis of the DMC results of the 55 Klett unit suspensions. The relationship between turbidity and DMC/ml is indicated in Figure 1.

Determination of Optimum pH of Brucella Medium Supplemented with $FeSO_4$ $7H_2O$, Sodium Bisulfite, and Pyruvic Acid

Past experience in our laboratory has indicated (22) that the stimulation of growth seen in *Brucella* medium supplemented with these three additives was more pronounced at a slightly alkaline pH (7.2) than at a slightly acid pH (6.8). Therefore, the growth responses of three reference strains, H840, 1510 MB, and 998, were examined at various pH values. Inocula were grown in 50 ml of *Brucella* broth (pH 7.4) incubated under 6% O_2, 2.5% CO_2 at 37 C with agitation. After reaching a turbidity of 50-55 Klett units, a 1:10^5 dilution was made in *Brucella* broth and 1 ml (ca. 10^4 cells) was inoculated into 50 ml volumes of broth (per 250 ml flask) of differing pH values. These test broths contained 0.025% $FeSO_4 \cdot 7H_2O$, 0.050% sodium bisulfite, and 0.025% pyruvic acid, the levels previously found to be satisfactory for strain H840 (20). The inoculated media were then incubated as described above. The growth responses at different pH values were determined turbidimetrically after 26 hours of incubation.

Following this determination of the optimum pH for growth in a supplemented *Brucella* medium, the growth responses obtained with various modifications of this medium were evaluated as described below.

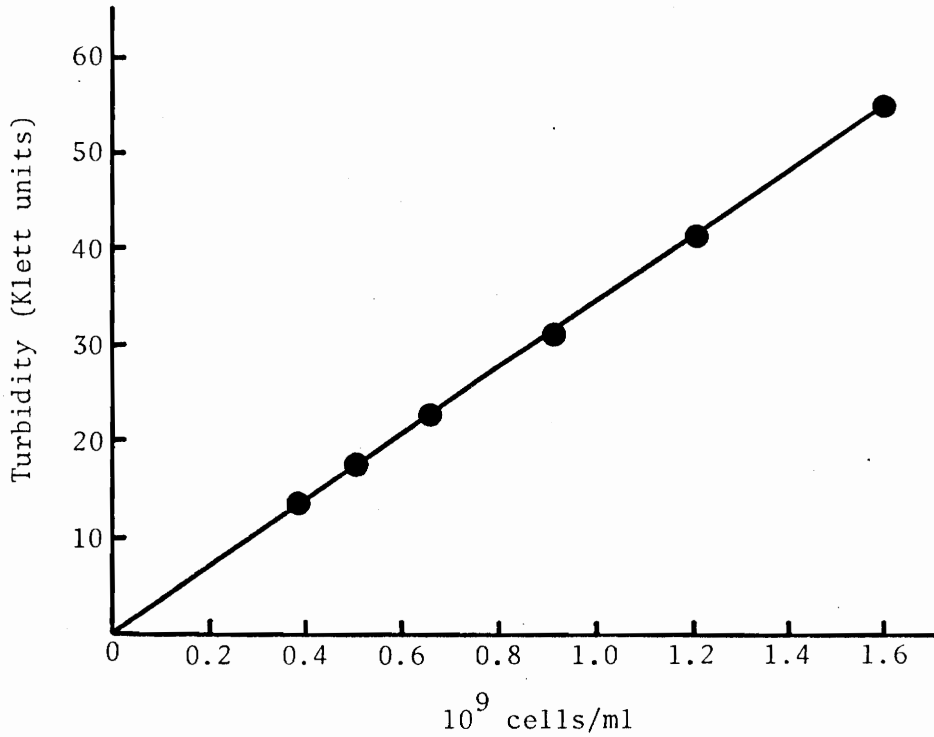

Figure 1. Relationship of turbidity (Klett units) to cells/ml of Brucella broth or Brucella broth supplemented with $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid. Cells/ml were estimated by direct microscopic counts using a Petroff-Hauser chamber. Turbidities were estimated using a Klett colorimeter with the red (660 nm) filter and 16 mm cuvettes.

Growth on Agar Plates

Initial experiments were aimed at determining optimum levels of three basic supplements ($FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid) for the three reference strains indicated earlier. In these experiments, the concentration of one of the additives was varied while the other two were kept constant at the levels previously found suitable for strain H840.

In all determinations of growth responses on agar plates, the same protocol was used. A loopful of a 1 to 3 day old semisolid stock culture was inoculated into 50 ml of *Brucella* broth (pH 7.4) and incubated under 6% O_2, 2.5% CO_2 at 37 C with agitation. The turbidity was allowed to reach 50-55 Klett units. Decimal dilutions were made through $1:10^6$ in *Brucella* broth, and 0.1 ml was inoculated onto the surface of the test plate. The inoculum was spread with a glass spreading rod. This would generally yield ca. 80-250 colonies per plate, assuming optimum recovery. All platings were done in triplicate and incubated at 6, 17, and 21% O_2, all with 2.5% CO_2 (see "Gas Tensions"). Plates were usually incubated for 4 days; however, it was found that some strains required slightly longer incubations for measurable growth.

Growth responses were determined by colony counts and colony diameters. Colony counts for triplicate plates were averaged. Colony diameters were measured with a filar ocular micrometer attached to a dissecting microscope. The diameters of 6 colonies taken at random were measured on each of the triplicate plates and the results averaged. Results for growth on agar plates were expressed as a Relative Growth

Index, which is defined as the results (colony counts or colony diameters) obtained under the indicated conditions divided by the corresponding results obtained on control (unsupplemented) Brucella agar. For example, a result of 1.30 for the colony count of a particular strain when incubated at 17% O_2, 2.5% CO_2 on a supplemented medium, indicates that the counts obtained under these conditions were 1.3 times greater than the counts obtained on Brucella agar at 6% O_2, 2.5% CO_2. A similar system was used to express colony diameters.

Growth in Broth

Inocula for this series of experiments were grown as indicated above. After reaching a turbidity of 50-55 Klett units, a $1:10^5$ dilution was made in Brucella broth and 1.0 ml of this dilution (ca. 10^4 cells) was inoculated into 50 ml of test broth (contained in square "milk dilution" bottles with loosened screw caps; these vessels hold approximately 160 ml and the surface:volume ratio approximates that of a Difco blood culture bottle). Duplicate bottles were incubated statically at 37 C under 21% O_2, 2.5% CO_2 (see "Gas Tensions"). After 72 hours, the turbidity was determined. Sterile broths which had been incubated under identical conditions served as blanks. Results were again expressed as a Relative Growth Index, in this case defined as the turbidity seen in the test borth divided by the turbidity seen in the control (unsupplemented) Brucella broth.

Initial experiments to determine optimum levels of the three basic supplements were conducted in broth media as indicated in the protocol for the growth response on agar; i.e., one of the three

components was varied while the other two were kept at a constant level. The same three reference srrains were again used.

Gas Tensions

Three primary gas tensions were used in this study: 6% O_2 (microaerobic conditions), 17% O_2 (candle jar conditions), and 21% O_2 (atmospheric oxygen conditions). In all cases, 2.5% CO_2 was added, as this is the reported CO_2 content of a candle jar (42, 46). At sea level, barometric pressure is 29.9" Hg of which 21% is oxygen with virtually no CO_2. Because Blacksburg, Virginia is 2,200 feet above sea level, the molar oxygen content per unit volume of air is less than at sea level. To standardize conditions, daily barometric pressures were determined and the various gas tensions used were corrected to sea level values manometrically. For 6% O_2 conditions, a premixed gas cylinder of 6% O_2, 94% N_2 was used. In all cases, vessels were evacuated, the 2.5% CO_2 added, followed by the appropriate gas mixtures. When producing 17% O_2 conditions, a slight negative pressure remains in the vessel after the addition of air; in this case, the pressure was equilibrated with N_2. All gas cylinders were purchased from Airco Industrial Gas Co., New York.

Effect of Light Exposure on Brucella Medium

In the course of this study, the effect of light exposure on sterile *Brucella* was investigated. The growth of strain H840 on media previously exposed to light (with and without supplements) was then determined using the dilution plating procedures otulined above. This series of experiments was conducted by incubating sterile agar plates

at 25 C adjacent to a window for 8 to 11 hours during daylight prior to inoculation. The plates were not exposed to direct sunlight.

RESULTS

The results of this study are divided into the following sections: (i) the determination of optimum pH and composition of Brucella medium supplemented with $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid, (ii) the growth response of the strains of C. fetus to the new formulation, (iii) the effect of light, superoxide dismutase, and catalase on the growth and aerotolerance of strain H840, and (iv) the effect of natural iron chelators on the growth and aerotolerance of strain H840.

Determination of Optimum pH and Composition of Brucella Medium Supplemented with $FeSO_4 \cdot 7H_2O$, Sodium Bisulfite, and Pyruvic Acid

Determination of optimum pH. For the three reference strains used, the optimum pH for growth in Brucella broth supplemented with these additives was found to be pH 7.4 (see Table 5). For strains H840 and 1510 MB, the optimum was seen both when inocula were grown at pH 7.4 and 7.0. Strain 998 also grew best at pH 7.4 when the inoculum was grown at pH 7.4; however, when the inoculum was grown at pH 7.0, good growth was seen at both pH 7.4 and 7.0. In all subsequent experiments, including media for growth of inocula and for maintenance of stock cultures, Brucella media with or without additives were adjusted to pH 7.4.

Determination of optimum levels of the additives in agar media. As indicated earlier, the growth response on agar plates was evaluated in

Table 5. Growth response of three reference strains of C. fetus to different pH values in Brucella broth supplemented with $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid.[a]

pH of supplemented Brucella broth	Turbidity reached after 26 hours incubation (Klett units)		
	H840	1510 MB	998
Inocula grown at pH 7.0			
6.8	32	37	24
7.0	37	40	29
7.2	38	44	29
7.4	42	45	28
7.6	36	40	22
Inocula grown at pH 7.4			
6.8	34	36	26
7.0	37	42	28
7.2	38	44	29
7.4	40	47	31
7.6	37	43	27

[a]Brucella broth was supplemented with 0.025% $FeSO_4 \cdot 7H_2)$, 0.050% sodium bisulfite, and 0.025% pyruvic acid.

two ways: by colony counts and by colony diameters. Results are expressed as a Relative Growth Index, defined as the result (colony count or colony diameter) obtained under the indicated conditions divided by the corresponding result obtained on unsupplemented *Brucella* agar at 6% O_2. The choice of the final optimum levels of the media additives was based primarily upon colony counts; however, when no differences were apparent, colony diameters were used as the criterion for selection. Certainly the most critical consideration for the utility of a medium for the isolation of an organism is its ability to support any growth, and in this regard colony counts were felt to be the more significant.

To determine the optimum levels of $FeSO_4 \cdot 7H_2O$ in agar medium, the level of this compound was varied while the levels of sodium bisulfite and pyruvic acid were kept constant (0.050% and 0.025%, respectively). Based on colony count, the optimum level of $FeSO_4 \cdot 7H_2O$ for strain H840 was 0.025% at 6%, 17%, and 21% O_2 (see Table 6). For this strain, the largest colony diameters were obtained at 0.010% $FeSO_4 \cdot 7H_2O$ for all oxygen tensions (see Table 7). In the case of strain 1510 MB, colony counts at 6% O_2 were highest with 0.040%; at 17% O_2, they were highest at 0.025%; and at 21% O_2, they were highest at 0.010% $FeSO_4 \cdot 7H_2O$ (see Table 8). Colony diameters for strain 1510 MB at 6% O_2 were largest at 0.040% $FeSO_4 \cdot 7H_2O$; at 17% O_2, they were largest at 0.010% and 0.025%; and at 21% O_2, they were largest at 0.010% $FeSO_4 \cdot 7H_2O$ (see Table 9). In the case of strain 998, colony counts were highest at 6% O_2 and 21% O_2 at 0.025% $FeSO_4 \cdot 7H_2O$; and at 17% O_2, they were highest at 0.025% and 0.040% $FeSO_4 \cdot 7H_2O$ (see Table 10). Colony diameters of

Table 6. Colony counts of C. fetus strain H840 on Brucella agar supplemented with different levels of $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid.

Supplement[a]	Relative Growth Index[b] at 1:10^6 dilution:		
	6% O_2, 2.5% CO_2	17% O_2, 2.5% CO_2	21% O_2, 2.5% CO_2
% $FeSO_4 \cdot 7H_2O$			
0.000	1.18	1.06	0.98
0.010	1.41	1.24	1.00
0.025	1.53	1.34	1.05
0.040	1.14	1.27	0.92
0.070	1.10	1.01	0.85
0.100	1.01	0.93	0.83
control[c]	1.00	0.16	0.00
% sodium bisulfite			
0.000	1.02	0.84	0.72
0.010	1.24	1.25	1.10
0.025	1.30	1.37	1.00
0.050	1.26	1.22	0.93
0.100	1.27	1.19	0.90
control	1.00	0.22	0.00
% pyruvic acid			
0.000	1.02	0.91	0.88
0.010	1.04	1.09	1.02
0.025	1.12	1.06	1.03
0.040	0.95	1.03	0.97
0.070	0.95	1.02	0.90
0.100	0.97	0.92	0.83
control	1.00	0.18	0.00

[a]The level of one of the additives was varied while the other two were kept constant (0.025% $FeSO_4$ $7H_2$), 0.050% sodium bisulfite, and 0.025% pyruvic acid).

[b]Relative Growth Index is defined as the colony counts obtained under the indicated conditions divided by the colony counts obtained on unsupplemented Brucella agar at 6% O_2, 2.5% CO_2. The results were calculated from the mean of triplicate plates.

[c]Control medium is unsupplemented Brucella agar.

Table 7. Colony diameters of C. fetus strain H840 on Brucella agar supplemented with different levels of $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid.

Supplement[a]	Relative Growth Index[b] at $1:10^6$ dilution		
	6% O_2, 2.5% CO_2	17% O_2, 2.5% CO_2	21% O_2, 2.5% CO_2
% $FeSO_4 \cdot 7H_2O$			
0.000	1.02	1.04	0.90
0.010	1.30	1.14	0.94
0.025	1.22	1.11	0.87
0.040	1.17	1.06	0.85
0.070	1.03	0.95	0.82
0.100	1.00	0.95	0.78
control[c]	1.00	0.62	0.00
% sodium bisulfite			
0.000	1.16	1.18	0.83
0.010	1.24	1.31	0.99
0.025	1.45	1.22	1.04
0.050	1.29	1.14	0.94
0.100	1.11	1.10	0.93
control	1.00	0.49	0.00
% pyruvic acid			
0.000	1.20	1.29	1.00
0.010	1.23	1.33	0.99
0.025	1.18	1.42	0.97
0.040	1.14	1.28	0.83
0.070	1.14	1.27	0.80
0.100	1.08	1.18	0.78
control	1.00	0.56	0.00

[a]The level of one of the additives was varied while the other two were kept constant (0.025% $FeSO_4$ $7H_2O$, 0.050% sodium bisulfite, 0.025% pyruvic adid).

[b]Relative Growth Index is defined as the colony diameters obtained under the indicate conditions divided by the colony counts obtained under the Brucella agar at 6% O_2. The results were calculated from mean diameters of 18 colonies.

[c]Control medium is unsupplemented Brucella agar.

Table 8. Colony counts of C. fetus strain 1510 MB on Brucella agar supplemented with different levels of $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid.

	Relative Growth Index at $1:10^6$ dilution		
Supplement	6% O_2, 2.5% CO_2	17% O_2, 2.5% CO_2	21% O_2, 2.5% CO_2
% $FeSO_4 \cdot 7H_2O$			
0.000	1.12	0.84	0.63
0.010	1.16	1.01	0.89
0.025	1.27	1.23	0.82
0.040	1.29	1.19	0.84
0.070	1.28	1.17	0.80
0.100	1.26	1.15	0.81
control	1.00	0.00	0.00
% sodium bisulfite			
0.000	1.07	0.81	0.78
0.010	1.01	1.00	1.01
0.025	1.05	0.99	1.00
0.050	1.06	1.02	0.73
0.100	1.05	0.47	0.53
control	1.00	0.00	0.00
% pyruvic acid			
0.000	1.00	1.17	0.83
0.010	1.05	1.33	1.09
0.025	1.05	1.28	1.09
0.040	1.08	1.20	1.08
0.070	1.04	1.21	1.05
0.100	1.07	1.22	1.03
control	1.00	0.00	0.00

NOTE: For additional explanatory material, see footnotes to Table 6 or Table 7.

Table 9. Colony diameters of C. fetus strain 1510 MB obtained on Brucella agar supplemented with different levels of $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid.

Supplement	Relative Growth Index at 1:10^6 dilution		
	6% O_2, 2.5% CO_2	17% O_2, 2.5% CO_2	21% O_2, 2.5% CO_2
% $FeSO_4 \cdot 7H_2O$			
0.000	0.92	1.07	0.50
0.010	0.88	1.12	0.58
0.025	0.95	1.12	0.49
0.040	0.91	1.01	0.48
0.070	0.84	0.87	0.44
0.100	0.83	0.85	0.44
control	1.00	0.00	0.00
% sodium bisulfite			
0.000	1.06	0.70	0.69
0.010	1.00	0.70	0.74
0.025	0.99	0.73	0.68
0.050	0.88	0.66	0.59
0.100	0.84	0.49	0.45
control	1.00	0.00	0.00
% pyruvic acid			
0.000	0.71	0.54	0.52
0.010	0.77	0.73	0.71
0.025	0.75	0.66	0.68
0.040	0.74	0.61	0.68
0.070	0.74	0.56	0.58
0.100	0.67	0.52	0.56
control	1.00	0.00	0.00

NOTE: For additional explanatory material, see footnotes to Table 6 or Table 7.

Table 10. Colony counts of C. fetus strain 998 obtained on Brucella strain with different levels of $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid.

Supplement	Relative Growth Index at $1:10^6$ dilution		
	6% O_2, 2.5% CO_2	17% O_2, 2.5% CO_2	21% O_2, 2.5% CO_2
% $FeSO_4 \cdot 7H_2O$			
0.000	0.91	0.00	0.00
0.010	1.23	0.22	0.00
0.025	1.37	0.30	0.27
0.040	1.34	0.30	0.25
0.070	1.21	0.24	0.00
0.100	0.95	0.24	0.00
control	1.00	0.00	0.00
% sodium bisulfite			
0.000	1.11	0.18	0.00
0.010	1.24	0.40	0.18
0.025	1.40	0.39	0.24
0.050	1.30	0.32	0.00
0.100	1.15	0.00	0.00
control	1.00	0.00	0.00
% pyruvic acid			
0.000	0.99	0.11	0.11
0.010	1.38	0.13	0.06
0.025	1.45	0.28	0.13
0.040	1.37	0.25	0.13
0.070	1.25	0.20	0.10
0.100	1.25	0.11	0.00
control	1.00	0.00	0.00

NOTE: For additional explanatory material, see footnotes to Table 6 or Table 7.

strain 998 were largest at 6%, 17%, and 21% O_2 at 0.025% $FeSO_4 \cdot 7H_2O$ (see Table 11). On the basis of colony counts and colony diameters, the results for all three strains indicated an optimum level of $FeSO_4 \cdot 7H_2O$ of 0.025%, as this was the most frequently occurring maximum value. On this basis, this level was chosen for use in the media formulation.

To determine the optimum level of sodium bisulfite in agar medium, the level of this compound was varied while the levels of $FeSO_4 \cdot 7H_2O$ and pyruvic acid were kept constant (0.025% each). On the basis of colony counts, the optimum level of sodium bisulfite for strain H840 was found to be 0.025% at 6% O_2 and 21% O_2, and 0.010% at 17% O_2 (see Table 6). The colony diameters obtained with this strain were found to be largest at 6% O_2 and 21% O_2 with 0.025% sodium bisulfite, and at 17% O_2 with 0.010% sodium bisulfite (see Table 7). Strain 1510 MB yielded the highest colony counts at 6% and 17% O_2 with the concentration of sodium bisulfite at a level of 0.050%, and at 21% O_2, at a level of 0.010% (see Table 8). Colony diameters for this strain at 6% O_2 were largest in the absence of sodium bisulfite, i.e., when the *Brucella* medium was supplemented with only $FeSO_4 \cdot 7H_2O$ and pyruvic acid (see Table 9). At 17% O_2 the largest colony diameters were obtained with 0.025% sodium bisulfite, and at 21% O_2 the largest diameters were obtained with 0.010% sodium bisulfite. Colony count results of strain 998 at 6% O_2 and 21% O_2 indicated an optimum level of 0.025% sodium bisulfite, while at 17% O_2, the optimum level was 0.010% (see Table 10). Colony diameter results of strain 998

Table 11. Colony diameters of C. fetus strain 998 obtained on Brucella agar supplemented with different levels of $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid.

Supplement	Relative Growth Index at $1:10^6$ dilution		
	6% O_2, 2.5% CO_2	17% O_2, 2.5% CO_2	21% O_2, 2.5% CO_2
% $FeSO_4 \cdot 7H_2O$			
0.000	0.94	0.00	0.00
0.010	0.95	0.45	0.00
0.025	0.99	0.57	0.45
0.040	0.88	0.51	0.43
0.070	0.87	0.50	0.00
0.100	0.84	0.50	0.00
control	1.00	0.00	0.00
% sodium bisulfite			
0.000	1.15	0.44	0.00
0.010	1.18	0.56	0.56
0.025	1.22	0.53	0.48
0.050	1.00	0.46	0.00
0.100	0.98	0.00	0.00
control	1.00	0.00	0.00
% pyruvic acid			
0.000	0.92	0.60	0.00
0.010	0.91	0.54	0.42
0.025	0.95	0.51	0.53
0.040	0.86	0.49	0.53
0.070	0.82	0.52	0.49
0.100	0.82	0.50	0.00
control	1.00	0.00	0.00

NOTE: For additional explanatory material, see footnotes to Table 6 or Table 7.

indicated an optimum level of 0.010% for this additive at 17% and 21% O_2; and of 0.025% at 6% O_2 (see Table 11). Colony count and colony diameter results of the three reference strains indicated optimum levels of sodium bisulfite to be 0.010%, 0.025%, and 0.040% with approximately equal distribution. On this basis, an intermediate value of 0.025% was chosen for use in the media formulation.

To determine the optimum levels of pyruvic acid in agar medium, the level of this additive was varied while the levels of $FeSO_4 \cdot 7H_2O$ and sodium bisulfite were kept constant (0.025% and 0.050%, respectively). Strain H840 exhibited maximum colony counts with pyruvic acid presernt at a level of 0.025% for all oxygen tensions (see Table 6). At 6% O_2, the largest colony diameters for this strain were seen with pyruvic acid present at a level of 0.010%; at 17% O_2, a maximum was seen at 0.025%, and at 21% O_2, the largest colony diameters were seen in the absence of pyruvic acid (see Table 7). In the case of strain 1510 MB, colony counts at 6% O_2 were highest with 0.040% pyruvic acid; at 17% O_2 they were highest at 0.010% pyruvic acid, and at 21% O_2, they were highest at the 0.010% and 0.025% levels (see Table 8). Colony diameters with this strain were greatest at 0.010% pyruvic acid for all oxygen tensions (see Table 9). Strain 998 demonstrated maximum colony counts at 6% and 17% O_2 with 0.025% pyruvic acid, while at 21% O_2, the highest counts were obtained with 0.025% and 0.040% pyruvic acid (see Table 10). Colony diameters for this strain at 6% O_2 were largest with 0.025% pyruvic acid; at 17% O_2 they were greatest at 0.010% pyruvic acid, and at 21% O_2, they were greatest at 0.025% and 0.040% pyruvic acid (see Table 11). As in the case of variations in

the concentration of sodium bisulfite, a distribution of both colony count and colony diameter optimum levels for pyruvic acid was seen between 0.010% and 0.040%. Again, an intermediate value of 0.025% was chosen as a satisfactory compromise for the results obtained with all three strains.

Therefore, the new agar formulation, hereafter termed FBPA agar, consisted of Brucella agar supplemented with 0.025% (each) $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid.

Determination of the optimum levels of the additives in broth medium. Using the same protocol of varying one of the additives while maintaining the remaining two at constant levels, the optimum concentrations of the three supplements were found to be different in broth media than on agar plates (see Table 12). The most striking finding was a much greater tolerance to high levels of $FeSO_4 \cdot 7H_2O$ under these conditions. The optimum level for this supplement was shown to be 0.2% for the three reference strains.

The optimum level for sodium bisulfite was similar to that found on agar plates. Strain 998 exhibited a sensitivity to this compound not demonstrated by the other two strains. The level of sodium bisulfite used in subsequent experiments was 0.025%, intermediate between the optimum levels for strains H840 and 1510 MB (0.050%), and strain 998 (0.013%).

The optimum level of pyruvic acid common to all three strains was shown to be 0.050%. Therefore, the new broth formulation, hereafter termed FBPB broth, consisted of Brucella broth supplemented with 0.2% $FeSO_4 \cdot 7H_2O$, 0.025% sodium bisulfite, and 0.050% pyruvic acid.

Table 12. Growth responses of three strains of *C. fetus* in *Brucella* broth supplemented with different levels of $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid.[a]

Supplement	Turbidity at 72 h[b]		
	H840	1510 MB	998
% $FeSO_4 \cdot 7H_2O$			
0.000	10	11	9
0.050	11	13	9
0.100	11	14	11
0.125	11	14	14
0.150	13	15	15
0.175	14	16	16
0.200	21	24	19
0.250	18	21	17
control[c]	7	8	0
% sodium bisulfite			
0.000	8	8	12
0.0032	8	9	12
0.0063	8	8	13
0.013	7	11	15
0.025	8	14	12
0.050	13	10	11
0.100	6	9	7
control	8	5	0
% pyruvic acid			
0.000	14	13	12
0.0032	15	13	12
0.0063	13	13	12
0.013	14	18	12
0.025	16	20	12
0.050	19	20	15
0.100	11	16	11
control	15	16	0

[a]Broth media was incubated statistically at 21% O_2, 2.5% CO_2.

[b]Klett units (red filter, 16 mm cuvettes).

[c]Control broth is unsupplemental *Brucella* broth.

Growth Responses of Other Strains of C. Fetus to the New Formulations

A total of 62 strains of *C. fetus*, representing each subspecies, were tested for their growth resyonses to FBPA agar and FBPB broth. The new formulations were found to be stimulatory for the growth and aerotolerance of many strains.

Growth responses on FBPA agar. Colony count and colony diameter data for *C. fetus* subsp. *intestinalis* grown at 6%, 17%, and 21% O_2 are given in Tables 13, 14, and 15, respectively. The results for *C. fetus* subsp. jejuni grown at 6%, 17%, and 21% O_2 are given in Tables 16, 17, and 18, respectively. The results for *C. fetus* subsp. *fetus* grown at all three oxygen tensions are given in Table 19. A summary of results is given in Table 20.

About 66% of the 62 strains tested were capable of growth at 17% O_2, on unsupplemented *Brucella* agar; however, of these s-rains, nearly 50% gave Relative Growth Indices of less than 0.50. This indicates that the colony counts under these conditions were less than half of those obtained on *Brucella* agar at 6% O_2. At 21% O_2, ca. 39% of the strains were capable of growth on *Brucella* agar, and of these strains, ca. 50% gave Relative Growth Indices of less than 0.50. Strains which were capable of growth at these oxygen tensions on unsupplemented *Brucella* agar generally exhibited higher colony counts but somewhat smaller colony diameters on FBPA agar.

About 82% of the strains grew on FBPA agar at 17% O_2, and of these, less than 2% of the strains gave Relative Growth Indices of less than 0.50. This indicates that the majority of the strains which grew at

Table 13. Growth responses of strains of C. fetus subsp. intestinalis on FBPA agar at 6% O_2, 2.5% CO_2.[a]

Strain	RGI (colony counts) at 1:10^6 dilution[b]	RGI (colony diameters) at 1:10^6 dilution[b]
2221	0.95	1.08
Nelson 1	4.50	1.75
10951	1.14	0.99
18151-B-76	1.12	0.85
Keith 76-1435	1.15	0.99
319-A-76	1.06	0.91
10451	0.86	1.11
10148	0.99	0.89
Pacemaker	0.98	0.76
Pedro	1.17	1.22
11669	1.00	0.89
13161	1.01	0.86
HCB	1.24	0.92
10296	3.18	1.01
11164	0.97	0.83
PB 1/76	6.64	0.97
11324-A-76	1.05	0.78
8391-B-76	1.03	0.91
W5-184	NA (42)[c]	NA (1.14)[c]
11529	1.03	0.89
11639-B-76	1.52	0.99
Grant	0.97	1.23
Langford	0.97	0.87
DT-74	0.82	0.99
702	1.20	1.03
PB 1/35	0.85	1.31
B6286	0.91	0.99
HF1	1.04	0.90
6339	NA (49)	NA (1.68)
PB 1/1	NA (39)	NA (0.77)
4440	1.12	0.88
EMB 1545	1.14	1.04
273	0.99	0.97
1925	0.88	1.28
Barr	1.06	0.89
10583	2.24	0.98
436	NA (0)	NA (0)

[a]FBPA agar - Brucella agar supplemented with 0.025% (each) $FeSO_4 \cdot 7H_2$), sodium bisulfite, and pyruvic acid.

Table 13, continued

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[b] Relative Growth Index (colony counts) is defined at the colony counts obtained under the indicated conditions divided by the colony counts obtained on Brucella agar at 6% O_2. Relative Growth Index (colony diameters) is defined as the colony diameters obtained under the indicated conditions divided by the dolony diamters obtained on Brucella agar at 6% O_2.

[c] NA = not applicable, because no growth occurred on Brucella agar at 6% O_2. In the case of dolony counts, the value given in parentheses is the number of colonies per plate at a 1:10^6 dilution under the indicated conditions. Results were calculated from the mean of triplicate plates. In the case of colony diameters, the value given in parentheses is the colony diameter in millimeters at a 1:10^6 dilution under the indicated conditions. Results were calculated from the mean diameters of 18 colonies.

Table 14. Growth responses of strains of C. fetus subsp. intestinalis on Brucella agar and FBPA agar at 17% O_2, 2.5% CO_2.

Strain	RGI (colony counts) at 1:10^6 dilution		RGI (colony diameters) at 1:10^6 dilution	
	Brucella agar	FBPA agar	Brucella agar	FBPA agar
2221	0.64	0.96	1.26	1.15
Nelson 1	0.00	0.00	0.00	0.00
10951	1.02	1.07	1.15	0.86
18151-B-76	1.23	1.35	0.56	0.66
Keith 76-1435	0.00	0.85	0.00	0.93
319-A-76	1.17	1.20	0.37	0.37
10451	0.96	1.06	0.71	0.73
10148	0.00	0.00	0.00	0.00
Pacemaker	0.79	1.01	1.12	0.84
Pedro	1.17	1.16	1.12	1.12
11669	0.97	1.04	1.20	0.89
13161	0.60	1.23	0.42	0.56
HCB	0.76	1.55	0.94	0.75
10296	0.00	3.36	0.00	0.69
11164	0.65	0.86	0.73	0.59
PB 1/76	0.00	0.00	0.00	0.00
11324-A-76	1.06	1.11	0.81	0.78
8391-B-76	1.12	1.10	1.01	1.37
W5-184	NA (0)	NA (0)	NA (0.00)	NA (0.00)
11529	0.42	1.07	0.98	0.77
11639-B-76	0.50	1.61	0.90	0.75
Grant	0.00	0.00	0.00	0.00
Langford	0.00	0.70	0.00	0.78
DT-74	0.03	0.18	0.88	1.46
702	0.00	0.00	0.00	0.00
PB 1/35	0.00	0.00	0.00	0.00
B6286	0.30	1.05	1.11	0.92
HF1	0.00	0.00	0.00	0.00
6339	NA (0)	NA (0)	NA (0.00)	NA (0.00)
PB 1/1	NA (0)	NA (0)	NA (0.00)	NA (0.00)
4440	0.60	0.93	0.79	0.77
EMB 1545	0.00	0.95	0.00	0.67
273	0.05	0.94	0.94	0.77
1925	0.00	0.00	0.00	0.00
Barr	0.17	0.94	0.78	0.53
10583	0.00	2.34	0.00	0.78
436	NA (0)	NA (0)	NA (0.00)	NA (0.00)

NOTE: For additional explanatory material, see footnotes to Table 13.

Table 15. Growth responses of strains of C. fetus subsp. intestinalis on FBPA agar and Brucella agar at 21% O_2, 2.5% CO_2.

Strain	RGI (colony counts) at 1:10^6 dilution		RGI (colony diameters) at 1:10^6 dilution	
	Brucella agar	FBPA agar	Brucella agar	FBPA agar
2221	0.00	0.00	0.00	0.00
Nelson 1	0.00	0.00	0.00	0.00
10951	0.46	0.44	0.87	0.49
18151-B-76	0.00	0.00	0.00	0.00
Keith 76-1435	0.00	0.79	0.00	0.53
319-A-76	0.00	0.00	0.00	0.00
10451	0.00	0.17	0.00	0.44
10148	0.00	0.00	0.00	0.00
Pacemaker	0.00	0.80	0.00	0.52
Pedro	1.16	1.17	1.11	1.27
11669	1.03	1.07	1.14	0.80
13161	0.00	0.78	0.00	0.26
HCB	0.81	0.89	0.80	0.81
10296	0.00	3.36	0.00	0.74
11164	0.00	0.00	0.00	0.00
PB 1/76	0.00	0.00	0.00	0.00
11324-A-76	0.58	1.05	0.52	0.46
8391-B-76	1.11	1.01	0.84	0.91
W5-184	NA (0)	NA (0)	NA (0.00)	NA (0.00)
11529	0.00	1.06	0.00	0.77
11639-B-76	0.00	1.54	0.00	0.58
Grant	0.00	0.00	0.00	0.00
Langford	0.00	0.59	0.00	0.44
DT-74	0.00	0.00	0.00	0.00
702	0.00	0.00	0.00	0.00
PB 1/35	0.00	0.00	0.00	0.00
B6286	0.00	0.84	0.00	0.72
HF1	0.00	0.00	0.00	0.00
6339	0.00	NA (0)	NA (0.00)	NA (0.00)
PB 1/1	0.00	NA (0)	0.00	0.00
4440	0.00	0.59	0.00	0.59
EMB 1545	0.00	0.00	0.00	0.00
273	0.00	0.84	0.00	0.85
1925	0.00	0.00	0.00	0.00
Barr	0.00	0.10	0.00	0.42
10583	0.00	1.48	0.00	0.75
436	NA (0)	NA (0)	NA (0.00)	NA (0.00)

NOTE: For additional explanatory material, see footnotes to Table 13.

Table 16. Growth responses of strains of C. fetus subsp. jejuni on FBPA agar at 6% O_2, 2.5% CO_2.

Strain	RGI (colony counts) at 1:10^6 dilution	RGI (colony diameters) at 1:10^6 dilution
Wang III	1.00	1.05
C14	1.25	1.00
Otis P.	1.03	1.03
8945-A-76	0.75	0.95
6963	0.99	0.99
8916	1.07	0.76
H641	0.98	1.22
B7619	1.18	1.25
29A	0.98	1.01
13136	1.12	1.15
Smith	1.02	0.85
4849	1.14	1.00
H550	0.89	0.94
11642-B-76	1.05	1.17
H325	0.97	1.00
11641-B-76	1.04	0.98
Holy Cross	1.10	1.38

NOTE: For additional explanatory material, see footnotes to Table 13.

Table 17. Growth responses of strains of C. fetus subsup. jejuni on FBPA agar and Brucella agar at 17% O_2, 2.5% CO_2.

Strain	RGI (colony counts) at 1:10^6 dilution		RGI (colony diameters) at 1:10^6 dilution	
	Brucella agar	FBPA agar	Brucella agar	FBPA agar
Wang III	0.05	0.90	1.07	0.74
C14	0.57	1.59	1.30	1.16
Otis P.	1.44	1.43	1.42	1.58
8945-A-76	0.06	0.71	1.09	1.13
6963	0.90	1.06	2.43	2.72
8916	0.78	1.09	0.56	0.68
H641	0.89	0.95	1.15	1.64
B7619	0.23	1.28	1.89	1.40
29A	0.98	0.91	3.03	3.00
13136	0.73	0.84	2.65	2.83
Smith	0.88	1.06	1.13	0.97
4849	0.00	0.86	0.00	0.69
H550	1.32	1.29	1.02	0.90
11642-B-76	0.91	0.97	1.25	1.31
H325	0.83	0.74	1.00	0.96
11641-B-76	0.75	0.92	2.39	1.78
Holy Cross	0.60	0.89	1.60	1.61

NOTE: For additional explanatory material, see footnotes to Table 13.

Table 18. Growth responses of strains of C. fetus subsp. jejuni on FBPA agar and Brucella agar at 21% O_2, 2.5% CO_2.

Strain	RGI (colony counts) at $1:10^6$ dilution		RGI (colony diameters) at $1:10^6$ dilution	
	Brucella agar	FBPA agar	Brucella agar	FBPA agar
Wang III	0.00	0.81	0.00	0.62
C14	0.43	1.09	0.77	1.33
Otis P.	0.00	1.10	0.00	0.88
8945-A-76	0.00	0.25	0.00	1.28
6963	0.04	0.72	3.26	3.04
8916	0.23	0.79	0.53	0.52
H641	0.66	0.77	1.02	1.74
B7619	0.08	1.06	2.44	1.60
29A	0.48	0.87	3.48	3.47
13136	0.01	0.25	2.78	3.22
Smith	1.00	1.06	1.10	0.97
4849	0.00	0.34	0.00	0.22
H550	1.00	0.86	0.97	0.97
11642-B-76	0.77	0.94	1.24	1.27
H325	0.40	0.76	0.84	1.01
11641-B-76	0.20	0.72	2.92	2.28
Holy Cross	0.20	0.65	2.01	1.92

NOTE: For additional explanatory material, see footnotes to Table 13.

Table 19. Growth responses of strains of C. fetus subsp. fetus on FBPA agar and Brucella agar at different oxygen tensions.

Gaseous Conditions	Strain	RGI (colony counts) at 1:10^6 dilution		RGI (colony diameters) at 1:10^6 dilution	
		Brucella agar	FBPA agar	Brucella agar	FBPA agar
6% O_2, 2.5% CO_2					
	21085	1.00	1.12	1.00	1.25
	14093	1.00	0.92	1.00	1.06
	7721	1.00	1.41	1.00	0.90
	75-183	1.00	3.21	1.00	0.91
	13841	1.00	1.35	1.00	1.00
	14664	1.00	1.96	1.00	1.03
	14701	1.00	1.53	1.00	1.11
17% O_2, 2.5% CO_2					
	21085	0.00	0.00	0.00	0.00
	14093	0.26	0.94	1.02	0.93
	7721	0.00	0.71	0.00	0.75
	75-183	0.00	2.14	0.00	0.95
	13841	0.00	0.90	0.00	0.72
	14664	0.00	1.08	0.00	0.65
	14701	0.00	0.00	0.00	0.00
21% O_2, 2.5% CO_2					
	21085	0.00	0.00	0.00	0.00
	14093	0.04	0.92	0.86	0.87
	7721	0.00	0.26	0.00	0.64
	75-183	0.00	1.07	0.00	0.92
	13841	0.00	0.41	0.00	0.58
	14664	0.00	0.00	0.00	0.00
	14701	0.00	0.00	0.00	0.00

NOTE: For additional explanatory material, see footnotes to Table 13.

Table 20. Summary of growth responses of strains of C. fetus grown on Brucella agar and on FBP agar at various oxygen tensions.

Supspecies	Gaseous conditions	Medium	Relative Growth Index for dilution platings[a]				
			0.00	0.01-0.49	0.50-0.99	≥1.00	NA[b]
intestinalis	17% O_2, 2.5% CO_2	Brucella agar	14/37[c]	5/37	9/37	6/37	3/37
	17% O_2, 2.5% CO_2	FBPA agar	9/37	1/37	8/37	16/37	3/37
	21% O_2, 2.5% CO_2	Brucella agar	28/37	1/37	2/37	3/37	3/37
	21% O_2, 2.5% CO_2	FBPA agar	15/37	3/37	8/37	8/37	3/37
jejuni	17% O_2, 2.5% CO_2	Brucella agar	1/18	4/18	11/18	2/18	0/18
	17% O_2, 2.5% CO_2	FBPA agar	0/18	0/18	10/18	8/18	0/18
	21% O_2, 2.5% CO_2	Brucella agar	4/18	10/18	2/18	2/18	0/18
	21% O_2, 2.5% CO_2	FBPA agar	0/18	3/18	10/18	5/18	0/18
fetus	17% O_2, 2.5% CO_2	Brucella agar	6/7	1/7	0/7	0/7	0/7
	17% O_2, 2.5% CO_2	FBPA agar	2/7	0/7	3/7	2/7	0/7
	21% O_2, 2.5% CO_2	Brucella agar	6/7	1/7	0/7	0/7	0/7
	21% O_2, 2.5% CO_2	FBPA agar	3/7	2/7	1/7	1/7	0/7

[a]Relative Growth Index = no. of colonies at $1:10^6$ dilution plating under the stated condition divided by the no. of colonies at $1:10^6$ dilution plating on Brucella agar at 6% O_2, 2.5% CO_2.

[b]NA = not applicable, because no growth occurs at $1:10^6$ dilution plating on Brucella agar at 6% O_2, 2.5% CO_2. Two of the three strains indicated did grow at $1:10^6$ dilution plating on FBPA agar at 6% O_2, 2.5% CO_2 but not a higher levels of O_2. The remaining strain grew only on FBPA agar at 1% O_2, 2.5% CO_2 but not on Brucella agar under similar conditions.

[c]The values are expressed as the ratio of the number of strains having the particular Relative Growth Index to the total number of strains tested.

this oxygen tension on FBPA agar had colony counts which were at least half of those obtained on Brucella agar at 6% O_2. At 21% O_2, 71% of the strains grew on FBPA agar, and of these, ca. 18% had Relative Growth Indices of less than 0.50. Therefore, not only did a higher percentage of the strains grow on FBPA agar at 17% and 21% O_2 than on Brucella agar, but, in addition, the colony counts obtained at these oxygen tensions were higher on the supplemented medium.

Growth responses in FBPB broth. The results for growth of strains of *C. fetus* subsp. *intestinalis* in FBPB broth are given in Table 21. The results for growth of strains of *C. fetus* subsp. *jejuni* and *fetus* are given in Table 22. With the single exception of strain 436 of subspecies *intestinalis*, all strains were capable of growth in FBPB broth at 21% O_2 in static culture. In contrast, ca. 23% of the strains failed to grow in Brucella broth alone under these conditions. In those cases where growth did occur in Brucella broth, growth rates were consistently more rapid in FBPB broth.

Effect of Light, Superoxide Dismutase, and Catalase on the Growth and Aerotolerance of C. fetus strain H840

The growth of strain H840 was shown to be inhibited at 6% O_2 on Brucella agar plates which had been exposed to light prior to inoculation (see Table 23). Sterile plates which had been stored adjacent to a window for 8 hours during daylight ("light" plates) and which were subsequently inoculated and incubated at 6% O_2, showed a 36% reduction in colony counts compared to similar plates not

Table 21. Growth response of strains of *C. fetus* subsp. *intestinalis* in FBPB broth at 21% O_2, 2.5% CO_2.

Strain	Relative Growth Index[a]	Strain	Relative Growth Index[a]
2221	1.93	Nelson 1	NA (24)[b]
10951	1.06	18151-B-76	1.26
Keith 76-1435	3.00	319-A-76	1.58
10451	1.70	10148	1.81
Pacemaker	NA (22)	Pedro	2.25
11669	1.74	13161	1.26
HCB	NA (5)	10296	1.35
11164	1.38	PB 1/76	NA (13)
11324-A-76	1.69	8391-B-76	1.18
W5-184	3.00	11529	1.35
639-B-76	1.32	Grant	NA (10)
Langford	NA (13)	DT-74	NA (15)
702	1.20	PB 1/35	1.38
B6286	1.16	HF1	7.00
6339	NA (24)	PB 1/1	NA (21)
4440	1.76	EMB 1545	NA (13)
273	1.22	1925	NA (17)
Barr	1.64	10583	2.50
436	NA (0)		

[a]The Relative Growth Index is defined as the turbidity reached in FBPB broth at 72 hours divided by the turbidity reached in control (unsupplemented) *Brucella* broth.

[b]NA = not appliciable, because no growth occurred in *Brucella* broth. The values given in parentheses are the turbidities in Klett units in FBPB broth.

Table 22. Growth responses of strain of C. fetus subsp. jejuni and C. fetus subsp. fetus in FBPB broth at 21% O_2, 2.5% CO_2.

Strain	RGI	Strain	RGI
C. fetus subsp. jejuni		C. fetus subsp. fetus	
Wang III	1.41	21085	NA (14)
C14	1.83	14093	2.18
Otis P.	3.00	7721	1.37
8945-A-76	1.18	75-183	2.13
6963	1.52	13841	NA (13)
8916	1.03	14664	2.25
H641	1.38	14701	NA (25)
B7619	1.53		
29A	1.25		
13136	1.24		
Smith	1.43		
4849	NA (11)		
H550	1.44		
11642-B-76	2.25		
H325	1.36		
11641-B-76	2.27		
Holy Cross	3.22		

NOTE: For additional explanatory material, see footnotes to Table 21.

Table 23. Growth response of C. fetus strain H840 on Brucella agar and FBPA agar previously exposed to light.[a]

Medium	Colony counts at $1:10^6$ dilution when incubated at:[b]		
	6% O_2, 2.5% CO_2	17% O_2, 2.5% CO_2	21% O_2, 2.5% CO_2
Light			
Brucella agar	48	0	0
FBPA agar	87	35	0
Dark			
Brucella agar	75	0	0
FBPA agar	94	86	88

[a]Sterile agar plates were incubated aerobically at 25 C adjacent to a window for 8 hours during daylight. They were not exposed to direct sunlight.

[b]Values represent the mean of triplicate plates.

exposed to the illumination ("dark" plates). When "light" plates containing ferrous sulfate + sodium bisulfite + pyruvic acid (FBS) were used, no reduction in colony counts occurred, indicating that the FBP was providing protection against the inhibition. When either "light" or "dark" plates of Brucella agar were incubated at 17% or 21% O_2, no colonies were formed. If FBP had been present in the medium, colony formation did occur on both the "light" and "dark" plates; however, there were 41% fewer colonies on the "light" plates. This indicated that at 17% O_2, FBP could only partially reverse the inhibition caused by illumination. At 21% O_2, the FBP was not effective at all in reversing the inhibition: a good growth response occurred on the "dark" plates but no colonies were formed on the "light" plates.

This procedure was then repeated using the three media additives separately to determine if one or more than one of the additives were responsible for the protective effect (see Table 24). In this case, sterile agar plates were incubated aerobically adjacent to a window for 11 hours during daylight. The longer period of illumination resulted in nearly complete inhibition of growth on unsupplemented Brucella agar at 6% O_2. "Light" plates containing 0.025% $FeSO_4 \cdot 7H_2O$, 0.025% sodium bisulfite, or 0.025% pyruvic acid demonstrated only 12%, 17%, and 8% reduction in colony counts, respectively, at 6% O_2 compared with corresponding plates not exposed to light. This indicated that each of the three media supplements was capable of providing the protective effect. At 17% O_2, no growth was seen on Brucella agar or Brucella agar supplemented with sodium bisulfite or pyruvic acid which had been exposed to light. Only on "light" plates containing $FeSO_4 \cdot 7H_2O$

Table 24. Growth response of *C. fetus* strain H840 on media previously exposed to light.

Experimental conditions	Supplement added to *Brucella* agar	Colony counts at 1:10^6 dilution when incubated at:[b]		
		6% O_2, 2.5% CO_2	17% O_2, 2.5% CO_2	21% O_2, 2.5% CO_2
Light	none	9	0	0
	0.025% $FeSO_4$ $7H_2O$	125	40	0
	0.025% sodium bisulfite	114	0	0
	0.025% pyruvic acid	118	0	0
Dark	none	103	0	0
	0.025% $FeSO_4$ $7H_2O$	142	88	81
	0.025% sodium bisulfite	137	47	38
	0.025% pyruvic acid	128	70	59

[a]Sterile agar plates were incubated aerobically at 25 C adjacent to a window for 11 hours during daylight. They were not exposed to direct sunlight.

[b]Values represent the mean colony counts from triplicate plates.

were colonies observed at 17% O_2, and these represented a 55% reduction in colony counts compared to the "dark" plates containing $FeSO_4 \cdot 7H_2O$ at 17% O_2. "Dark" plates of Brucella agar did not support growth at 17% O_2; however, with the addition of sodium bisulfite or pyruvic acid, some colony formation did occur. "Light" plates incubated at 21% O_2 did not support growth regardless of the supplement added; however, each of the three supplements supported some degree of colony formation on "dark" plates incubated at 21% O_2.

It was also demonstrated that the presence of oxygen during illumination was essential for the inhibition of growth to occur (see Table 25). Brucella agar plates were prepared under a nitrogen atmosphere and exposed to light under a nitrogen atmosphere (Brucella-N_2 agar); other plates were prepared aerobically and exposed to light aerobically. Both kinds of plates were incubated at 25 C adjacent to a window (not in direct sunlight) for 11 hours during daylight. Nearly complete inhibition of growth at 6% O_2 occurred on Brucella agar exposed to light under aerobic conditions prior to inoculation. However, Brucella-N_2 agar demonstrated no inhibition of growth following illumination. Brucella agar and Brucella-N_2 agar gave comparable colony counts when not exposed to light. No growth was seen at 17% O_2 or 21% O_2 on either medium.

The effects of superoxide dismutase (SOD) and catalase on Brucella agar exposed to light prior to inoculation were also investigated (see Table 26). Unsupplemented Brucella agar exposed to light failed to support growth of strain H840 at 6% O_2. However, in the presence of 70 U/ml SOD or catalase, moderate growth did occur under

Table 25. Growth response of C. fetus strain H840 on media previously exposed to light under aerobic and anaerobic conditions.

Medium[a]	Colony counts at 1:10^6 dilution when incubated at:[b]		
	6% O_2, 2.5% CO_2	17% O_2, 2.5% CO_2	21% O_2, 2.5% CO_2
Light			
Brucella agar	3	0	0
Brucella-N_2 agar	113	0	0
Dark			
Brucella agar	119	0	0
Brucella-N_2 agar	115	0	0

[a]Sterile *Brucella* agar plates were prepared and exposed to light under aerobic conditions. Sterile *Brucella*-N_2 agar were prepared and exposed to light under N_2. Plates were incubated at 25 C adjacent to a window for 11 hours during daylight. They were not exposed to direct sunlight.

[b]Values represent the mean of triplicate plates.

Table 26. Growth response of C. fetus strain H840 on media previously exposed to light in the presence of SOD and catalase.[a]

Medium[b]	Colony counts at 1:10^6 dilution when incubated at:[c]		
	6% O_2, 2.5% CO_2	17% O_2, 2.5% CO_2	21% O_2, 2.5% CO_2
Light			
Brucella agar	0	0	0
+70 U/ml SOD	67	5	0
+70 U/ml catalase	53	0	0
+inactivated SOD	1	0	0
+inactivated catalase	4	0	0
+0.006 mg/ml BSA	4	0	0
Dark			
Brucella agar	115	4	0
+70 U/ml SOD	125	100	45
+70 U/ml catalase	131	37	0
+inactivated SOD	110	0	0
+inactivated catalase	117	1	0
+0.006 mg/ml BSA	118	0	0

[a]Sterile agar plates were incubated aerobically at 25 C adjacent to a window for 11 hours during daylight. The plates were not exposed to direct sunlight.

[b]Superoxide dimutase (SOD), catalase, and bovine serum albumin were prepared as stock solutions in distilled water and sterlized by filtration. Heat-inactivated enzymes were prepared by placing these stock solutions in boiling water bath for 20 minutes. Stock solutions of enzymes were added to sterile Brucella agar at 45 C to give a final concentration of 70 U/ml (each). Bovine serum albumin (BSA) was added at a level approximating the mg protein/ml of Brucella agar supplemented with the enzymes.

[c]Values represent the mean of triplicate plates.

these conditions. "Light" plates of Brucella agar supplemented with SOD exhibited a 46% reduction in colony counts at 6% O_2 compared with counts obtained on the corresponding "dark" plates. Similarly, a 60% reduction in colony counts at 6% O_2 occurred on "light" plates containing catalase. "Light" plates containing heat-inactivated enzymes or bovine serum albumin showed nearly complete inhibition of growth at 6% O_2. "Dark" plates containing SOD or catalase gave colony counts slightly higher at 6% O_2 than those on "dark" plates without these supplements. "Dark" plates containing heat-inactivated enzymes or bovine serum albumin yielded counts comparable to those on unsupplemented plates at 6% O_2. "Light" plates containing SOD supported slight growth at 17% O_2, but supported a relatively good growth response on "dark" plates at 17% O_2. "Light" plates containing catalase exhibited no growth at 17% O_2, but some growth did occur on "dark" plates at this oxygen tension. At 21% O_2, "dark" plates containing SOD exhibited some growth, but no growth occurred on "light" plates under similar conditions.

On the basis of the foregoing data, it appeared that the inhibition of growth at 6% O_2 of strain H840 on Brucella agar previously exposed to light could be partially reversed by $FeSO_4 \cdot 7H_2O$, sodium bisulfite, pyruvic acid, SOD, or catalase. Of the enzymes used, SOD provided the greatest protection. Moreover, SOD was shown to enhance the aerotolerance of strain H840 at 17% and 21% O_2 on Brucella medium not exposed to light. Ordinarily, no growth or only very slight growth in the absence of SOD occurred under such conditions. It was of great interest that those

additives found to be most effective in providing protection against the inhibitory effects of light were also the same substances found to enhance the aerotolerance of strain H840.

Effect of Natural Iron Chelators on the Growth and Aerotolerance of Strain H840

As suggested by Bowdre *et al.* (3), the stimulatory effects of dihydroxyphenyl compounds on the aerotolerance of *S. volutans* and *C. fetus* might be due to their ability to chelate ferric iron and make it more available for growth. Accordingly, two iron chelators of microbial origin were tested for their ability to enhance the aerotolerance of *C. fetus* strain H840 (see Table 27). Both enterochelin and ferrichrome were shown to be effective when present at a level of 2×10^{-4}M. Colony counts obtained at 6% O_2 were higher in the presence of either additive than on unsupplemented *Brucella* agar. No growth was seen on unsupplemented *Brucella* agar at 17 or 21% O_2. Colony counts obtained at 17% O_2 on media supplemented with enterochelin were comparable to those obtained on *Brucella* agar at 6% O_2; ferrichrome was less effective, showing a 30% reduction in counts compared with those obtained on *Brucella* agar at 6% O_2. Colony counts at 21% O_2 showed 5% and 43% reductions for media containing enterochelin or ferrichrome, respectively, as compared with those obtained on *Brucella* agar at 6% O_2. At these levels, enterochelin appeared to be the more effective compound. No attempt was made to determine the optimum levels for these additives.

Table 27. Effect of natural iron chelators on the aerotolerance of C. fetus strain H840.

Medium[a]	Colony counts at 1:10^6 dilution when incubated at:[b]		
	6% O_2, 2.5% CO_2	17% O_2, 2.5% CO_2	21% O_2, 2.5% CO_2
Brucella agar	152	0	0
+2 × 10^{-4} M enterochelin	186	155	143
+2 × 10^{14} M ferrichrome	177	106	87

[a]Enterochelin and ferrichrome were prepared as stock solutions in distilled water, sterilized by filtration, and added to sterile Brucella agar at 45 C.

[b]Values represent the mean of triplicate plates.

DISCUSSION

This discussion is divided into the following sections: (i) growth of C. fetus on FBPA agar and in FBPB broth, and (ii) microaerophilism of C. fetus.

Growth of C. fetus on FBPA Agar and in FBPB Broth

Microaerophilic organisms can grow in an aerobic atmosphere providing that oxygen tensions are lower than those found in air. In the case of C. fetus, the optimum concentration for growth on agar plates is usually ca. 5-6% O_2 (21, 28). Bowdre et al. (3) and Hoffman (20) found a combination of $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid to be stimulatory for the growth and aerotolerance of several strains of C. fetus subsp. jejuni. In the present study, the optimum levels for these compounds were determined for three strains of C. fetus, one from each subspecies. The growth responses of additional strains of each subspecies to the new formulations was also determined.

Growth of C. fetus on FBPA agar. An examination of the growth responses of 62 strains of C. fetus has revealed a spectrum of oxygen tolerance on both unsupplemented Brucella agar and FBPA agar. Growth responses were seen to vary from strains capable of growth only at 1% O_2 on FBPA agar to those which could grow at 21% O_2 on unsupplemented Brucella agar.

Bovine isolates of subsp. fetus were generally the most difficult to grow. Colonies on agar plates at 6% O_2 on either medium were consistently smaller than those of the other two subspecies. Strains of subsp. fetus also appeared to be the most consistently oxygen sensitive. Unlike subsp. intestinalis and jejuni, no strains were capable of growth at 17 or 21% O_2 on unsupplemented media. Nevertheless, even these strains responded well to the new formulation; 5 of the 7 strains grew at 17% O_2 on FBPA agar, all with Relative Growth Indices of ≥ 0.70.

Strains of subsp. intestinalis from human as well as animal sources were found to vary greatly with regard to aerotolerance and stimulation by the media supplements. The most oxygen-sensitive as well as the most aerotolerant strains were found within this subspecies. Of the animal strains (bovine or ovine) tested within subsp. intestinalis, ca. 29% gave Relative Growth Indices of $\geq$ 17% O_2. A somewhat higher percentage (40%) of the human strains within this subspecies gave comparable results. Indeed, it is interesting that the most aerotolerant isolates examined, i.e., those giving Relative Growth Indices of ≥ 1.00 on Brucella agar at 21% O_2, were all obtained from human sources.

The tendency toward greater oxygen tolerance seen with human strains is difficult to interpret. General recognition of C. fetus as a microaerophilic pathogen of doemstic animals has long existed in the veterinary community. As a result, recovery of the organism

from animal sources has been maximized by considerations given for its microaerophilic nature. Only in recent years, however, has C. fetus been recognized as an opportunistic human pathogen. Without a general awareness of the unusual oxygen requirements of the organism, isolation from human sources has been rather fortuitous; it is likely that the more aerotolerant isolates would be recovered under these conditions. Therefore, the human strains examined here may reflect a biased sampling with regard to aerotolerance. A truly random sampling of strains associated with human disease, not available at present, may not reveal a tendency toward greater oxygen tolerance. In any case, it is evident that regardless of the source, aerotolerant as well as oxygen-sensitive isolates may be found.

Strains of subsp. jejuni, also pathogenic for humans, appeared to be the most consistently aerotolerant. Ca. 72% of the strains of this subspecies gave Relative Growth Indices of ≥ 0.50 on Brucella agar at 17% O_2; unlike strains of subsp. fetus and intestinalis, all strains gave Relative Growth Indices of ≥ 0.70 on FBPA agar incubated under these conditions. This indicated that for strains of this subspecies, at least 70% of the colonies appearing on Brucella agar at 6% O_2 could be recovered at 17% O_2 on FBPA agar. Since many of the strains of this subspecies were obtained from human sources, conclusions regarding consistently greater oxygen tolerance are subject to the same limitations indicated above.

No correlation of aerotolerance could be seen among human strains isolated from fecal as opposed to blood sources. Since strains of C. fetus may differ greatly with regard to aerotolerance, one might

expect isolates to tolerate levels of oxygen comparable to those found in the environment from which they were recovered. For example, strains isolated from fecal sources, obtained from a highly anaerobic environment, might be expected to be generally more oxygen-sensitive. Similarly, strains isolated from the blood might be expected to be more generally aerotolerant. However, aerotolerant as well as oxygen-sensitive strains were recovered from both sources with approximately the same frequency. Indeed, it is not known if C. fetus does actually grow in the gut or in the blood; the primary foci of infection might well be different from the sites from which the organism is isolated. For example, in the case of blood isolation, it is possible that C. fetus might grow in macrophages, as is the case with Salmonella typhi. Therefore, a bacteremia rather than a septicemia may occur. Similarly, fecal isolates might grow in regional lymph nodes following penetration of the intestinal wall, rather than in the lumen. Moreover, it is not presently clear how C. fetus can grow in the anaerobic environment of the gut. Although nitrate can be used as a terminal electron acceptor, it is unlikely that any would be available in the gut for growth. An alternate electron acceptor, unidentified at present, would seem to be necessary for growth under these conditions. Thus, predictions regarding oxygen sensitivities of strains obtained from different sites of isolation are complicated by the lack of knowledge concerning the pathogenesis of human campylobacteriosis.

For the majority of the 62 strains tested, oxygen present at a level of 6% still appeared to be toxic. Thus, higher colony counts

were generally obtained on FBPA agar than on Brucella agar under these conditions. However, it was frequently found that the colonies appeared to be somewhat smaller on FBPA agar. As indicated earlier, it was felt that the most critical consideration for the utility of a medium for the isolation of an organism is its ability to support any growth, and in this regard, the higher colony counts obtained on FBPA agar are desirable.

In general, it was seen that FBPA agar supported better growth of C. fetus regardless of the oxygen level. With such a spectrum of aerotolerance occurring on Brucella agar, it is difficult to imagine that a single medium could allow growth of all strains at 17 or 21% O_2. Even if a particular strain were not rendered more aerotolerant, the higher colony counts obtained on FBPA under conditions where the organism could grow, indicate that it is a more useful medium than Brucella agar.

The dilution plating procedures used to evaluate growth are certainly the most stringent conditions which could have been used from at least two standpoints. First, C. fetus characteristically exhibits inoculum-dependent growth. Streaked plates of strain H840 show a slight growth on Brucella agar at 17 and 21% O_2 in the area of heaviest inoculum; under similar conditions, diluation plates show little or no growth. Therefore, under conditions where a single isolated cell is not able to divide, several cells proximal to each other might be ablt to initiate growth. Second, the optimum concentration of CO_2 for cultivation of bovine strains of C. fetus has been found to be 5-10% (28). In this study, 2.5% CO_2 was used as this

is the level reported to be present in a candle jar (42, 46). Concentrations higher than 2.5% CO_2 were not used because of the desirability of designing a simple cultivation system such as a candle jar, rather than employing special gas mixtures, vacuum pumps, or other apparatus not readily available in most clinical laboratories.

Growth of C. fetus in FBPB broth. The stimulatory effect of the media additives was shown to be more consistent in broth media than on agar plates. Under the conditions used, 61 of the 62 strains were capable of initiating growth in FBPB broth when incubated statically at 21% O_2, 2.5% CO_2. Ca. 23% of the strains did not grow in unsupplemented Brucella broth under similar conditions. Strains which were capable of growth in Brucella broth demonstrated more rapid growth rates in FBPB broth.

In general, strains giving the highest turbidities (> 35 Klett units after 72 hours incubation) were those which had been isolated from human sources. It might be argued that the differences seen in turbidities of strains from human sources may only reflect larger cell sizes of these strains. However, with the exception of strains 436 of subsp. intestinalis, no apparent differences in cell size or cell morphology could be seen upon microscopic examination of any of the strains tested. Furthermore, direct microscopic counts of suspensions having identical turbidities of a strain which grew well in broth (strain Pedro) and a strain that did not (strain Grant) indicated comparable cell numbers. Therefore, the higher turbidities seen with human strains would indeed appear to be attributable to higher cell

densities. As previously seen, however, strains which grew well in broth as well as strains which did not had been isolated from both human and animal sources.

It is difficult to directly compare growth responses on agar plates and those in broth media. Generally, strains which failed to grow in Brucella broth also did not grow on unsupplemented Brucella agar at 17 or 21% O_2. However, other strains which grew on Brucella agar only at 6% O_2 grew well in Brucella broth at 21% O_2. It seems likely that static incubation of broth cultures provides a gradient of oxygen concentration within the medium. Therefore, strains which appear to be oxygen-sensitive on agar plates may still grow well in broth media; similarly, it would not be expected that a strain would be shown aerotolerant on agar and not in broth.

The finding that strains of C. fetus could tolerate higher levels of $FeSO_4 \cdot 7H_2O$ in broth media than on agar plates is difficult to explain. The level of this additive used in FBPB broth (0.2%) was shown to be highly inhibitory on agar plates. Whatever role the media supplements play in enhancing aerotolerance, one might suspect that a higher level of the compounds would be required for growth on an exposed agar surface than in liquid media. Clearly, then, it is not the high level of these compounds per se which is responsible for the toxicity of the compounds seen on the agar plates. However, the mean concentration of dissolved oxygen on the surface of an agar plate is certainly greater than in the depths of liquid media. Since iron forms insoluble precipitates under aerobic rather than anaerobic conditions, it seems liekly that there would be a greater tendency for iron to precipitate

on the surface of an agar plate. Directly or indirectly, this might possibly be responsible for the toxic effect seen in agar and not broth media.

Recommendation for use of FBPA agar and FBPB broth. Due to the apparent diversity of aerotolerance among strains of C. fetus, the successful recovery of any particular isolate on FBPA agar is still, to some degree, dictated by the oxygen tension used for cultivation. As indicated earlier, FBPA agar generally supported better growth than Brucella agar, regardless of conditions. The new agar formulation was shown to support growth of ca. 82% of the strain under simulated candle jar conditions; of these strains, only 2% had Relative Growth Index of less than 0.50. In contrast, ca. 66% of the strains were capable of growth on Brucella agar under similar conditions; of these strains, nearly 50% had Relative Growth Indices of less than 0.50. Therefore, if a candle jar is the only means available for reducing oxygen tensions, then the new agar media can be expected to recover a higher percentage of isolates, with higher growth yields, than Brucella agar.

Because the new formulation supported growth of many but not all strains at 17% O_2, lower oxygen tensions should ideally be used to insure recovery. Of the 62 strains, only one failed to grow on FBPA agar at 6% O_2. While the great majority of strains also grew on Brucella agar under these conditions, higher colony counts were generally obtained on FBPA agar.

FBPA agar would be most useful in the primary isolation of C. fetus from specimens with mixed microbial flora, such as from the

gastrointestinal and reproductive tracts. The incorporation of antibiotics, previously used by other workers (50, 52, 56, 58), would seem to be desirable. Also, the new agar formulation would be useful in obtaining axenically pure cultures from enrichment media.

FBPB broth would be useful as an enrichment medium for isolation of C. fetus in instances where few contaminating organisms might be expected, such as from blood or cerebrospinal fluid. For 61 of the 62 isolates, an inoculum of ca. 10^4 cells, added to 50 ml of FBPB broth, initiated growth at 21% O_2, 2.5% CO_2 with static incubation. Although not examined in this study, smaller inocula may also be sufficient to initiate growth.

Once pure cultures have been obtained, the most convenient system for the maintenance of stock cultures is semisolid media. Generally, cultures may be incubated aerobically without CO_2; however, at least one strain appears to require CO_2 even for growth in semisolid media (R. M. Smibert, personal communication). Smibert (personal communication) has indicated that the addition of 0.02% $FeSO_4 \cdot 7H_2O$ to semisolid Brucella media is useful for maintenance of stock cultures and for recovery of strains following mailing from distant sources. It is also likely that semisolid Brucella medium supplemented with $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid would be advantageous for recovery of C. fetus from clinical samples where pure cultures would be expected.

Microaerophilism of C. fetus

Bowdre et al. (3) established that the addition of high levels of iron salts or ferric chelates to Brucella medium enhanced the

aerotolerance of _C. fetus_ strain H840. In view of the known physical properties of iron, at first it seemed likely that the function of these additives was to make iron more available to the cell.

Under aerobic conditions at neutral pH, ferrous iron is rapidly oxidized to the ferric state. In an aqueous environment, ferric iron tends to form extremely insoluble precipitates of varying composition (44). Aerobic organisms have developed specific transport systems to sequester iron from an aerobic environment which involve low molecular weight chelators with high affinity for ferric iron. These iron transport compounds, or siderophores, have been classified as phenolate or hydroxamate chelators on the basis of the functional group serving as an iron ligand. Phenolate chelators often consist of o-di-hydroxyphenyl groups linked to an amino acid residue. Among the gram-negative bacteria, the most widely studied iron transport system has been that of _E. coli_ and its siderophore, enterochelin. This compound, the cyclic triester of 2,3-dihydroxybenzoylserine, is formed from a branch in the pathway for aromatic biosynthesis. The synthesis of natural iron chelators is derepressed by growing organisms on iron-deficient media, and an operon system has been proposed (44).

Because of the structural similarities and chelating properties shared between naturally occurring phenolate iron chelators and the dihydroxyphenyl compounds found to be stimulatory for aerotolerance, Bowdre _et al._ (3) proposed that the phenomenon of microaerophilism may reflect the inability of an organism to extract iron from its environment in sufficient amounts to permit adequate levels of iron-containing cell components necessary to initiate growth at higher

oxygen tensions. In a microaerobic environment, iron-containing enzymes such as catalase, cytochromes, and succinic dehydrogenase might not be needed in large concentrations to keep oxygen from reaching the interior of the cell. Survival at higher oxygen tensions, however, may depend upon the immediate availability of iron to permit adequate levels of any, or all, of these cell components. Thus, microaerophilic organisms such as C. fetus might not be able to synthesize ferric iron-binding compounds quickly enough or in sufficient quantities to initiate growth at 17 or 21% O_2. Bowdre et al. (3) also suggested that the need for additional iron for growth at higher oxygen tensions migth also reflect the need for higher levels of a ferri-superoxide dismutase, such as that found in the periplasmic space of E. coli (15).

Supporting this hypothesis, Kiggins and Plastridge (28) found catalase levels in C. fetus to decrease with incubation at higher oxygen tensions, i.e., under conditions where the greatest amount of H_2O_2 may be produced, less catalase was available. In addition, Cole and Rittenberg (7) demonstrated lower levels of cytochromes and tricarboxylic acid cycle enzymes in S. volutans than in two aerobic spirilla.

However plausible this hypothesis appears, another interpretation of the results can be made, viz., the additives might exert a direct effect on the medium. Ferric iron-EDTA complexes have been shown to possess superoxide dismutase activity (18). Bisulfite is known to degrade hydrogen peroxide (51), and sulfite, formed upon the addition of bisulfite to water, can scavenge superoxide radicals under certain

conditions (34). In addition, pyruvic acid can degrade hydrogen peroxide, producing acetate, CO_2, and water (61). Hematin, found by Border (2) to be stimulatory for growth of C. fetus under microaerobic conditions, is also capable of degrading peroxide (26, 27) and superoxide (18).

Thus, the media supplements might function in two ways: (i) to supply cells of C. fetus with sufficient iron to detoxify oxygen more efficiently, or (ii) to protect the cells against the toxic effects of oxygen or its byproducts by direct action on the culture medium. To examine these possibilities, Hoffman (20) has measured a number of physiological parameters of C. fetus strain H840 after growth at different oxygen tensions, with and without the media supplements.

On the basis of $^{55}FeCl_3$ uptake experiments, Hoffman found that nor-epinephrine did indeed make iron more available to the cell. Cells grown at 21% O_2 in the presence of the media additives showed higher rates of iron uptake in the presence of this chelator than it its absence. In view of the tendency for iron to form insoluble precipitates in aqueous systems, small molecular weight chelators such as nor-epinephrine would be expected to have this effect. These results tended to support the hypothesis that the availability of iron was related to aerotolerance. However, Hoffman also found that the supplemented medium did not cause any increase in the cytochrome levels of strain H840, and also that respiration rates with various tricarboxylic acid cycle intermediates were either the same or slightly lower. Determinations of the specific activities of tricarboxylic acid cycle enzymes gave similar results, and catalase levels were shown to

actually decrease. The only enzyme activity which was stimulated by the additives was superoxide dismutase (SOD), which had nearly doubled its activity in cells grown with $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid.

Although SOD activity in strain H840 increased in the presence of $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid, Hoffman nevertheless concluded that total SOD activity was probably not related to aerotolerance because of the following evidence: (i) when grown in the presence of the stimulatory compounds, H840 possessed the same level of SOD activity as *E. coli*, an oxygen-tolerant organism, (ii) a variant of H840 was found to have nearly double the SOD activity of the parent strain, yet was still microaerophilic, and (iii) although strain 436 of *C. fetus* was one of the more oxygen-sensitive strains, being unable to grow even at 6% O_2, this strain had a specific SOD activity as high as that of strain H840 which was more aerotolerant.

These results led Hoffman to consider the second hypothesis given above, viz., that the stimulatory compounds were exerting some direct action on the culture medium rather than enhancing some physiological process in the cells. Hoffman demonstrated that the supplemented *Brucella* medium possessed superoxide scavenging activity. whereas the unsupplemented medium possessed no such activity. Moreover, Hoffman showed that iron/nor-epinephrine complexes also had considerable superoxide scavenging ability. When superoxide radicals were generated in *Brucella* broth by photochemical means, as indicated by the reduction of nitroblue tetrazolium, a combination of iron and nor-epinephrine was able to inhibit the reduction of the dye. With *Brucella* agar,

$FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid similarly inhibited reduction of the dye. In *Brucella* broth where superoxide radicals had been generated photochemically, the growth of strain H840 was inhibited, but the media supplements alleviated this inhibition. Thus, the compounds which could scavenge superoxide radicals sere the same compounds which caused enhancement of aerotolerance in *C. fetus*. Therefore, Hoffman concluded that the action of these compounds was directed mainly toward the culture medium rather than toward *C. fetus* itself.

In the present study, powerful support for this hypothesis can be found in the results with SOD. When bovine SOD (i.e., SOD of the Cu-Zn type rather than the Fe or Mn type) was added to *Brucella* agar, the aerotolerance of *C. fetus* was greatly enhanced. Here, there was no addition of iron salts; moreover, the enzyme, being a protein, would not be able to penetrate cells of *C. fetus*. Consequently, its action must have been directed toward the culture medium. Additional support for this hypothesis is indicated by the alleviation of the inhibitory effect of light on the medium not only by SOD or by $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid, but also by nor-epinephrine and, to a much lesser extent, by catalase. Moreover, the inhibitory effect of light appeared to be oxygen-dependent, because illumination under anaerobic conditions did not result in inhibition of growth. On this basis, it seems likely that the inhibition of growth results from the production of oxygen-related compounds capable of reacting with SOD, presumably the superoxide radical itself. Furthermore, it appeared that the inhibition of

growth following illumination was not due to the accumulation of stable toxic compound in the medium since the addition of lyophilized *Brucella* broth which had been exposed to intense illumination and added to fresh *Brucella* agar at 45 C failed to inhibit growth. Similarly, the inhibition of growth did not appear to result from the destruction of a needed nutrient in the medium since *Brucella* broth which had been exposed to intense illumination and added to sterile agar (no nutrients) at 45 C, also did not inhibit growth.

In the present study, two iron chelators of microbial origin, ferrichrome and enterochelin, were shown to enhance the aerotolerance of strain H840. Using the dye-reduction assays indicated earlier, Hoffman (20) has shown that ferrichrome-iron complexes have weak superoxide scavenging ability. Although no activity was seen in assays of enterochelin, it is likely that once added to *Brucella* agar at 45 C and incubated at 37 C, enterochelin degradation products are produced. If partial or complete degradation were to occur under these conditions, the breakdown products of enterochelin, such as its monomer, dihydroxybenzoylserine, closely resemble nor-epinephrine in structure and therefore might be expected to have superoxide scavenging activity. In the case of ferrichrome, it is interesting that this compound had only weak superoxide scavenging activity and that it was the weaker natural chelator with regard to enhancement of aerotolerance.

C. fetus provides a unique and useful tool for the study of mechanisms of oxygen toxicity. It appears certain that oxygen radicals are involved in the aerotolerance of *C. fetus*; however, it is not known why this organism differs from other aerobes with regard to

sensitivity to these agents. Although no apparent correlation can be made between total SOD activity of cell-free extracts and the ability to tolerate oxygen, it is possible that initiation of growth at higher oxygen tensions is contingent upon not the quantity of total SOD available but the precise location of one of the isozymes of SOD within the cell. It seems likely that an isozyme within the periplasmic space would be highly beneficial in affording protection against exogenous superoxide radicals. In the case of strain 436, highly oxygen-sensitive yet having total SOD activity similar to that of more aerotolerant strains, the cells may not have the correct distribution of isozymes at the locations involved in oxygen damage.

Britton and Fridovich (4) have recently reevaluated the intracellular locations of SOD isozymes in E. coli. Determinations of SOD activities obtained following osmotic shock treatment and spheroplast formation failed to confirm previous reports that the Fe-containing SOD of E. coli was located in the periplasmic space. Similarly, treatment of intact cells with diazo-NDS, a reagent shown to inactivate some enzymes which are external, or are attached, to the plasma membrane, failed to inactivate the Fe-SOD; SOD isozymes from cell-free extracts were readily inactivated. Nevertheless, increased levels of the Fe-SOD have been correlated with increased resistance to exogenous superoxide radicals (17), regardless of its intracellular location. Assuming such a function for this isozyme, it seems possible that the Fe-SOD may be embedded in the plasma membrane, insensitive to diazo-NDS and not released by osmotic shock, yet still able to afford protection against exogenous superoxide radicals. In

the case of _C. fetus_, an increase in one of the isozymes may therefore be significant, as protection against exogenous radicals appears to be a critical factor associated with growth at higher oxygen tensions.

SUMMARY

In this study, the stimulatory effects of various compounds added to Brucella medium were tested for 62 strains of C. fetus, representing each subspecies, to test the desirability of such a supplemented medium for the clinical isolation of C. fetus.

A survey of the growth responses of these strains revealed a spectrum of oxygen tolerance within the species. Strains varied in aerotolerance from those capable of growth only at 6% O_2 on the supplemented medium to those capable of growth at 21% O_2 on unsupplemented Brucella agar. Determiniations of colony counts and colony diameters obtained after growth at different oxygen tensions indicated that Brucella agar supplemented with 0.025% (each) $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid (FBPA agar) supports better growth of C. fetus regardless of oxygen levels. Ca. 82% of the strains tested were capable of growth on FBPA agar under simulated candle jar conditions; of these strains, 98% gave colony counts at least half of those obtained on Brucella agar at 6% O_2. Although not all strains were capable of growth at oxygen tensions above 6% O_2 on FBPA agar, better growth was seen with this medium than with Brucella agar under conditions where growth did occur.

Brucella broth supplemented with $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid (0.2%, 0.025%, and 0.050%, respectively, FBPB broth) supported growth of 61 of 62 strains of C. fetus at 21% O_2 with static incubation. Ca. 23% of the strains failed to grow in unsupplemented

Brucella broth under these conditions. Where growth did occur in Brucella broth, more rapid growth rates were seen in FBPB broth.

Therefore, FBPA agar and FBPB broth are recommended for the clinical isolation of C. fetus. Although isolation from clinical sources is still dictated to some extent by the oxygen tension used for cultivation, improved recovery of C. fetus may be expected, regardless of facilities or equipment available.

Two iron chelators of microbial origin, ferrichrome and enterochelin, as well as SOD, were shown to be stimulatory for growth and aerotolerance of C. fetus strain H840. Also, Brucella agar supplemented with SOD, or with $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid, afforded protection against the oxygen-dependent inhibitory effect of light on Brucella medium. The results obtained support the hypothesis that the stimulatory effect of the media additives in enhancement of aerotolerance results from a direct action on the culture medium, and that toxic derivatives of oxygen, such as the superoxide radical or hydrogen peroxide, are involved in the microaerophilism of C. fetus.

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VITA

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Hugh A. George

FORMULATION OF IMPROVED MEDIA FOR ISOLATION

AND CULTIVATION OF CAMPYLOBACTER FETUS

by

Hugh A. George

(ABSTRACT)

Campylobacter fetus, a microaerophilic, Gram-negative rod, is a well-known cause of contagious abortion and infertility in cattle and sheep and is gaining increasing recognition as an opportunistic human pathogen. In the past, the unusual oxygen requirements of the organism have complicated its recovery from clinical sources; optimum recovery necessitates the use of special gas mixtures, vacuum pumps, etc., not routinely used in most laboratories. In this study, the stimulatory effects of compounds found to enhance aerotolerance and growth of C. fetus were tested for 62 strains of C. fetus, representing each subspecies, to test the desirability of supplementing conventional media with these additives. Brucella agar supplemented with 0.025% (each) $FeSO_4 \cdot 7H_2O$, sodium bisulfite, and pyruvic acid (FBPA agar) supported growth of 82% of the strains tested under simulated candle jar condtions. Brucella broth supplemented with 0.2% $FeSO_4 \cdot 7H_2O$, 0.025% sodium bisulfite, and 0.050% pyruvic acid (FBPB broth) supported growth of 61 of 62 strains at 21% O_2, 2.5% CO_2 with static incubation.

Therefore, FBPA agar and FBPB broth are recommended for the isolation and cultivation of C. fetus. Although isolation from

clinical sources is still dictated to some extent by the oxygen tension used for cultivation, improved recovery may be expected regardless of equipment or facilities available.

Another compound found to enhance aerotolerance of *C. fetus* was SOD. This finding supports the hypothesis that the stimulatory effect of the media additives results from a direct action on the culture medium by degrading toxic derivatives of oxygen, such as the superoxide radical and hydrogen peroxide.