

**EFFECT OF INCUBATION TEMPERATURE AND COMPOSITION OF BRUCELLA AGAR ON
GROWTH OF CAMPYLOBACTER JEJUNI**

by

Mann-Hsi Tso Lee

Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Microbiology

APPROVED:

Noel R. Krieg, Chairman

Robert M. Smibert

Joseph O. Falkinham III

June, 1987

Blacksburg, Virginia

921 928 31

**EFFECT OF INCUBATION TEMPERATURE AND COMPOSITION OF BRUCELLA AGAR ON
GROWTH OF CAMPYLOBACTER JEJUNI**

by

Mann-Hsi Tso Lee

Noel R. Krieg, Committee Chairman

(ABSTRACT)

Aerotolerance of Campylobacter jejuni ATCC 29428 and one of its aerotolerant mutants (strain MC711-01) was measured at 37°C and 42°C. The aerotolerance of C. jejuni was higher at 42°C than at 37°C.

Three different lots of Gibco dehydrated Brucella broth were used to prepare Brucella agar. The agar media were then tested to see if they differed in their ability to support growth of C. jejuni. However, only slight differences in viable counts of C. jejuni were obtained between lots.

Ageing of dehydrated Brucella medium for 2½ months and hydrated Brucella medium for 1½ months greatly affected the growth of C. jejuni and decreased its aerotolerance. This is probably due to the deterioration of the sodium bisulfite in Brucella medium during storage, because addition of 0.01% sodium bisulfite (the same amount as contained in the Brucella medium) to the aged medium (dehydrated or hydrated form) restored the ability of the medium to support growth of C. jejuni under various O₂ levels equivalent to or even better than that obtained with fresh Brucella medium. Moreover, when Brucella agar was prepared from

the individual chemical and peptone components, only the medium containing the 0.01% bisulfite yielded colony counts of C. jejuni similar to that obtained on fresh commercial Brucella medium. When sodium bisulfite was omitted, viable counts and aerotolerance were decreased.

ACKNOWLEDGEMENTS

I would like to give my deepest appreciation and admiration to my major professor and a true scholar and scientist who generously devoted his time and wisdom to his students. I am grateful for his guidance, support and encouragement throughout my graduate study. I would also like to thank for their helpful suggestions and encouragement as members of my graduate committee.

I am thankful to my parents, and my parents in law, for the support, understanding and encouragement they provided me, my husband and my children.

I would like to thank all my friends including the technical staff in this and other departments for their immeasurable help and friendship. They made life as a graduate student enjoyable.

Special thanks go to a graduate student in the statistics department, who did an excellent job in the statistical analyses for my research.

Above all, I thank my dear husband, and my lovely daughters, for their love, understanding and sacrifices.

Table of Contents

	Page
Abstract	ii
Acknowledgements.....	iv
List of Tables.....	vii
List of Figures.....	x
Introduction.....	1
Literature Cited.....	3
Literature Review.....	4
Characteristics of <u>Campylobacter jejuni</u>	4
Pathogenicity and Epidemiology of <u>Campylobacter jejuni</u>	5
Isolation and Cultivation of <u>Campylobacter jejuni</u>	7
Microaerophily and Oxygen Toxicity of <u>Campylobacter jejuni</u> ...	12
Literature Cited.....	14
<u>Campylobacter jejuni</u> : Microaerophile or Aerobe?.....	21
Introduction.....	22
Materials and Methods.....	23
Results.....	26
Discussion.....	36
Acknowledgements.....	40
Literature Cited.....	41
Appendix A. Growth Curves of <u>Campylobacter jejuni</u>	43
Appendix B. Additional Results.....	46
Appendix C. Statistical Analysis.....	58
Appendix D. Production of Different Gas Mixtures.....	63
Vita	65
	v

List of Tables

	page
Literature Review	
Table 1. Selective media used for isolation of <u>Campylobacter jejuni</u>	9
<u>Campylobacter jejuni</u> : Microaerophile or aerobe?	
Table 1. Effect of different lots of Brucella medium (GIBCO) on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 on Brucella agar plates incubated at 42°C at various oxygen levels (Test 1)..	27
Table 2 Effect of ageing of dehydrated Brucella medium on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 incubated at 42°C at various oxygen levels (Test 1).....	29
Table 3. Effect of ageing of dehydrated Brucella medium on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 incubated at 37°C at various oxygen levels (Test 1).....	30
Table 4. Effect of ageing of hydrated Brucella agar on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 incubated at 42°C at various oxygen levels (Test 1).....	32
Table 5. Effect of 0.01% sodium bisulfite in Brucella agar on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 incubated at 42°C at various oxygen levels (Test 1).....	35

Appendix B. Additional Results

Table B1. Effect of different lots of Brucella medium (GIBCO) on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 on brucella agar plates incubated at 42°C at various oxygen levels (Test 2)	47
Table B2. Effect of different lots of Brucella medium (GIBCO) on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 on brucella agar plates incubated at 37°C at various oxygen levels (Test 1)	48
Table B3. Effect of different lots of Brucella medium (GIBCO) on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 on brucella agar plates incubated at 37°C at various oxygen levels (Test 2)	49
Table B4. Effect of ageing of dehydrated Brucella medium on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 incubated at 42°C at various oxygen levels (Test 2).....	50
Table B5. Effect of ageing of dehydrated Brucella medium on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 incubated at 37°C at various oxygen levels (Test 2).....	51
Table B6. Effect of ageing of hydrated Brucella agar on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the	

mutant MC711-01 incubated at 42°C at various oxygen levels (Test 2).....	52
Table B7. Effect of ageing of hydrated Brucella agar on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 incubated at 37°C at various oxygen levels (Test 1).....	53
Table B8. Effect of ageing of hydrated Brucella agar on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 incubated at 37°C at various oxygen levels (Test 2).....	54
Table B9. Effect of 0.01% sodium bisulfite in Brucella agar on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 incubated at 42°C at various oxygen levels (Test 2).....	55
Table B10. Effect of 0.01% sodium bisulfite in Brucella agar on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 incubated at 37°C at various oxygen levels (Test 1).....	56
Table B11. Effect of 0.01% sodium bisulfite in Brucella agar on viable counts of <u>C. jejuni</u> ATCC 29428 the wild type and the mutant MC711-01 incubated at 37°C at various oxygen levels (Test 2).....	57
Appendix C. Statistical Analysis	
Table C1. Statistical analysis of effect of different lots of dehydrated Brucella broth (GIBCO) on viable counts of <u>C. jejuni</u>	59

<u>C. jejuni</u>	59
Table C2. Statistical analysis of effect of ageing of dehydrated Brucella broth on viable counts of <u>C.</u> <u>jejuni</u>	60
Table C3. Statistical analysis of effect of ageing of hydrated Brucella agar on viable counts of <u>C. jejuni</u>	61
Table C4. Statistical analysis of effect of 0.01% sodium bisulfite in Brucella agar on viable counts of <u>C.</u> <u>jejuni</u>	62

List of Figures

	page
Appendix A. Growth Curves of <u>Campylobacter jejuni</u>	
Figure A1. The growth curves of <u>C. jejuni</u> ATCC 29428 and its aerotolerant mutant MC711-01.....	45

INTRODUCTION

Campylobacters have been known to veterinarians since 1913 as important pathogens of domestic animals, causing infertility and abortion in cattle and sheep (Smibert 1984). Some species of Campylobacter were discovered to cause blood infections in humans and, at the time, it was suspected that they might also cause diarrhea in humans (King 1957; Levy 1946; Vincent et al. 1947). However, this was not proven until 1972 when the species Campylobacter jejuni was first isolated from human diarrheic stools by a selective method employing antibiotics (DeKeyser et al. 1972). Today, improved methods for the isolation and cultivation of this species have led to the isolation of hundreds of strains and have revealed that the species C. jejuni is a major cause of diarrhea in humans (Butzler et al. 1973; Skirrow 1977).

The late recognition of the clinical significance of C. jejuni was not only due to the lack of suitable selective methods but also to the special gas requirements for cultivation of this organism. C. jejuni is a microaerophile, growing only under an atmosphere containing a lower level of oxygen than that in air (Smibert 1984). The organism needs oxygen as a terminal electron acceptor to respire, but is poisoned by too much oxygen. The fundamental basis of the sensitivity to oxygen is not yet known, but it is known that the organism is highly sensitive to low levels of hydrogen peroxide or superoxide radicals in the culture medium (Hoffman et al. 1979). If aerotolerant mutants of C. jejuni could be obtained, then a comparison of the physiological differences between

the aerotolerant mutants and the microaerophilic wild type would be a useful approach for investigating the fundamental nature of microaerophily and oxygen toxicity in this species.

I was able to isolate several mutants of C. jejuni ATCC 29428 that were capable of growing aerobically on Brucella agar, a medium that is widely used to grow campylobacters. While assessing the degree of aerotolerance of the mutants and the wild type, I found that the viable counts (and thus my measurements of aerotolerance) varied considerably from one experiment to another. The basis for this inability to obtain reproducible results was unknown. Unless consistent viable counts could have been obtained, it would be difficult to measure aerotolerance and measure the survival of the wild type and aerotolerant mutants treated with hydrogen peroxide or superoxide radicals. Consequently, the objective of my research was to identify the factors responsible for the variation in viable counts and to determine the cultural conditions to obtain reproducible viable counts of Campylobacter jejuni.

Literature Cited

- BUTZLER, J. P., P. DEKEYSER, M. DETRAIN, AND F. DEHAEN. 1973. Related vibrio in stools. *J. Pediatr.* 82:493-495.
- DEKEYSER, P., M. GOUSSUIN-DETRAIN, J. P. BUTZLER AND J. STERNON. 1972. Acute enteritis due to related Vibrio: first positive stool culture. *J. Infect. Dis.* 125:390-392.
- HOFFMAN, P. S., H. A. GEORGE, N. R. KRIEG AND R. M. SMIBERT. 1979. Studies of the microaerophilic nature of Campylobacter fetus subsp. jejuni. II. Role of exogenous superoxide anions and hydrogen peroxide. *Can. J. Microbiol.* 25:8-16.
- KING, E. O. 1957. Human infections with Vibrio fetus and a closely related vibrio. *J. Infect. Dis.* 101:119-128.
- LEVY, A. J. 1946. A gastro-enteritis outbreak probably due to a bovine strain of vibrio. *Yale J. Biol. Med.* 18:243-258.
- SKIRROW, M. B. 1977. Campylobacter enteritis: a "new" disease. *Br. Med. J.* 2:9-11.
- SMIBERT, R. M. 1984. Genus Campylobacter (Sebald and Véron 1963). In Bergey's Manual of Systematic Bacteriology. Vol. 1. Edited by N. R. Krieg and J. G. Holt. The Williams and Wilkins Co., Baltimore, Maryland. pp. 111-118.
- VINZENT, R., J. DUMAS, and N. PICARD. 1947. Septicémie grave au cours de al grossesse due à un Vibrion. Avortement consécutif. *Bull. Acad. Natl. Méd. Paris* 131:90-92.

LITERATURE REVIEW

I. Characteristics of Campylobacter jejuni

Morphology. Campylobacter jejuni cells are small gram-negative, nonsporeforming, spirally curved rods. Cells range from 0.2-0.5 μm in width and 0.5-5.0 μm or longer in length and usually appear as comma-, S-, or gull-shaped. The longer forms are rare in log phase cultures growing in rich medium and they are often spiral-shaped and are comprised of a chain of single curved cells. C. jejuni cells exhibit a characteristic corkscrew-like darting motility by means of a single polar flagellum at one or both ends of the cell (Smibert 1984). When cultures enter stationary phase, the cells rapidly degenerate to nonculturable coccoid forms (Ogg 1962; Ng et al. 1985; Smibert 1984; Rollins et al. 1986). Temperature, pH and aeration of the medium significantly influence the formation of coccoid bodies (Ogg 1962; Smibert 1984). Rollins and Colwell (1986) believe that these coccoid bodies, although nonculturable, are viable, and that they are important in survival in the natural aquatic environment and may play important role in epidemiology.

Colonies are generally round, 1-2 mm in diameter flat to convex, tan to brownish, smooth and glistening. When media are wet or have a low agar concentration, swarming growth may occur that results in a thin, translucent veil of growth with an irregular edge (Smibert 1984).

Taxonomy and physiology. Campylobacters were once considered to belong to the genus Vibrio but are now known to be quite unrelated to

the members of this genus (Smibert 1984). Unlike Vibrio species, campylobacters are microaerophilic with a strictly respiratory type of metabolism (Smibert 1984). They require low level of O₂, usually between 1-20%, and 3-5% CO₂ for growth. They do not ferment or oxidize carbohydrates. Amino acids and tricarboxylic acid cycle intermediates are the primary energy sources. The mol% G + C of the DNA ranges from 30 to 38 (Smibert 1963, 1984).

Campylobacter jejuni grows best at 42°C to 45°C but cannot grow at 25°C. It is oxidase- and catalase-positive, sensitive to nalidixic acid (30 µg/disk), but resistant to cephalothin (30 µg/disk), hydrolyzes hippurate (Harvey 1980), cannot grow in Roop's minimal medium (Roop et al. 1984), and is unable to produce H₂S on TSI slants (Roop et al. 1984). The last three tests are thought to be the most reliable and allow differentiation of C. jejuni from the related species C. coli (Roop et al. 1984).

II. Pathogenicity and epidemiology of Campylobacter jejuni

Campylobacter jejuni causes infectious abortion in sheep and now is recognized as the leading cause of bacterial gastroenteritis in humans (Smibert 1984). Since its initial isolation from diarrheic stools by DeKeyser et al. (1972), it has been isolated with increasing frequency in clinical laboratories all over the world. A recent study also indicates C. jejuni causes travellers' diarrhea, especially in tropic areas (Speelman et al. 1984).

The symptoms and signs of a C. jejuni infection are not distinctive enough to permit diagnosis on those grounds alone. A wide

spectrum of signs and symptoms are presented by the patients. These signs and symptoms vary from asymptomatic infection or mild diarrhea lasting only 1-2 days to an enterocolitis with abdominal pain and bloody diarrhea which may last for several weeks (Butzler et al. 1973, Skirrow 1977, Mandal et al. 1984). Fever, vomiting and nausea may occur. Some complications such as reactive arthritis (Berden et al. 1979), erythema nodosum (Lambert et al. 1980), meningitis (Norrby et al. 1980) and urinary tract infection (Davies et al. 1979) associated with campylobacter enteritis have been reported. The variation in symptoms may be due to the pathogenicity of the strain involved (Itoh et al. 1982) or it may be related to the immune status of the host and the number of microorganisms ingested (Mouton et al. 1982).

Symptoms begin one to seven days after exposure to C. jejuni (Mandal et al. 1984). The organisms colonize and grow mainly in the distal ileum and colon. Transient bacteremia may occur in the early stage of the enteritis (Newell 1984, Mandal et al. 1984). Invasive capacity and activities of enterotoxin and cytotoxin of C. jejuni all have been noted, but the significance of these factors in the pathogenic mechanism requires further investigation (Newell 1984).

Most cases of campylobacter enteritis are self-limited and therapy is unnecessary. Severe cases such as prolonged or recurrent diarrhea as well as enterocolitis may require administration of fluids and chemotherapy. Erythromycin is the drug of choice since it eliminates campylobacters from patients' stools rapidly (Mandal et al. 1984). However, instances of erythromycin resistance have been reported (Vanhoof et al. 1982, Taylor et al. 1982). Gentamicin and

chloramphenicol are good alternatives to erythromycin but penicillin and cephalosporin are ineffective (Mandal et al. 1984).

Campylobacter enteritis is known to occur in all parts of the world and in all age group (Blaser et al. 1984). The incidence of the disease appears to be greater in young children especially in developing countries (Blaser et al. 1984), and it shows a seasonal peak of infection in warm weather (Smibert 1984). Although the organisms have been found in the intestinal tract of many wild and domestic warm blood animals, and outbreaks have been associated with the consumption of contaminated water, milk and food and especially poultry , the route of transmission still is questioned (Blaser et al. 1984). Most believe the organisms are transmitted by the fecal-oral route (Smibert 1984). Pathogenicity markers and a universally acceptable serotyping system are needed in order to gaining a better understanding of epidemiology of campylobacter enteritis.

III. Isolation and cultivation of Campylobacter jejuni

The isolation of C. jejuni from cases of enteritis is relatively simple if selective methods that suppress the growth of competing fecal flora are used and if microaerobic conditions are provided. Initially, DeKeyser et al. (1972) used a selective filtration technique (mean pore size of 0.65 μm) and cultured the filtrate on a selective medium containing antibiotics. Although the filtration procedure allowed only relatively small bacteria such as campylobacters to pass through, it was very time-consuming. Skirrow (1977) developed a highly selective medium

with multiple antibiotics which allowed successful isolation of C. jejuni directly without filtration.

Currently, several types of selective media are used widely (Table 1). Although different basal media are used, all the selective media contain various combinations of antibiotics to inhibit the normal fecal flora. Evaluation of some of these media for isolating C. jejuni have been the subjects of several recent publications (Well et al. 1982, Merino et al. 1986, Goossens et al. 1986). The isolation rates of campylobacters are usually similar among the tested media. However the various combinations of antibiotics influence the growth of competing fecal flora to different extents and therefore influence the ease of detection of suspect colonies. Goossens et al. (1986) suggested that a good selective medium for C. jejuni should contain antimicrobial agents against gram-negative bacteria (e.g., cefoperazone), gram-positive bacteria (e.g., rifampin or vancomycin) and yeasts (e.g., amphotericin B or cycloheximide). They also indicated that the blood-free Preston medium was as effective as blood-containing ones and would be valuable for use in developing countries, where sterile blood is difficult to get.

Ng and colleagues (1985) indicated that some Campylobacter strains were somewhat susceptible to antibiotics present in the selective media. Accordingly, a selective culture technique independent of antibiotics was proposed. This involved a filtration system (Steele et al. 1984, Goossens et al. 1986) in which the sample was merely placed on top of a 0.45- μ m membrane filter laying on an agar plate. However, the sensitivity of this method was low and it could not detect small numbers

Table 1. Selective Media Used for Isolation of Campylobacter jejuni

Name	Basal medium	Antibiotics	Other Supplements	Reference
Skirrow's medium	Blood agar base No. 2	Vancomycin, 10 µg/ml Polymyxin B, 2.5 IU/ml Trimethoprim, 5 µg/ml	5% lysed defibrinated horse blood	Skirrow (1977)
Butzler's medium	Thioglycolate medium + 1.05% agar	Bacitracin, 25 IU/ml Novobiocin, 5 µg/ml Actidione, 50 µg/ml Colistin, 10 IU/ml Cefazolin, 5 µg/ml	5-7% sheep blood	Butzler et al. (1979)
Campy-BAP (Blaser medium)	Brucella agar base	vancomycin, 10 µg/ml trimethoprim, 5 µg/ml polymyxin B, 2.5 IU/ml amphotericin B, 2 µg/ml cephalothin, 15 µg/ml	10% sheep blood	Blaser et al. (1979)
Preston medium	Nutrient broth No.2 + 1.2% New Zealand agar	rifampicin, 10 µg/ml polymyxin B, 5 IU/ml trimethoprim, 10 µg/ml cyclohexamide, 2 µg/ml	5% lysed defibrinated horse blood	Bolton et al. (1982)

TABLE 1, continued.....

Name	Basal medium	Antibiotics	Other Supplements	Reference
Butzler's medium Virion	Columbia agar base	cefoperazone, 15 µg/ml rifampicin, 10 µg/ml colistin, 10 IU/ml amphotericin B, 2 µg/ml	5-7% sheep blood	Goossens et al. (1983)
Preston blood-free medium	Nutrient broth No.2 + 1.2% New Zealand agar	cefoperazone, 32 µg/ml	charcoal 4 mg/ml ferrous sulfate, 0.25 mg/ml sodium pyruvate, 0.25 mg/ml casein hydrolysate, 3 mg/ml sodium deoxycholate, 1 mg/ml	Bolton et al. (1984)

of campylobacters in stools (Goosens et al. 1986).

Some enrichment media have been developed, such as Campy-Thio (Blaser et al. 1979), Bruce-Zochowsky medium (Hodge et al. 1984), alkaline peptone water (Philips et al. 1985) and semisolid enrichment medium (Chan et al. 1982, Shimada et al. 1986). These media have increased the ability to recover small numbers of campylobacters from foods and feces. The GAM semisolid medium developed by Shimada et al. (1986) can even recover as few as 3-8 cells per gram of feces or ml of milk aerobically.

The work of Agulla et al. (1987) showed that isolation rates with or without an enrichment procedure did not differ significantly. They suggested that enrichment media were not useful for the isolation of C. jejuni from fresh samples from acute diarrheal patients, but might be useful as transport media, or for specimens which contained very few organisms such as milk, food or water. Therefore, enrichment media may be of great value in epidemiological studies.

For cultivating C. jejuni, most basal media used to prepare the Campylobacter selective media are satisfactory. Blood or other supplements are not needed; however, blood or FBP supplemented agar plates give higher recovery rates and better growth (George et al. 1978, Smibert 1981). A chemically defined medium has been formulated by Smibert (1963). It contains 18 amino acids, salts, nicotinic acid and other B vitamins. However, a large inoculum (0.1 ml of a suspension having an optical density of 0.3 at 420 nm) was used to obtain growth in this defined medium.

C. jejuni is microaerophilic and capnophilic. An atmosphere containing 3-6% O₂ and 2-5% CO₂ is optimal for growth (Smibert 1981). Commercial gas-evolving generators (Campy Pak II gas generator system, Poly-bag system), evacuation-replacement system, candle jars and a system that utilizes Fortner's principle (Karmali et al. 1979) all have been used to generate the microaerobic atmosphere required for incubation of broth cultures and agar plates (Goossens et al. 1984).

For isolating C. jejuni, an incubation temperature of 42-43°C is better than 37°C (Smibert 1984). The isolation rate is lower at 37°C than at 42°C when the O₂ concentration is higher than 15%, such as occurs with a candle jar system (17% O₂ and 3% CO₂) (Wang et al. 1983). C. jejuni grows faster and colonies are larger at 42-43°C than 37°C, and the growth of many competing microorganisms is inhibited at 42-43°C (Goossens et al. 1984).

IV. Microaerophily and oxygen toxicity of Campylobacter jejuni

Oxygen is essential for many living organisms. However, many toxic derivatives of oxygen such as superoxide anions, hydroxyl radicals, singlet oxygen and hydrogen peroxide can be produced during cellular metabolism, or by autooxidation or photooxidation in the growth environment (Hoffman et al. 1979). Organisms can be damaged or killed if they do not have suitable mechanisms to cope with these toxic derivatives of oxygen.

C. jejuni is a microaerophile. It requires oxygen for growth, yet it is poisoned by the oxygen concentration in the air. Although the cells of C. jejuni possess catalase and superoxide dismutase (SOD)

activities, these enzymes seem unable to protect the organisms against the toxic effects of oxygen.

The physiological basis of microaerophily in C. jejuni is still unknown, but it is known that the organism is more sensitive to the toxic derivatives of oxygen generated in the growth medium than are aerotolerant organisms (Krieg and Hoffman 1986).

A variety of agents have been added to the growth medium of C. jejuni to increase the aerotolerance of this organism. These agents include nor-epinephrine (Bowdre et al. 1976); ferrous sulfate, sodium bisulfite and sodium pyruvate (George et al. 1978); catalase or SOD (Hoffman et al. 1979); lysed blood (Bolton et al. 1983); charcoal, ferrous sulfate and sodium pyruvate (Bolton et al. 1983); and sodium dithionite or histidine (Juven et al. 1985). Uninoculated Brucella medium contains some toxic forms of oxygen such as hydrogen peroxide and superoxide radicals probably generated during storage (Hoffman et al. 1979). All the supplements which can enhance the aerotolerance of C. jejuni are able to destroy some of these toxic forms of oxygen (Hoffman 1979, Bolton et al. 1984, Juven et al. 1985).

Literature Cited

- AGULLA, A. , F. J. MERINO, P. A. VILLASANTE, J. V. SAZ, A. DIAZ AND A. C. VELASCO. 1987. Evaluation of four enrichment media for isolation of Campylobacter jejuni. J. Clin. Microbiol. 25:174-175.
- BERDEN, J. H., H. I. MUYTJENS AND I, B. A. VAN DE PUTTE. 1979. Reactive arthritis associated with Campylobacter jejuni enteritis. Br. Med. J., 1:380-381.
- BLASER, M. J., I. D. BERKOWITZ, F. M. LAFORCE, J. CRAVENS, L. B. RELLER AND W. L. WANG. 1979. Campylobacter enteritis: clinical and epidemiological features. Ann. Intern. Med. 91:179-185.
- BLASER, M. J., D. N. TAYLOR AND R. A. FELDMAN. 1984. Epidemiology of Campylobacter infections. In Campylobacter infection in man and animals. Edited by J. P. Butzler. CRC Press, Inc., Boca Raton, FL. pp. 143-161.
- BOLTON, F. J. AND L. ROBERTSON. 1982. A selective medium for isolating Campylobacter jejuni/coli. J. Clin. Pathol. 35:462-467.
- BOLTON, F. J., D. COATES. 1983. Development of a blood-free Campylobacter medium: screening tests on basal media and supplements, and the ability of selected supplements to facilitate aerotolerance. J. Appl. Bacteriol. 54:115-125.
- BOLTON, F. J., D. COATES AND D. N. HUTCHINSON. 1984. The ability of campylobacter media supplements to neutralize photochemically induced toxicity and hydrogen peroxide. J. Appl. Bacteriol. 56:151-157.

- BOLTON, F. J., D. N. HUTCHINSON AND D. COATES. 1984. Blood-free selective medium for isolation of Campylobacter jejuni from feces. J. Clin. Microbiol. 19:169-171.
- BOWDRE, J. H., N. R. KRIEG, P. S. HOFFMAN AND R. M. SMIBERT. 1976. Stimulatory effect of dihydroxyphenyl compounds on the aerotolerance of Spirillum volutans and Campylobacter fetus spp. jejuni. Appl. Environ. Microbiol. 31:127-133.
- BUTZLER, J. P., P. DEKEYSER, M. DETRAIN AND F. DEHAEN. 1973. Related vibrio in stools. J. Pediatr., 82:493-495.
- BUTZLER, J. P. AND M. B. SKIRROW. 1979. Campylobacter enteritis. Clin. Gastroenterol. 8:737-765.
- CHAN, F. T. H. AND A. M. R. MACKENZIE. 1982. Enrichment medium and control system for isolation of Campylobacter fetus spp. jejuni from stools. J. Clin. Microbiol. 15:12-15.
- DAVIES, J. S. AND J. B. PENFOLD. 1979. Campylobacter urinary infection. Lancet. 1:1091-1092.
- DEKEYSER, P., M. GOUSSUIN-DETRAIN, J. P. BUTZLER AND J. STERNON. 1972. Acute enteritis due to related Vibrio: first positive stool culture. J. Infect. Dis. 125:390-392.
- GEORGE, H. A., P. S. HOFFMAN, R. M. SMIBERT AND N. R. KRIEG. 1978. Improved media for growth and aerotolerance of Campylobacter fetus. J. Clin. Microbiol. 8:36-41.
- GOOSSENS, H., M. DE BOECK AND J. P. BUTZLER. 1983. A new selective medium for the isolation of Campylobacter jejuni from human feces. Eur. J. Clin. Microbiol. 2:389-393.

- GOOSSENS, H., M. D. BOECK, H. V. LANDUYT AND J. P. BUTZLER. 1984. Isolation of Campylobacter jejuni from human feces. In Campylobacter infection in man and animals. Edited by J. P. Butzler. CRC Press, Inc., Boca Raton, FL. pp. 39-50.
- GOOSSENS, H., M. DE BROECK, H. COIGNAU, L. VLAES, C. V. BORRE AND J. P. BUTZLER. 1986. Modified selective medium for isolation of Campylobacter spp. from feces: comparison with Preston medium, a blood-free medium, and a filtration system. J. Clin. Microbiol. 24:840-843.
- HODGE, D. J. AND R. TERRO. 1984. Comparative efficacy of enrichment medium for isolation of Campylobacter jejuni. J. Clin. Microbiol. 19:434.
- HOFFMAN, P. S., H. A. GEORGE, N. R. KRIEG AND R. M. SMIBERT. 1979. Studies of the microaerophilic nature of Campylobacter fetus subsp. jejuni. II. Role of exogenous superoxide anions and hydrogen peroxide. Can. J. Microbiol. 25:8-16.
- ITOH, T., K. SAITO, Y. YANAGAWA, S. SAKAI AND M. OHASHI. 1982. Campylobacter enteritis in Tokyo. In Campylobacter: epidemiology, pathogenesis and biochemistry. Edited by D. G. Newell. MTP Press Ltd., Lancaster. pp. 5-12.
- JUVEN, B. J. AND I. ROSENTHAL. 1985. Effect of free-radical and oxygen scavengers on photochemically generated oxygen toxicity and on the aerotolerance of Campylobacter jejuni. J. Appl. Bacteriol. 59:413-419.

- KARMALI, M. A. AND P. C. FLEMING. 1979. Application of the Fortner principle to isolation of campylobacter from stools. *J. Clin. Microbiol.* 10:245-247.
- KRIEG, N. R. AND P. S. HOFFMAN. 1986. Microaerophily and oxygen toxicity. *Annu. Rev. Microbiol.* 40:107-130.
- LAMBERT, M., E. MARION, E. COCHE AND J. P. BUTZLER. 1982. Campylobacter enteritis and erythema nodosum. *Lancet.* 1:1409.
- MANDAL, B. K., P. DE MOL AND J. P. BUTZLER. 1984. Clinical aspects of Campylobacter infections in humans. In Campylobacter infection in man and animals. Edited by J. P. Butzler. CRC Press, Inc., Boca Raton, FL. pp. 21-32.
- MERINO, F. J., A. AGULLA, P. A. VILLASANTE, A. DIAZ, J. V. SAZ AND A. C. VELASCO. 1986. Comparative efficacy of seven selective media for isolating Campylobacter jejuni. *J. Clin. Microbiol.* 24:451-452.
- MOUTON, R. P., J. J. VELTKAMP, S. LAUWERS AND J. P. BUTZLER. 1982. Analysis of a small outbreak of campylobacter infections with high morbidity. In Campylobacter: epidemiology, pathogenesis and biochemistry. Edited by D. G. Newell. MTP Press Ltd., Lancaster. pp. 129-134.
- NEWELL, D. G. 1984. Experimental Studies of Campylobacter enteritis. In Campylobacter infection in man and animals. Edited by J. P. Butzler. CRC Press, Inc., Boca Raton, FL. pp. 113-132.
- NG, L.-K., R. SHERBURNE, D. E. TAYLOR AND M. E. STILES. 1985. Morphological forms and viability of Campylobacter species studied by electron microscopy. *J. Bacteriol.* 164:338-343.

- NG, L.-K., M. E. STILES AND D. E. TAYLOR. 1985. Inhibition of Campylobacter coli and Campylobacter jejuni by antibiotics used in selective growth media. J. Clin. Microbiol. 22:510-514.
- NORRBY, R., G. MCCLOSKEY, G. ZACKRISSON AND E. FALSEN. 1980. Meningitis caused by Campylobacter fetus spp. jejuni. Br. Med. J. 280:1164.
- OGG, J. E. 1962. Studies on the coccoid form of ovine Vibrio fetus I. cultural and serologic investigation. Am. J. Vet. Res. 23:354-358.
- PHILLIPS, E. AND P. NASH. 1985. Culture media. In: Manual of clinical microbiology. Edited by E. H. Lennette, A. Balows, W. J. Hausler and H. J. Shadomy. 4th ed. American society for Microbiology, Washington, D. C. pp. 1051-1092.
- ROLLINS, D. M. AND R. R. COLWELL. 1986. Viable but nonculturable stage of Campylobacter jejuni and its role in survival in the natural aquatic environment. Appl. Environ. Microbiol. 52:531-538.
- ROOP, R. M., II, R. M. SMIBERT, J. L. JOHNSON AND N. R. KRIEG. 1984. Differential characteristics of catalase-positive campylobacters correlated with DNA homology groups. Can. J. Microbiol. 30:938-951.
- SHIMADA, K. AND H. TSUJI. 1986. Enrichment for detection of Campylobacter jejuni. J. Clin. Microbiol. 23:887-890.
- SKIRROW, M. B. 1977. Campylobacter enteritis, a new disease. Br. Med.J., 2:9-11.
- SMIBERT, R. M. 1963. Nutrition of Vibrio fetus. J. Bacteriol. 85:394-398
- SMIBERT, R. M. 1978. The genus Campylobacter. Annu. Rev. Microbiol. 32:673-709.

- SMIBERT, R. M.** 1981. The genus Campylobacter. In: The prokaryotes: a handbook on habitats, isolation, and identification of bacteria. Edited by: M. P. Stolp, H. G. Trüper, A. Balows and H. G. Schlegel. Springer-Verlag, New York. pp. 609-617.
- SMIBERT, R. M.** 1984. Genus Campylobacter (Sebald and Véron 1963). In Bergey's Manual of Systematic Bacteriology. Vol. 1. Edited by N. R. Krieg and J. G. Holt. The Williams and Wilkins Co., Baltimore, Maryland. pp. 111-118.
- SPEELMAN, P. AND M. J. STRUELENS.** 1984. Campylobacter jejuni in travellers' diarrhea. In Campylobacter infection in man and animals. Edited by J. P. Butzler. CRC Press, Inc., Boca Raton, FL. pp. 33-38.
- STEELE, T. W. AND S. N. MEDERMOTT.** 1984. The use of membrane filters applied directly to the surface of agar plates for the isolation of Campylobacter jejuni from feces. Pathology 16:263-265.
- TAYLOR, D. E., S. A. DE GRANDIS, M. A. KARMALI, F. C. FLEMING, R. VANHOOF AND J. P. BUTZLER.** 1982. Erythromycin resistance in Campylobacter jejuni. In Campylobacter: epidemiology, pathogenesis and biochemistry. Edited by D. G. Newell. MTP Press Ltd., Lancaster. pp. 211-213.
- VANHOOF, R., H. COIGNAU, G. STAS AND J. P. BUTZLER.** 1982. Resistance pattern of different antimicrobial agent in Campylobacter jejuni from man and animals. In Campylobacter: epidemiology, pathogenesis and biochemistry. Edited by D. G. Newell. MTP Press Ltd., Lancaster. p. 155.

WANG, W. L. AND N. W. LUECHTEFELD. 1983. Effect of incubation atmosphere and temperature on isolation of Campylobacter jejuni from human stools. Can. J. Microbiol. 29:468-470.

WELLS, J. G., C. A. BOPP AND M. J. BLASER. 1982. Evaluation of selective medium for the isolation of Campylobacter jejuni. In Campylobacter: epidemiology, pathogenesis and biochemistry. Edited by D. G. Newell. MTP Press Ltd., Lancaster. pp. 80-82.

Campylobacter jejuni: Microaerophile or Aerobe?

MANN-HSI T. LEE

Microbiology & Immunology Section, Department of Biology, Virginia
Polytechnic Institute & State University, Blacksburg, VA, U.S.A. 24061

ROBERT M. SMIBERT

Department of Anaerobic Microbiology, Virginia Polytechnic Institute &
State University, Blacksburg, VA, U.S.A. 24061

AND

NOEL R. KRIEG¹

Microbiology & Immunology Section, Department of Biology, Virginia
Polytechnic Institute & State University, Blacksburg, VA, U.S.A. 24061

¹Author to whom all correspondence should be addressed.

INTRODUCTION

Campylobacter jejuni is a microaerophile, growing only under an atmosphere containing a lower level of oxygen than that in air. The organism needs oxygen as a terminal electron acceptor to respire, but is poisoned by too much oxygen (Smibert 1984). The fundamental basis of the sensitivity to oxygen is not yet known, but it is known that the organism is highly sensitive to low levels of hydrogen peroxide or superoxide radicals in the culture medium (Hoffman et al. 1979). If aerotolerant mutants of C. jejuni could be obtained, then a comparison of the physiological characteristics between the mutants and the microaerophilic wild type would be a useful approach for investigating the fundamental nature of microaerophily and oxygen toxicity.

Several aerotolerant mutants of C. jejuni ATCC 29428 were isolated on Brucella agar. However, the measurements of aerotolerance (by viable counts with incubation under different conditions of aerobiosis) of the mutants and the wild type, i.e., the viable counts varied considerably from one experiment to another. Because the basis for this inability to obtain reproducible results was unknown and consistent viable counts were required to assess aerotolerance, the present study had as its goal the identification of the factors responsible for the variation in viable counts and aerotolerance of Campylobacter jejuni.

MATERIALS AND METHODS

Organisms and selection of an aerotolerant mutant

Campylobacter jejuni ATCC 29438 and its aerotolerant mutant MC711-01 were used in this study. The aerotolerant mutant was selected by use of the mutagen ethylmethanesulfonate (EMS). A 10-ml 24-hr-old broth culture was incubated with 4% EMS for 1 h, centrifuged at 12,000 X g for 5 min, and cells were washed twice with 10-ml portions of Brucella broth (Gibco). The cell suspension was incubated at 37°C under microaerobic condition (i.e. 6% O₂, 3.6% CO₂, 91.4% N₂) for 24 h. After incubation, mutants were selected by inoculating the culture into 100 ml of Brucella broth stirred under an atmosphere of 21% O₂, 3.6% O₂ and 75.4% N₂ at 37°C for 24 h. Serial passages were made daily, except that the inoculum was decreased each time until only 0.05 ml was used as inoculum. Despite the decreased inoculum size the culture continued to become turbid in 24 h. In addition the O₂ level was increased by 2.5% for each transfer, until the atmosphere contained 33.5% O₂. Finally the culture was streaked onto Brucella agar and incubated under 21% O₂ or higher O₂ level to select the aerotolerant mutants. Mutant MC711-01 was from a colony isolated on a Brucella agar plate which had been incubated under 33.5% O₂, 3.6% CO₂ and 63% N₂ at 37°C.

In order to confirm MC711-01 was an aerotolerant mutant, not a stable adaptation, it was transferred three times under microaerobic (nonselective) conditions. Similar plate counts on Brucella agar plates were subsequently obtained under both 6% O₂ and 21% O₂.

The identity of MC711-01 as C. jejuni was confirmed by appropriate biochemical tests (Roop et al. 1984).

Stock cultures of the wild type and mutant were maintained by weekly transfer in semisolid Brucella medium at 37°C.

Gaseous condition

All cultures were incubated under gas atmospheres containing 3.6% CO₂ and various levels of O₂ and N₂. O₂ concentrations were verified with an oxygen electrode.

Viable counts

Measurements of viable counts were done in triplicate for all experiments. Each experiment was performed twice on two consecutive days (called test 1 and test 2) in order to confirm the reproducibility of each result.

The medium for estimation of viable counts was Brucella broth (Gibco) solidified with 1.5% (w/v) agar (Gibco). In some instances Brucella agar was prepared from individual components obtained from Gibco. In order to have same amount of ingredients (such as the amount of sodium bisulfite added to the aged medium) in Brucella agar of test 1 and test 2, the Brucella agar of test 1 and test 2 was prepared at same time. The media were autoclaved and allowed to solidify in 100-ml square dilution bottles. The media for test 1 were melted and 20-ml portions were dispensed into 100 x 15-mm Petri plates on the next day; the media for test 2 were melted and dispensed one day later. All plates were

allowed to dry at room temperature in the dark for two days prior to use.

Cultures were grown at 37°C without agitation in Brucella broth under 6% O₂ to the midlog phase [20 nephelometer turbidity units (NTU) for the wild-type culture and 14 NTU for the aerotolerant mutant]. Phase microscopy indicated that these cultures contained < 1% coccoid bodies. The cultures were diluted to 1:10⁵ dilution in Brucella broth and 0.1 ml was spread onto the surface of Brucella agar plates. All plates were incubated at 37°C and 42°C.

Statistical analysis

Two way ANOVA with Fisher's least significant difference (LSD) (a multiple comparison procedure) was used to compare the groups within each test for each organism. The interaction between O₂ levels and groups of the two way ANOVA was used to test the pattern over test 1 and test 2. General linear contrast was used to do the comparison between two special sets of data within each test for each organism.

RESULTS

The results from test 1 and test 2 for any given experiment showed a similar pattern in all cases; therefore, only the results of test 1 are presented here. Test 2 sometimes showed lower colony counts than test 1 at the highest O₂ level (21% O₂ for the wild type or 26% O₂ for the mutant). This is probably because that the Brucella agar of test 1 and test 2 was prepared at the same time, the hydrated medium of test 2 was one day older than that of test 1. However, the pattern of response to temperature, O₂ level, or other factors being tested was similar to that of test 1.

In estimating the effect of an incubation temperature of 37°C vs. 42°C on the aerotolerance of the wild type and its aerotolerant mutant of C. jejuni, a consistent pattern in all tests was observed. A greater degree of aerotolerance occurred at 42°C. Consequently, this effect of incubation temperature is shown only once in this section of the thesis, viz., in the experiment dealing with the effect of ageing of dehydrated Brucella medium on growth. For other experiments only the results of 42°C are presented.

Effect of different lots of dehydrated Brucella broth (Gibco) on viable counts of C. jejuni

Viable counts were obtained using Brucella agar which was prepared from freshly opened jars of three different lots of dehydrated Brucella broth (Gibco): lot # 580147 ("lot 1"), lot # 580483 ("lot 2") and lot # 680108 ("lot 3"). The inoculated plates were incubated at 42°C under four different O₂ concentrations as shown in Table 1. The three lots

TABLE 1. Effect of different lots of Brucella medium (GIBCO) on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 42°C at various oxygen levels (Test 1)

Dehydrated Brucella medium from freshly opened jar of different lots	Mean CFU/ml \pm standard deviation $\times 10^{-6}$ with incubation at oxygen levels of:			
	Strain ATCC 29428			
	6%	15%	21%	26%
Lot # 580147 (lot 1)	207 \pm 31	219 \pm 10	139 \pm 50	0 \pm 0
Lot # 580483 (lot 2)	215 \pm 30	201 \pm 25	93 \pm 9	0 \pm 0
Lot # 680108 (lot 3)	195 \pm 26	175 \pm 23	130 \pm 13	0 \pm 0
	Strain MC711-01			
	6%	15%	21%	26%
Lot # 580147 (lot 1)	157 \pm 43	148 \pm 37	152 \pm 21	157 \pm 10
Lot # 580483 (lot 2)	161 \pm 18	198 \pm 15	173 \pm 16	131 \pm 33
Lot # 680108 (lot 3)	157 \pm 15	150 \pm 19	159 \pm 38	163 \pm 16

exhibited only slight differences in viable counts of the wild type C. jejuni. Colony counts of the wild type differed significantly between lots 1 and 2 when plates were incubated under 21% O₂. By contrast, colony counts of the aerotolerant mutant showed no significant differences between the three different lots of medium ($\alpha = 0.05$).

Effect of ageing of dehydrated Brucella broth (Gibco) on viable counts of C. jejuni

In this experiment three differently aged batches of Brucella agar were used. Three batches all were made from the lot 1 dehydrate Brucella broth (Gibco) of Table 1. The first batch was prepared from a freshly opened (i.e. at the time of experiment) jar. The dehydrated media of batches 2 and 3 had been stored in a foil-covered beaker for 2.5 months, and mixed one or two times each week. The third batch differed from the second in that sodium bisulfite (the same amount as contained in the Brucella medium) was added into the stored dehydrated medium to a final concentration of 0.01% at the time of preparation of the hydrated Brucella agar. After preparation of the media and pouring the plates in the dark, the plates were dried for 48 h, inoculated, and incubated under four different O₂ levels at 42°C (Table 2) and at 37°C (Table 3).

Ageing of dehydrated Brucella medium greatly affected the aerotolerance and viable counts of C. jejuni. At 42°C (Table 2), the wild type grew poorly at 15% O₂ and not at all at 21% O₂ on the medium prepared from batch # 2 (aged 2.5 months). The aerotolerant mutant also grew poorly at 21% O₂ and hardly at all at 26% O₂ on this medium. When plates were incubated at 37°C rather than 42°C, the inhibitory effect of

TABLE 2. Effect of ageing of dehydrated Brucella medium on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 42°C at various oxygen levels (Test 1)

Treatment of dehydrated Brucella broth (GIBCO) ^b	Mean CFU/ml ± standard deviation x 10 ⁻⁶ with incubation at oxygen levels of:			
	Strain ATCC 29428			
	6%	15%	21%	26%
Batch 1: control Direct from freshly opened jar	206 ± 30	176 ± 27	2 ± 2 ^a	0 ± 0
Batch 2 Stored in foil-covered beaker for 2½ months	253 ± 18	32 ± 20 ^a	0 ± 0	0 ± 0
Batch 3 Stored in foil-covered beaker for 2½ months and supplemented with 0.01% bisulfite	214 ± 23	224 ± 36	94 ± 17 ^a	0 ± 0
	Strain MC711-01			
	6%	15%	21%	26%
Batch 1: control Direct from freshly opened jar	126 ± 7	168 ± 25	165 ± 20	175 ± 9
Batch 2 Stored in foil-covered beaker for 2½ months	112 ± 12 ^a	131 ± 25	89 ± 8 ^a	1 ± 1 ^a
Batch 3 Stored in foil-covered beaker for 2½ months and supplemented with 0.01% bisulfite	145 ± 21 ^a	169 ± 18	136 ± 11	179 ± 16

^a Colonies were smaller (≤ 0.6 mm diameter) than usual (0.7 to 1.5 mm diameter at 42°C).

^b Batches 1, 2 and 3 were made from the dehydrated lot 1 Brucella broth (Gibco) of Table 1.

TABLE 3. Effect of ageing of dehydrated Brucella medium on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 37°C at various oxygen levels (Test 1)

Treatment of dehydrated Brucella broth (GIBCO) ^b	Mean CFU/ml ± standard deviation x 10 ⁻⁶ with incubation at oxygen levels of:			
	Strain ATCC 29428			
	3%	6%	15%	21%
Batch 1: control Direct from freshly opened jar	211 ± 31	223 ± 23	0 ± 0	0 ± 0
Batch 2 Stored in foil-covered beaker for 2½ months	163 ± 31 ^a	0 ± 0	0 ± 0	0 ± 0
Batch 3 Stored in foil-covered beaker for 2½ months and supplemented with 0.01% bisulfite	207 ± 10	190 ± 16	87 ± 5 ^a	0 ± 0
	Strain MC711-01			
	6%	15%	21%	26%
Batch 1: control Direct from freshly opened jar	126 ± 4	130 ± 7	82 ± 15	0 ± 0
Batch 2 Stored in foil-covered beaker for 2½ months	141 ± 9	0 ± 0	0 ± 0	0 ± 0
Batch 3 Stored in foil-covered beaker for 2½ months and supplemented with 0.01% bisulfite	157 ± 11	133 ± 20	88 ± 18	23 ± 5 ^a

^a Colonies were smaller (≤ 0.6 mm diameter) than usual (0.7 to 1.0 mm diameter at 37°C).

^b Batches 1, 2 and 3 were made from the dehydrated lot 1 Brucella broth (Gibco) of Table 1.

batch # 2 was even more evident. Although 6% O₂ has been considered as the optimal O₂ concentration for cultivating C. jejuni, the wild type plated on batch # 2 medium and incubated at 37°C, failed to form colonies under 6% O₂. Under the same conditions, the aerotolerant mutant grew under 6% O₂ and not at all at 15% O₂ or higher. Addition of 0.01% sodium bisulfite to the aged medium restored the ability of the medium to support growth of C. jejuni equivalent to or even better than that obtained with the fresh Brucella medium in the case of both the wild type and the mutant (Tables 2 and 3).

Effect of incubation temperature

C. jejuni was found to exhibit a higher degree of aerotolerance at 42°C than at 37°C (compare Table 2 with Table 3). At 42°C, on the freshly prepared medium (batch # 1) the wild type grew well at 15% O₂ (Table 2) and even formed a few colonies on 21% O₂. However, when grown at 37°C (Table 3), its aerotolerance decreased and it grew well only at 6% O₂, with no growth occurring at 15% and 21% O₂. In the case of the aerotolerant mutant, a similar effect was shown: at 42°C the mutant grew well at 26% O₂, whereas at 37°C it only grew at 15% O₂, poorly at 21% O₂, and not at all at 26% O₂. The beneficial effect of the 42°C incubation temperature was also evident with the other two batches of media (batches # 2 and # 3).

Effect of ageing of hydrated Brucella agar on viable counts of C. jejuni

TABLE 4. Effect of ageing of hydrated Brucella agar on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 42°C at various oxygen levels (Test 1)

Treatment of hydrated Brucella agar	Mean CFU/ml \pm standard deviation $\times 10^{-6}$ with incubation at oxygen levels of:			
	Strain ATCC 29428			
	6%	15%	21%	26%
Batch 1: control	238 \pm 23	223 \pm 23	76 \pm 13 ^a	0 \pm 0
Batch 2: (D) ^b	242 \pm 37	148 \pm 3	0 \pm 0	0 \pm 0
Batch 3: (D+b) ^b	229 \pm 52	200 \pm 26	245 \pm 6	0 \pm 0
Batch 4: (L) ^b	237 \pm 29	173 \pm 19	0 \pm 0	0 \pm 0
Batch 5: (L+b) ^b	221 \pm 24	193 \pm 24	103 \pm 17 ^a	0 \pm 0
	Strain MC711-01			
	6%	15%	21%	26%
Batch 1: control	159 \pm 22	162 \pm 36	171 \pm 12	144 \pm 19
Batch 2: (D)	190 \pm 30	184 \pm 29	178 \pm 48	127 \pm 14
Batch 3: (D+b)	129 \pm 34	192 \pm 34	134 \pm 33	134 \pm 6
Batch 4: (L)	132 \pm 28	168 \pm 18	163 \pm 35	0 \pm 0
Batch 5: (L+b)	56 \pm 5 ^a	156 \pm 9	144 \pm 21	12 \pm 6 ^a

^a Colonies were smaller (≤ 0.6 mm diameter) than usual (0.7 to 1.5 mm diameter at 42°C).

^b D: The hydrated Brucella agar had been stored in dark for 1½ month.
 D+b: The batch 2 medium was added filter-sterilized 0.01% sodium bisulfite before it was melted to prepare the agar plates.
 L: The hydrated Brucella agar had been stored under diffused sun light for 1½ month.
 L+b: The batch 4 medium was added filter-sterilized 0.01% sodium bisulfite before it was melted to prepare the agar plates.

This experiment was done to determine the effect of ageing the hydrated medium rather than dehydrated powder, on growth of C. jejuni. Five batches of Brucella agar were prepared (Table 4). For batch # 1, Brucella agar was freshly prepared at the time of experiment. For batches 2 to 5, the medium was prepared 1½ months earlier. The agar medium was stored in square dilution bottles with the cap screwed on tightly. Batches 2 and 3 were stored in dark, whereas batches 4 and 5 were stored under diffused sun light (on a window sill). At the time of experiment and before melting the agar, filter-sterilized sodium bisulfite was added to batches 3 and 5 to a final concentration of 0.01%. All the media for each experiment were melted and dispensed onto plates in the dark at same time. The inoculated plates were incubated under four different O₂ levels as shown in Table 4.

Ageing of the hydrated medium affected growth of the wild type C. jejuni adversely. Decreased colony counts were obtained on batches 2 and 4 at 15% O₂ and no growth occurred at 21% O₂. It made little difference whether the medium had been stored in the light or the dark. When the aged medium was supplemented with 0.01% sodium bisulfite, the inhibitory effect of ageing was reversed. The plate counts of the wild type were similar to those on the control medium (batch # 1) or even greater. In the case of the aerotolerant mutant, somewhat different results were obtained. Ageing of hydrated Brucella agar in the light did decrease the the growth of the mutant (e.g. no colonies grew at 26% O₂); however, addition of 0.01% sodium bisulfite produced only a slight alleviation of the inhibition instead of the much greater alleviation it had exhibited with the wild type. Further, ageing of hydrated Brucella agar in the dark

did not reduce viable counts, whereas such ageing did cause significant inhibition in the case of the wild type.

Effect of 0.01% sodium bisulfite in Brucella agar on viable counts of C. jejuni

Sodium bisulfite is a normal component of Brucella medium and is present at a concentration of 0.01%. In order to study the effect of this small amount of sodium bisulfite on the growth and aerotolerance of C. jejuni, three different batches of Brucella agar were prepared. The first batch was prepared from dehydrated Brucella broth (Gibco). Batches 2 and 3 were prepared "from scratch", i.e., from the individual components of Brucella broth (Gibco peptone 20, 15.0 g/l; Gibco peptone 100, 5.0 g/l; dextrose, 1.0 g/l; Gibco yeast extract, 2.0 g/l; sodium chloride, 5.0 g/l; sodium bisulfite, 0.1 g/l; sodium citrate, 1.0 g/l). Batch 3 did not contain the sodium bisulfite, whereas batch 2 did contain this component.

The inoculated plates were incubated at four different O₂ levels as shown in Table 5. The presence of the 0.01% sodium bisulfite greatly increased the aerotolerance of both the wild type and the mutant strain C. jejuni. The wild type, did not grow at 15% and 21% O₂ unless the bisulfite was present. The mutant showed markedly decreased plate counts in the absence of bisulfite when incubated at 21% and 26% O₂. When the medium contained sodium bisulfite, it supported growth of the mutant to a level similar to that for the control; the wild type grew even better than on the control medium when the bisulfite was present.

TABLE 5. Effect of 0.01% sodium bisulfite in Brucella agar on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 42°C at various oxygen levels (Test 1)

Type of Brucella agar	Mean CFU/ml \pm standard deviation $\times 10^{-6}$ with incubation at oxygen levels of:			
	Strain ATCC 29428			
	3%	6%	15%	21%
Batch 1: control Brucella agar from Gibco Brucella broth	139 \pm 14	169 \pm 23	152 \pm 14	72 \pm 13 ^a
Batch 2 Brucella agar from scratch with 0.01% sodium bisulfite	197 \pm 24	195 \pm 29	186 \pm 19	126 \pm 10 ^a
Batch 3 Brucella agar from scratch without 0.01% sodium bisulfite	174 \pm 38	160 \pm 16	0 \pm 0	0 \pm 0
	Strain MC711-01			
	6%	15%	21%	26%
Batch 1: control Brucella agar from Gibco Brucella broth	151 \pm 22	137 \pm 20	154 \pm 20	123 \pm 15
Batch 2 Brucella agar from scratch with 0.01% sodium bisulfite	159 \pm 22	149 \pm 15	145 \pm 37	146 \pm 24
Batch 3 Brucella agar from scratch without 0.01% sodium bisulfite	160 \pm 16	122 \pm 48	50 \pm 17 ^a	31 \pm 7 ^a

^a Colonies were smaller (≤ 0.6 mm diameter) than usual (0.7 to 1.5 mm diameter at 42°C).

DISCUSSION

For isolation C. jejuni, incubation at 42°C is better than 37°C (Smibert 1984). However, growth curves for C. jejuni grown in Brucella broth at 42°C and 37°C do not differ significantly (data shown in appendix A). This suggests that 42°C is better than 37°C only when growing C. jejuni on solid medium. The isolation rate of C. jejuni on Cam-BAP agar plates is lower at 37°C than at 42°C when the O₂ concentration is higher than 15%, such as occurs with a candle jar system (17% O₂ and 3% CO₂) (Wang et al. 1983). C. jejuni grows faster and colonies are larger at 42-43°C than 37°C on agar plates, and the growth of many competing microorganisms is inhibited at 42-43°C (Goossens et al. 1984). However the physiological basis for the beneficial effect of 42°C incubation on solid medium is not known. My results show that the higher incubation temperature increases the aerotolerance of C. jejuni. Although C. jejuni requires O₂ to respire, initiation of growth of microaerophiles on agar media is very O₂-sensitive (George et al. 1978; Padgett et al. 1982). Since the aerotolerance of C. jejuni is higher at 42°C than at 37°C, it is probable that the sensitivity of the isolation solid medium is improved at 42°C, especially when the O₂ concentration is relatively high, as with the use of a candle jar.

Brucella medium contains 0.01% sodium bisulfite as part of its normal formulation, although I have been unable to discover who originally devised the formulation and why it was used. It is known that the addition of sodium bisulfite to Brucella medium to give a total concentration of 0.035% greatly enhances the aerotolerance of C. jejuni (George et al. 1978). When the viable counts of C. jejuni on Brucella

agar varied from one experiment to another, it was suspected that the amount of sodium bisulfite in Brucella medium might have varied between lots and/or the sodium bisulfite might have been unstable during storage, thus causing the variation. Differences in the amount of the bisulfite included in the commercial medium might have occurred during its manufacture. The three different lots of Gibco dehydrated Brucella broth used in our experiment were doubtless prepared at different times by the company. How long they had been stored in their sealed bottles was unknown. However, in my experiments these lots exhibited only slight differences with regard to viable counts of C. jejuni. Thus, lot variation seems not very important, at least for the three different lots examined. It is possible, of course, that if more lots had been examined, a greater degree of variation in growth-promoting ability might have been found.

Another possible factor causing the variation in the viable counts of C. jejuni was that the sodium bisulfite might have deteriorated during laboratory storage, especially when the medium was exposed to air and moisture. Dehydrated Brucella medium is very hygroscopic, and sodium bisulfite is known to oxidize rapidly to sodium sulfate in the presence of moisture and O₂ (Windholz et al. 1983). My results showed that ageing of dehydrated and hydrated Brucella medium did affect the growth of C. jejuni and decreased its aerotolerance. Moreover, addition of 0.01% sodium bisulfite to the aged media (dehydrated and hydrated forms) restored their ability to support normal or even better growth. These results support the hypothesis that sodium bisulfite in Brucella medium deteriorates during storage and causes the decreased colony counts of C.

jejuni on Brucella agar. Sterile Brucella agar contains more toxic forms of O₂ if stored in light and air than in the dark or under an anaerobic atmosphere (Hoffman et al. 1979). Colony counts of C. jejuni were much reduced on plates stored in light and air than on plates stored in dark (Bolton et al. 1984; Juven et al. 1985). In my experiments, the colony counts of C. jejuni on plates prepared from aged hydrated Brucella medium stored in light and dark did not show the differences that we expected. This was probably because the hydrated agar was stored in dilution bottles with the cap screwed on tightly. Thus, these bottles contained little O₂ and the surface area exposed to the O₂ was much smaller compared to that of agar plates. Another possibility was that the toxic forms of O₂ generated by photooxidation were destroyed during the heating procedure when the agar was melted prior to pouring the plates.

When Brucella agar was prepared from "scratch", i.e., from the individual components, without the sodium bisulfite, decreased viable counts and aerotolerance of C. jejuni were obtained. However, when the Brucella agar was prepared with the 0.01% sodium bisulfite, it supported normal growth of C. jejuni, growth that was as good or better as that with the fresh commercial medium. These results again support the hypothesis that sodium bisulfite in Brucella agar may deteriorate during storage and thus affect the growth of C. jejuni. Moreover, the results confirm that the small amount of sodium bisulfite that is normally a component of Brucella medium is very important for the ability of Brucella medium to support the growth of C. jejuni. It strongly influences the aerotolerance of C. jejuni. It is known that the

uninoculated growth medium contains some toxic forms of O_2 such as H_2O_2 and superoxide radicals probably generated during storage (Hoffman et al. 1979). All supplements added to campylobacter media which enhance aerotolerance do so by quenching the toxic forms of O_2 (Hoffman et al. 1979; Bolton et al. 1984). Sodium bisulfite is proposed to act with iron salt in the Brucella medium and has the ability to destroy superoxide radicals (Hoffman et al. 1979).

The effectiveness of bisulfite depends on the particular kind of medium to which it is added. Bolton et al. (1984) added 0.025% sodium bisulfite to nutrient agar and obtained enhanced growth and aerotolerance of C. jejuni; however, the bisulfite did not prevent or eliminate the toxicity generated in nutrient agar that had been subjected to illumination. Juven et al. (1985) added superoxide dismutase (SOD) to nutrient agar and found that this did not improve the growth of C. jejuni on the photo-oxidized medium. Thus superoxide radicals are probably not involved in photochemically damaged nutrient agar. However, in Brucella agar, SOD is highly effective in enhancing the growth of C. jejuni on illuminated medium (Hoffman et al. 1979).

My results suggest that the sodium bisulfite that is present in commercial dehydrated Brucella agar may deteriorate during storage. After the bottle of the dehydrated Brucella medium have been opened, and also after the hydrated form of the medium has been prepared, the shelf life is shortened. My results also indicated that, to counteract the effect of bisulfite deterioration, 0.01% sodium bisulfite should routinely be added to the medium during its preparation to ensure good growth of C. jejuni.

Acknowledgements

The statistical analysis of the study was done in cooperation with the VPI statistical consulting center. The author is thankful to Mike Rozum for the statistical analyses done by him.

- BOLTON, F. J., D. COATES AND D. N. HUTCHINSON. 1984. The ability of campylobacter media supplements to neutralize photochemically induced toxicity and hydrogen peroxide. *J. Appl. Bacteriol.* 56:151-157.
- GEORGE, H. A., P. S. HOFFMAN, R. M. SMIBERT AND N. R. KRIEG. 1978. Improved media for growth and aerotolerance of Campylobacter fetus. *J. Clin. Microbiol.* 8:36-41.
- GOOSSENS, H., M. D. BOECK, H. V. LANDUYT AND J. P. BUTZLER. 1984. Isolation of Campylobacter jejuni from human feces. In Campylobacter infection in man and animals. Edited by J. P. Butzler. CRC Press, Inc., Boca Raton, FL. pp. 39-50.
- HOFFMAN, P. S., H. A. GEORGE, N. R. KRIEG AND R. M. SMIBERT. 1979. Studies of the microaerophilic nature of Campylobacter fetus spp. jejuni. II. Role of exogenous superoxide anions and hydrogen peroxide. *Can. J. Microbiol.* 25:8-16.
- JUVEN, B. J. AND I. ROSENTHAL. 1985. Effect of free-radical and oxygen scavengers on photochemically generated oxygen toxicity and on the aerotolerance of Campylobacter jejuni. *J. Appl. Bacteriol.* 59:413-419.
- PADGETT, P. J., W. H. COVER, N. R. KRIEG. 1982. The microaerophile Spirillum volutans: cultivation on complex liquid and solid media. *Appl. Environ. Microbiol.* 43:469-477.
- ROOP, R. M., II, R. M. SMIBERT, J. L. JOHNSON AND N. R. KRIEG. 1984. Differential characteristics of catalase-positive campylobacters correlated with DNA homology groups. *Can. J. Microbiol.* 30:938-951.

- SMIBERT, R. M.** 1984. Genus Campylobacter (Sebald and Véron 1963). In Bergey's Manual of Systematic Bacteriology. Vol. 1. Edited by N. R. Krieg and J. G. Holt. The Williams and Wilkins Co., Baltimore, Maryland. pp. 111-118.
- WANG, W. L. AND N. W. LUECHTEFELD.** 1983. Effect of incubation atmosphere and temperature on isolation of Campylobacter jejuni from human stools. Can. J. Microbiol. 29:468-470.
- WINDHOLZ, M., S. BUDAVARI, R. F. BLUMETTI AND E. S. OTTERBEIN.** (Editors) 1983. The Merck Index: an Encyclopedia of Chemicals, Drugs and Biologicals. Tenth edition. Merck & Co., Rahway, NJ. U. S. A. p.1102.

Appendix A

Growth curves of Campylobacter jejuni

Growth Curve of Campylobacter jejuni

Seventy-five ml of Brucella broth contained in a 250-ml Erlenmeyer flask was used for growing the cultures. The flask had a 16 x 150-mm test tube attached to one side for making nephelometric measurements of culture turbidity. The flask also had a small side arm which was connected to a thick rubber hose with a glass tube containing cotton; this side arm was used for adjusting the gas atmosphere within the flask.

Initially the inoculum for a flask consisted of 0.1 ml of 10^{-2} dilution of a 48-h-old-culture grown in semisolid medium at 42°C. In experiments using the wild type, variation occurred in the length of the lag phase, even when replicate flasks were incubated at the same temperature. Since the inoculum had been taken from a culture grown in semisolid medium, the variation might have been due to differences in cell numbers in the inoculum. Regardless of the variation in lag phase, however, the turbidity of the wild type cultures at the mid log phase of growth was always 15 NTU.

To minimize the variation in lag phase, the inoculum was changed to 1.0 ml (instead of 0.1 ml) of a culture grown in Brucella broth (instead of semisolid medium) to a turbidity of 15 NTU. In two separate experiments in which this inoculum was used the results were consistent. The growth curves from one experiment are shown in Figure 1. As before, the turbidity of mid log phase cultures of the wild type grown at was 15 NTU. In contrast, the turbidity of mid log phase cultures of the aerotolerant mutant MC711-01 at 37°C was 10 NTU.

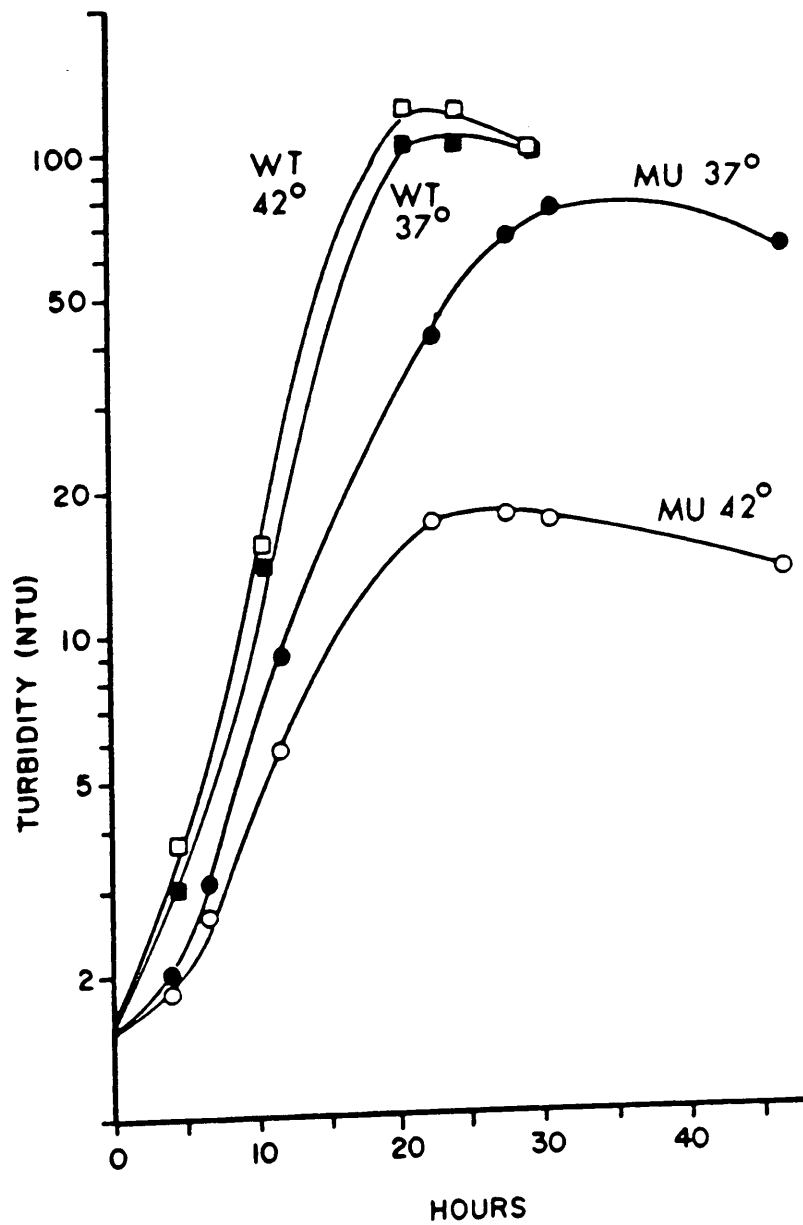


Figure 1. The growth curve of *C. jejuni* ATCC 29428 (■—■ at 37°C, □—□ at 42°) and its aerotolerant mutant MC711-01 (●—● at 37°C, ○—○ at 42°C)

Appendix B
Additional Results

TABLE B1. Effect of different lots of Brucella medium (GIBCO) on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 on brucella agar plates incubated at 42°C at various oxygen levels (Test 2)

Dehydrated Brucella medium from freshly opened jar of different lots	Mean CFU/ml \pm standard deviation $\times 10^{-6}$ with incubation at oxygen levels of:			
	The wild type			
	6%	15%	21%	26%
Lot # 580147 (lot 1)	204 \pm 20	187 \pm 42	89 \pm 30	0 \pm 0
Lot # 580483 (lot 2)	189 \pm 31	176 \pm 14	29 \pm 7	0 \pm 0
Lot # 680108 (lot 3)	149 \pm 28	183 \pm 6	21 \pm 12	0 \pm 0
	The mutant			
	6%	15%	21%	26%
Lot # 580147 (lot 1)	222 \pm 29	155 \pm 29	214 \pm 20	194 \pm 18
Lot # 580483 (lot 2)	225 \pm 1	202 \pm 28	224 \pm 38	189 \pm 33
Lot # 680108 (lot 3)	173 \pm 31	231 \pm 18	205 \pm 20	139 \pm 20

TABLE B2. Effect of different lots of Brucella medium (GIBCO) on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 on brucella agar plates incubated at 37°C at various oxygen levels (Test 1)

Dehydrated Brucella medium from freshly opened jar of different lots	Mean CFU/ml \pm standard deviation $\times 10^{-6}$ with incubation at oxygen levels of:			
	The wild type			
	6%	15%	21%	26%
Lot # 580147 (lot 1)	231 \pm 25	0 \pm 0	0 \pm 0	0 \pm 0
Lot # 580483 (lot 2)	213 \pm 8	0 \pm 0	0 \pm 0	0 \pm 0
Lot # 680108 (lot 3)	229 \pm 25	0 \pm 0	0 \pm 0	0 \pm 0
	The mutant			
	6%	15%	21%	26%
Lot # 580147 (lot 1)	100 \pm 5	119 \pm 21	127 \pm 11	0 \pm 1
Lot # 580483 (lot 2)	113 \pm 7	119 \pm 13	127 \pm 22	43 \pm 22 ^a
Lot # 680108 (lot 3)	107 \pm 12	109 \pm 9	108 \pm 14	90 \pm 8 ^a

^a Colonies were smaller (≤ 0.6 mm diameter) than usual (0.7-1.0 mm diameter at 37°C).

TABLE B3. Effect of different lots of Brucella medium (GIBCO) on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 on brucella agar plates incubated at 37°C at various oxygen levels (Test 2)

Dehydrated Brucella medium from freshly opened jar of different lots	Mean CFU/ml \pm standard deviation $\times 10^{-6}$ with incubation at oxygen levels of:			
	The wild type			
	6%	15%	21%	26%
Lot # 580147 (lot 1)	215 \pm 24	0 \pm 0	0 \pm 0	0 \pm 0
Lot # 580483 (lot 2)	243 \pm 31	5 \pm 6	0 \pm 0	0 \pm 0
Lot # 680108 (lot 3)	213 \pm 44	3 \pm 5	0 \pm 0	0 \pm 0
	The mutant			
	6%	15%	21%	26%
Lot # 580147 (lot 1)	111 \pm 17	153 \pm 21	115 \pm 14	9 \pm 4 ^a
Lot # 580483 (lot 2)	122 \pm 3	127 \pm 30	136 \pm 3	57 \pm 30 ^a
Lot # 680108 (lot 3)	138 \pm 8	123 \pm 18	142 \pm 34	127 \pm 51 ^a

^a Colonies were smaller (≤ 0.6 mm diameter) than usual (0.7-1.0 mm diameter at 37°C).

TABLE B4. Effect of ageing of dehydrated Brucella medium on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 42°C at various oxygen levels (Test 2)

Treatment of dehydrated Brucella broth (GIBCO) ^b	Mean CFU/ml ± standard deviation x 10 ⁻⁶ with incubation at oxygen levels of:			
	The wild type			
	6%	15%	21%	26%
Batch 1: control Direct from freshly opened jar	248 ± 33	234 ± 19	23 ± 6 ^a	0 ± 0
Batch 2 Stored in foil-covered beaker for 2½ months	238 ± 35	32 ± 2 ^a	0 ± 0	0 ± 0
Batch 3 Stored in foil-covered beaker for 2½ months and supplemented with 0.01% bisulfite	231 ± 19	222 ± 44	181 ± 23	0 ± 0
	The mutant			
	6%	15%	21%	26%
Batch 1: control Direct from freshly opened jar	122 ± 18	147 ± 23	160 ± 29	164 ± 32
Batch 2 Stored in foil-covered beaker for 2½ months	152 ± 30	162 ± 22	109 ± 16	19 ± 4 ^a
Batch 3 Stored in foil-covered beaker for 2½ months and supplemented with 0.01% bisulfite	151 ± 19	139 ± 12	152 ± 30	162 ± 11

^a Colonies were smaller (≤ 0.6 mm diameter) than usual (0.7-1.5 mm diameter at 42°C).

^b Batches 1, 2 and 3 were made from the dehydrated lot 1 Brucella broth (Gibco) of Table 1.

TABLE B5. Effect of ageing of dehydrated Brucella medium on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 37°C at various oxygen levels (Test 2)

Treatment of dehydrated Brucella broth (GIBCO) ^b	Mean CFU/ml ± standard deviation x 10 ⁻⁶ with incubation at oxygen levels of:			
	The wild type			
	3%	6%	15%	21%
Batch 1: control Direct from freshly opened jar	197 ± 15	219 ± 27	0 ± 0	0 ± 0
Batch 2 Stored in foil-covered beaker for 2½ months	144 ± 8 ^a	0 ± 0	0 ± 0	0 ± 0
Batch 3 Stored in foil-covered beaker for 2½ months and supplemented with 0.01% bisulfite	276 ± 8	283 ± 28	0 ± 0	0 ± 0
	The mutant			
	6%	15%	21%	26%
Batch 1: control Direct from freshly opened jar	161 ± 7	172 ± 4	125 ± 34	4 ± 4 ^a
Batch 2 Stored in foil-covered beaker for 2½ months	147 ± 27	0 ± 0	0 ± 0	0 ± 0
Batch 3 Stored in foil-covered beaker for 2½ months and supplemented with 0.01% bisulfite	179 ± 10	189 ± 2	132 ± 9	63 ± 13 ^a

^a Colonies were smaller (≤ 0.6 mm diameter) than usual (0.7-1.0 mm diameter at 37°C).

^b Batches 1, 2 and 3 were made from the dehydrated lot 1 Brucella broth (Gibco) of Table 1.

TABLE B6. Effect of ageing of hydrated Brucella agar on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 42°C at various oxygen levels (Test 2)

Treatment of hydrated Brucella agar	Mean CFU/ml \pm standard deviation $\times 10^{-6}$ with incubation at oxygen levels of:			
	The wild type			
	6%	15%	21%	26%
Batch 1: control	288 \pm 23	291 \pm 24	0 \pm 0	0 \pm 0
Batch 2: (D) ^b	309 \pm 33	58 \pm 29	0 \pm 0	0 \pm 0
Batch 3: (D+b) ^b	218 \pm 43	285 \pm 44	204 \pm 19	0 \pm 0
Batch 4: (L) ^b	189 \pm 77	100 \pm 19	0 \pm 0	0 \pm 0
Batch 5: (L+b) ^b	270 \pm 55	223 \pm 21	0 \pm 1	0 \pm 0
	The mutant			
	6%	15%	21%	26%
Batch 1: control	180 \pm 11	186 \pm 19	217 \pm 39	197 \pm 44
Batch 2: (D)	136 \pm 28	160 \pm 39	152 \pm 21	108 \pm 28
Batch 3: (D+b)	153 \pm 14	206 \pm 4	184 \pm 10	188 \pm 18
Batch 4: (L)	63 \pm 21	177 \pm 25	74 \pm 43	0 \pm 1
Batch 5: (L+b)	38 \pm 14 ^a	155 \pm 17	144 \pm 23	24 \pm 6 ^a

^a Colonies were smaller (≤ 0.6 mm diameter) than usual (0.7-1.5 mm diameter at 42°C).

^b D: The hydrated Brucella agar had been stored in dark for 1½ month.
D+b: The batch 2 medium was added filter sterilized 0.01% sodium bisulfite before it was melted to prepare the agar plates.
L: The hydrated Brucella agar had been stored under diffused sun light for 1½ month.
L+b: The batch 4 medium was added filter sterilized 0.01% sodium bisulfite before it was melted to prepare the agar plates.

TABLE B7. Effect of ageing of hydrated Brucella agar on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 37°C at various oxygen levels (Test 1)

Treatment of hydrated Brucella agar	Mean CFU/ml \pm standard deviation $\times 10^{-6}$ with incubation at oxygen levels of:			
	The wild type			
	3%	6%	15%	21%
Batch 1: control	219 \pm 15	235 \pm 34	0 \pm 0	0 \pm 0
Batch 2: (D) ^a	209 \pm 21	206 \pm 36	0 \pm 0	0 \pm 0
Batch 3: (D+b) ^a	167 \pm 36	176 \pm 12	178 \pm 18	48 \pm 13
Batch 4: (L) ^a	257 \pm 19	235 \pm 42	0 \pm 0	0 \pm 0
Batch 5: (L+b) ^a	158 \pm 31	159 \pm 27	141 \pm 26	0 \pm 0
	The mutant			
	6%	15%	21%	26%
Batch 1: control	142 \pm 35	173 \pm 21	192 \pm 29	61 \pm 5
Batch 2: (D)	117 \pm 2	130 \pm 20	51 \pm 14	4 \pm 2
Batch 3: (D+b)	118 \pm 14	145 \pm 19	173 \pm 10	144 \pm 2
Batch 4: (L)	152 \pm 31	169 \pm 38	46 \pm 5	0 \pm 0
Batch 5: (L+b)	121 \pm 19	162 \pm 22	178 \pm 27	50 \pm 15

- ^a D: The hydrated Brucella agar had been stored in dark for 1½ month.
D+b: The batch 2 medium was added filter sterilized 0.01% sodium bisulfite before it was melted to prepare the agar plates.
L: The hydrated Brucella agar had been stored under diffused sun light for 1½ month.
L+b: The batch 4 medium was added filter sterilized 0.01% sodium bisulfite before it was melted to prepare the agar plates.

TABLE B8. Effect of ageing of hydrated Brucella agar on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 37°C at various oxygen levels (Test 2)

Treatment of hydrated Brucella agar	Mean CFU/ml \pm standard deviation $\times 10^{-6}$ with incubation at oxygen levels of:			
	The wild type			
	3%	6%	15%	21%
Batch 1: control	273 \pm 19	316 \pm 30	0 \pm 0	0 \pm 0
Batch 2: (D) ^a	260 \pm 20	107 \pm 70	0 \pm 0	0 \pm 0
Batch 3: (D+b) ^a	267 \pm 9	237 \pm 33	284 \pm 25	151 \pm 29
Batch 4: (L) ^a	281 \pm 9	297 \pm 39	0 \pm 0	0 \pm 0
Batch 5: (L+b) ^a	179 \pm 9	251 \pm 53	252 \pm 49	0 \pm 0
	The mutant			
	6%	15%	21%	26%
Batch 1: control	156 \pm 34	156 \pm 14	141 \pm 30	11 \pm 6
Batch 2: (D)	113 \pm 6	81 \pm 30	6 \pm 2	0 \pm 0
Batch 3: (D+b)	138 \pm 20	147 \pm 23	153 \pm 27	118 \pm 8
Batch 4: (L)	118 \pm 15	105 \pm 25	12 \pm 6	0 \pm 0
Batch 5: (L+b)	112 \pm 24	128 \pm 37	120 \pm 21	8 \pm 2

- ^a D: The hydrated Brucella agar had been stored in dark for 1½ month.
D+b: The batch 2 medium was added filter sterilized 0.01% sodium bisulfite before it was melted to prepare the agar plates.
L: The hydrated Brucella agar had been stored under diffused sun light for 1½ month.
L+b: The batch 4 medium was added filter sterilized 0.01% sodium bisulfite before it was melted to prepare the agar plates.

TABLE B9. Effect of 0.01% sodium bisulfite in Brucella agar on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 42°C at various oxygen levels (Test 2)

Type of Brucella agar	Mean CFU/ml \pm standard deviation $\times 10^{-6}$ with incubation at oxygen levels of:			
	The wild type			
	3%	6%	15%	21%
Batch 1: control Brucella agar from Gibco Brucella broth	145 \pm 12	191 \pm 18	183 \pm 62	96 \pm 41 ^a
Batch 2 Brucella agar from scratch with 0.01% sodium bisulfite	44 \pm 17	174 \pm 20	195 \pm 32	105 \pm 22 ^a
Batch 3 Brucella agar from scratch without 0.01% sodium bisulfite	201 \pm 29	191 \pm 5	0 \pm 0	0 \pm 0
	The mutant			
	6%	15%	21%	26%
Batch 1: control Brucella agar from Gibco Brucella broth	162 \pm 18	138 \pm 18	149 \pm 36	156 \pm 32
Batch 2 Brucella agar from scratch with 0.01% sodium bisulfite	141 \pm 39	141 \pm 31	164 \pm 32	112 \pm 12
Batch 3 Brucella agar from scratch without 0.01% sodium bisulfite	153 \pm 40	36 \pm 38 ^a	8 \pm 2 ^a	0 \pm 0

^a Colonies were smaller (≤ 0.6 mm diameter) than usual (0.7-1.5 mm diameter at 42°C).

TABLE B10. Effect of 0.01% sodium bisulfite in Brucella agar on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 37°C at various oxygen levels (Test 1)

Type of Brucella agar	Mean CFU/ml \pm standard deviation $\times 10^{-6}$ with incubation at oxygen levels of:			
	The wild type			
	1.5%	3%	6%	15%
Batch 1: control Brucella agar from Gibco Brucella broth	171 \pm 31	212 \pm 10	268 \pm 38	141 \pm 31
Batch 2 Brucella agar from scratch with 0.01% sodium bisulfite	149 \pm 20	262 \pm 22	271 \pm 21	257 \pm 13
Batch 3 Brucella agar from scratch without 0.01% sodium bisulfite	9 \pm 15	85 \pm 140	0 \pm 0	0 \pm 0
	The mutant			
	3%	6%	15%	21%
Batch 1: control Brucella agar from Gibco Brucella broth	95 \pm 10	119 \pm 13	153 \pm 6	104 \pm 9
Batch 2 Brucella agar from scratch with 0.01% sodium bisulfite	120 \pm 11	151 \pm 8	165 \pm 22	134 \pm 1
Batch 3 Brucella agar from scratch without 0.01% sodium bisulfite	109 \pm 8	67 \pm 25 ^a	0 \pm 0	0 \pm 0

^a Colonies were smaller (≤ 0.6 mm diameter) than usual (0.7-1.0 mm diameter at 37°C).

TABLE B11. Effect of 0.01% sodium bisulfite in Brucella agar on viable counts of *C. jejuni* ATCC 29428 the wild type and the mutant MC711-01 incubated at 37°C at various oxygen levels (Test 2)

Type of Brucella agar	Mean CFU/ml \pm standard deviation $\times 10^{-6}$ with incubation at oxygen levels of:			
	The wild type			
	1.5%	3%	6%	15%
Batch 1: control Brucella agar from Gibco Brucella broth	206 \pm 39	236 \pm 46	235 \pm 29	0 \pm 0
Batch 2 Brucella agar from scratch with 0.01% sodium bisulfite	209 \pm 65	267 \pm 37	245 \pm 47	66 \pm 38
Batch 3 Brucella agar from scratch without 0.01% sodium bisulfite	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
	The mutant			
	3%	6%	15%	21%
Batch 1: control Brucella agar from Gibco Brucella broth	101 \pm 18	136 \pm 14	159 \pm 23	51 \pm 12
Batch 2 Brucella agar from scratch with 0.01% sodium bisulfite	127 \pm 26	150 \pm 18	147 \pm 5	124 \pm 12
Batch 3 Brucella agar from scratch without 0.01% sodium bisulfite	136 \pm 10	132 \pm 10 ^a	0 \pm 0	0 \pm 0

^a Colonies were smaller (≤ 0.6 mm diameter) than usual (0.7-1.0 mm diameter at 37°C).

Appendix C

Statistical analysis of each tested factor

Table C1. Statistical analysis of effect of different lots of dehydrated Brucella broth (GIBCO) on viable counts of C. jejuni

Culture	Temperature	Test	Comparison between groups ^a			Pattern over test 1 and test 2 ^b
ATCC 29428	42°C	1	1	<u>2</u>	3	1 = 1
						2 = 2
		2	1	<u>2</u>	3	3 ≠ 3
	37°C	1	<u>1</u>	2	3	1 = 1
						2 = 2
		2	<u>1</u>	2	3	3 = 3
MC711-01	42°C	1	<u>1</u>	2	3	1 = 1
						2 = 2
		2	<u>2</u>	<u>1</u>	3	3 ≠ 3
	37°C	1	1	<u>2</u>	3	1 = 1
						2 = 2
		2	<u>1</u>	<u>2</u>	3	3 = 3

^a The line under two numbers indicates there is no significant differences between these two groups at $\alpha = 0.05$ level. The group contains the higher colony counts is put in front.
 1 = 1 indicates the groups 1 of test 1 and test 2 have same patterns.
 3 ≠ 3 indicates the patterns of group 3 between test 1 and test 2 are different at $\alpha = 0.05$ level.

TABLE C2. Statistical analysis of effect of ageing of dehydrated Brucella broth (Gibco) on viable counts of C. jejuni

Culture	Temperature	Test	Comparison between groups ^a			Pattern over test 1 and test 2 ^b
ATCC 29428	42°C	1	3	1	2	1 = 1
						2 = 2
		2	3	1	2	3 ≠ 3
	37°C	1	<u>3</u>	<u>1</u>	2	1 = 1
						2 = 2
		2	3	1	2	3 ≠ 3
MC711-01	42°C	1	<u>3</u>	<u>1</u>	2	1 = 1
						2 = 2
		2	<u>3</u>	<u>1</u>	2	3 = 3
	37°C	1	<u>3</u>	<u>1</u>	2	1 = 1
						2 = 2
		2	3	1	2	3 = 3

^a The line under two numbers indicates there is no significant differences between these two groups, at $\alpha = 0.05$ level. The group contains the higher colony counts is put in front.

^b 1 = 1 indicates the groups 1 of test 1 and test 2 have same patterns. 3 ≠ 3 indicates the patterns of group 3 between test 1 and test 2 are different at $\alpha = 0.05$ level.

TABLE C3. Statistical analysis of effect of ageing of hydrated Brucella agar on viable counts of C. jejuni

Culture	Temperature	Test	Comparison between groups ^a				Pattern over test 1 and test 2 ^b		
ATCC 29428	42°C	1	3	<u>1</u>	<u>5</u>	<u>4</u>	<u>2</u>	1 ≠ 1 2 ≠ 2 3 ≠ 3 4 = 4 5 ≠ 5	
		2	3	<u>1</u>	<u>5</u>	<u>4</u>	<u>2</u>	5 ≠ 5	
		1	3	<u>4</u>	<u>5</u>	<u>1</u>	<u>2</u>	1 = 1 2 ≠ 2 3 = 3 4 = 4 5 = 5	
		2	3	<u>5</u>	<u>1</u>	<u>4</u>	<u>2</u>	5 = 5	
	37°C	1	3	<u>4</u>	<u>5</u>	<u>1</u>	<u>2</u>	1 = 1 2 ≠ 2 3 = 3 4 = 4 5 = 5	
		2	3	<u>5</u>	<u>1</u>	<u>4</u>	<u>2</u>	5 = 5	
		1	3	<u>3</u>	<u>1</u>	<u>5</u>	<u>4</u>	<u>2</u>	1 = 1 2 ≠ 2 3 = 3 4 = 4 5 = 5
		2	3	1	5	<u>4</u>	<u>2</u>	5 = 5	
MC711-01	42°C	1	<u>2</u>	<u>1</u>	<u>3</u>	4	5	1 = 1 2 = 2 3 = 3 4 ≠ 4 5 = 5	
		2	<u>1</u>	<u>3</u>	2	<u>5</u>	<u>4</u>	5 = 5	
	37°C	1	<u>3</u>	<u>1</u>	<u>5</u>	<u>4</u>	<u>2</u>	1 = 1 2 ≠ 2 3 = 3 4 = 4 5 = 5	
		2	3	1	5	<u>4</u>	<u>2</u>	5 = 5	

^a The line under two numbers indicates there is no significant differences between these two groups at $\alpha = 0.05$ level. The group contains the higher colony counts is put in front.

^b 1 = 1 indicates the groups 1 of test 1 and test 2 have same patterns. 3 ≠ 3 indicates the patterns of group 3 between test 1 and test 2 are different at $\alpha = 0.05$ level.

TABLE C4. Statistical analysis of effect of 0.01% sodium bisulfite in Brucella agar on viable counts of C. jejuni

Culture	Temperature	Test	Comparison between groups ^a			Pattern over test 1 and test 2 ^b
			2	1	3	
ATCC 29428	42°C	1	2	1	3	1 = 1 2 ≠ 2
		2	1	2	3	3 = 3
		1	<u>2</u>	1	3	1 ≠ 1 2 ≠ 2
	37°C	2	<u>2</u>	1	3	3 ≠ 3
		1	<u>2</u>	1	3	1 = 1 2 = 2
		2	<u>1</u>	2	3	3 = 3
MC711-01	42°C	1	<u>2</u>	1	3	1 = 1 2 = 2
		2	<u>1</u>	2	3	3 = 3
		1	2	1	3	1 ≠ 1 2 = 2
	37°C	2	2	1	3	3 = 3
		1	2	1	3	1 = 1 2 = 2
		2	2	1	3	3 = 3

^a The line under two numbers indicates there is no significant differences between these two groups at $\alpha = 0.05$ level. The group contains the higher colony counts is put in front.

^b 1 = 1 indicates the groups 1 of test 1 and test 2 have same patterns. 3 ≠ 3 indicates the patterns of group 3 between test 1 and test 2 are different at $\alpha = 0.05$ level.

Appendix D

Production of different gas mixtures

The special gas mixtures were produced inside a pressure cooker or an anaerobic jar which contained the broth culture flasks with the cotton plugs or the plates ; or it was directly produced inside a side-arm flask containing the broth culture. The regular atmospheric pressure was about 28 inches_{Hg}. No matter which gas chamber was used, the air inside was exhausted by a vacuum pump to 26 inches_{Hg}, 24 inches_{Hg}, 20 inches_{Hg} or 8 inches_{Hg}. Then the atmospheric pressure was restored by adding 1 inches_{Hg} CO₂ and then N₂. This resulted in O₂ concentrations of 1.5%, 3%, 6% or 15%.

To produce 21% O₂ or higher concentrations of O₂, the air inside the gas chamber was pumped out to 8 inches_{Hg}. The chamber was then refilled with 1 inches_{Hg} CO₂ and various amounts of pure O₂ (2.5% O₂ = 1 inches_{Hg} O₂) if higher O₂ concentrations were required. Then air was added to restore the atmospheric pressure. The CO₂ concentration in this study was always 3.6%. Besides the defined O₂ and CO₂ concentrations, the remainder of the gas phase consisted of N₂.

**The two page vita has been
removed from the scanned
document. Page 1 of 2**

**The two page vita has been
removed from the scanned
document. Page 2 of 2**