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Research Article

Media for aerobic resuscitation of *Campylobacter jejuni* supported by fumarate respiration

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Abstract

The human gastrointestinal pathogen *Campylobacter jejuni* is a microaerophilic bacterium with a respiratory metabolism. Although there have been numerous studies investigating the aerobic growth of *C. jejuni* supported by fumarate, relatively few have studied the resuscitation of stressed *C. jejuni* under these conditions. To establish an enrichment system of high efficiency for resuscitation of *Campylobacter jejuni* ATCC 35921 in Nutrient Broth No.2 (NB2) containing FBP (0.025% each of ferrous sulfate, sodium metabisulfite and sodium pyruvate) and 5% laked horse blood under an aerobic atmosphere, the effects of fumarate concentration (0, 20, 25 and 30 mM), formate (0, 5, 10 and 15 mM), inoculum levels (1 to 3 log cfu/ml), cell states (late exponential phase and stressed cells), and stress conditions (cold or heat temperature, starvation and acid treatment) were evaluated. Lag time (λ) and growth curve for each cell state and stress condition were determined. There was a significant effect ($P \leq 0.05$) of incorporating fumarate and formate in NB2 on the λ of stressed *C. jejuni* cells. Significant differences in the λs of low numbers (approximately 1, 2 and 3 log CFU/ml) of late exponential phase *C. jejuni* recovery were observed in NB2 incorporating formate ($P \leq 0.05$). However, the λ of cells grown aerobically in NB2 containing 30 mM of fumarate and 5 mM of formate was

the shortest and shorter than that obtained without fumarate and formate ($P \leq 0.05$). Enrichment in media incorporating 30 mM of fumarate and 5 mM of formate is a simple, convenient and time-saving method to replace microaerophilic incubation methods for resuscitation of *C. jejuni*.

Keywords: *Campylobacter jejuni*, aerobic growth, stressed cell, fumarate and formate

Introduction

Campylobacter jejuni is an enteropathogenic, food-borne bacterium which causes diarrhea and enteritis in humans. In recent years, a marked increase in the incidence of enteric campylobacteriosis has been reported in developed as well as developing countries [1, 2].

Under conditions that are unfavorable for growth, cells of *C. jejuni* change from their characteristic vibrioid morphology to a coccoid form [3]. During the conversion to coccoid morphology in *C. jejuni*, there is a rapid loss of viability as measured by plate counts on agar [4]. However, viability measured by other criteria may decline more slowly. For example, numbers of cells able to elongate in response to nutrients in the presence of an inhibitor of DNA synthesis, or that can reduce tetrazolium salts, are often orders of magnitude higher than those obtained by plate counts [4, 5]. This has led to the suggestion that coccoid cells are stressed to replicate on media that normally support growth [3].

Oxygen is both beneficial and deleterious for microaerophilic organisms. They show oxygen dependent growth and can use oxygen as a terminal electron acceptor, but at the oxygen concentration present in air they can not grow or grow poorly [6]. Most *Campylobacters* require oxygen for growth but grow best at reduced oxygen tensions and, although they have terminal electron transport cytochrome oxidase activity [7, 8, 9, 10], these bacteria also possess pathways characteristic of anaerobic metabolism. Campylobacters have mixed acid fermentation pathways [11] and obtain energy through the utilization of TCA cycle intermediates [12]. *C. jejuni* cannot grow under strictly anaerobic conditions, but, when oxygen supplies are limited, the addition of fumarate to the culture medium causes enhanced growth through the use of fumarate as a terminal electron acceptor instead of oxygen [13] and fumarate reductase (Frd) activity is increased when *C. jejuni* is grown in oxygen-limited cultures [14]. Both succinate dehydrogenase (*sdh*) and fumarate reductase (*frd*) genes are up-regulated under low oxygen conditions. The limited oxygen supplies have a greater effect on the expression levels of *dcuA*, *dcuB*, *dctA* (C4 dicarboxylic acid transporters) and *aspA*, (aspartate-ammonia lyase) than the presence of fumarate or succinate in microaerobic environments. This suggests that there are separate regulatory systems responding to oxygen and C_4-dicarboxylates in *C. jejuni* [15].

Previous studies have investigated media for the recovery of low numbers of late exponential phase *C. jejuni* [16, 17, 18, 19, 20, 21] and stressed *C. jejuni* (Tangwatcharin, Chanthachum, Khopaibool and Griffiths, unpublished results) under aerobic atmospheres. In this paper, we investigated the resuscitation of stressed cells of *C. jejuni* in media containing formate or fumarate under aerobic conditions.

Materials and Methods

Test strain, *C. jejuni* ATCC 35921 was obtained from the culture collection of the Canadian Research Institute for Food Safety (CRIFS), Guelph, ON, Canada. Cells were grown on Columbia agar (Oxoid Inc., Nepean, ON, Canada), supplemented with 5% lysed horse blood (Oxoid Inc.), for 24 h at 42^{o}C in a microaerobic atmosphere [22]. After 24 h, the cells were harvested and washed three times with NaCl (150 mM) by centrifugation at 12,000 *g* for 10 min at 4^{o}C. Washed cells were resuspended in NaCl to an optical density at 600 nm (OD_{600nm}) of 0.8 ± 0.1 (approximately 10^{9} to 10^{10} CFU /ml) [23].

Stress treatments of bacterial cells, as a result of stress upon exposure to cold, *C. jejuni* undergoes, in some cases, a change in morphology from spiral to coccoid form. *C. jejuni* was subjected to cold shock in water to obtain stressed cells according to previously published protocols [4, 25, 26]. After resuspension, 1 ml of cell suspension was transferred into bottles containing 500 ml of Brucella broth (BB; BD Diagnostic Systems, Sparks, MD, USA). The broth was adjusted to pH 7.0 ± 0.1 with 0.1 M NaOH and 0.1M HCl. The bottles were incubated at 4^{o}C with rotary shaking (100 rpm) for 15 days.

The stressed state was also induced by subjecting *C. jejuni* cells resuspended in Brain Heart Infusion broth (BHI broth; BD Diagnostic Systems) supplemented with 1% yeast extract (BHIYE; BD Diagnostic Systems) to a heat treatment of 60^{o}C for 10 min in a water bath.

C. jejuni was subjected to acid shock in water to obtain stressed cells according to previously published protocols [26]. After resuspension, 1 ml was transferred to 4 ml of Mueller-Hinton broth (MHB; BD Diagnostic Systems), which was adjusted to a pH of 4.00 ± 0.01 using formic acid (88%; Fisher Scientific Company, Ottawa, ON, Canada). The cultures were kept at 42^{o}C under microaerobic conditions for 1 h.

Starvation induced stress of *C. jejuni*, as indicated by a change in morphology from spiral to coccoid form, was elicited by transferring cells into bottles containing 500 ml of filter-sterilized (pore size, 0.2 μm) water (pH adjusted to 6.0 ± 0.1 with 0.1 M NaOH) [4, 25, 26]. The bottles were then incubated at 37^{o}C with rotary shaking (100 rpm) for 0 and 6 h.

Population density estimation, cells capable of growing on solid selective media were enumerated using spread plate count on blood free Campylobacter selective agar (CCDA; Oxoid Inc.) containing modified CCDA antibiotic (SR155; Oxoid Inc.) and Columbia agar containing 5% lysed horse blood (CBA) to assess the culturable (non-stressed cells) and total culturable (stressed and non-stressed cells) populations, respectively. A portion (0.1 ml or 1 ml) of each resuspension was plated onto CCDA and CBA. The cell numbers (CFU) were determined following incubation at 42^{o}C for 48 h under a microaerobic atmosphere [25]. Non-stressed and stressed cells were determined by the following equations [27].

Non-stressed cells	=	Culturable cells
Stressed cells	=	Total culturable – Culturable cells

Light Microscopy, for estimation of morphological changes, *C. jejuni* samples for light microscopy was centrifuged at 16,000 × *g* for 5 min and the supernatant was discarded. The pellets were washed 3 times with phosphate buffered saline (PBS). The final pellet

was re-suspended in 0.25 ml of PBS, and 5 µl of each suspension were fixed on a glass slide. The samples were stained by safranin (Gram stain kit; Fisher Scientific, Mississauga, Canada). An Olympus BH 2 epifluorescence microscope equipped with a BH 2-RFL-T2 100-W light source was used for examination of the filters at 1000 × magnification.

Supplement addition, Nutrient Broth No. 2 (Oxoid Inc.) incorporating FBP (0.025% each of ferrous sulfate, sodium metabisulfite and sodium pyruvate) and 5% laked horse blood (NB2) was modified by the addition of formate solution (0, 5, 10 and 15 mM) and fumarate solution (0, 20, 25 and 30 mM) as documented in Table 1. Growth in the supplemented medium during aerobic incubation was monitored and compared with aerobic or microaerobic (generated by the CampyGen gas generator; Oxoid Inc.) growth obtained in unsupplemented media. The cultures were incubated in a series of screw-capped test tubes each containing 30 ml of the appropriate medium. Cells of *C. jejuni* in late exponential phase or cells that had been stressed by exposure to heat, cold, formic acid or starvation were inoculated into each treatment medium at inoculum levels of 1, 2 or 3 log CFU/ml. The tubes were tightly closed or loosed lids for aerobic or microaerobic atmospheres, respectively. The cultures were incubated at 42^{o}C for 24 h in aerobic or microaerobic atmospheres. The numbers of non-stressed and total culturable cells were determined after incubation for 0, 3, 6, 9 and 24 h. The enrichment broths which exhibited the shortest time for the initial number to double were chosen for further study.

Dissolved Oxygen measurement (DO), DO measurement was performed as described by Wonglumsom et al. (2001) and Tuitemwong et al. (1995) [21, 28]. DO of cultures were as monitored by using a Benchtop BioFlo 3000® fermentor with Dissolved Oxygen probe (New Brunswick Scientific Co., Inc., Edison, NJ). After proper calibration of the DO meter as described in the manufacturer's manual, the probe was placed in a tube containing about 30 ml of samples that was withdrawn from liquid cultures. The DO values in (% air saturation) and temperature were recorded after both readings haved stabilized. Temperature compensation of the DO reading was determined automatically by the meter.

Table 1. Composition of media used in this study.

Treatment	FBP[a] (%)	Blood (%)	Formate (mM)	Fumarate (mM)	Atmosphere
C1	0.025	5	-	-	CampyGen
T1	0.025	5	-	-	Aerobic
T2	0.025	5	-	20	Aerobic
T3	0.025	5	-	25	Aerobic
T4	0.025	5	-	30	Aerobic
T5	0.025	5	5	-	Aerobic
T6	0.025	5	5	20	Aerobic
T7	0.025	5	5	25	Aerobic
T8	0.025	5	5	30	Aerobic
T9	0.025	5	10	-	Aerobic
T10	0.025	5	10	20	Aerobic
T11	0.025	5	10	25	Aerobic
T12	0.025	5	10	30	Aerobic
T13	0.025	5	15	-	Aerobic
T14	0.025	5	15	20	Aerobic
T15	0.025	5	15	25	Aerobic
T16	0.025	5	15	30	Aerobic

[a] FBP; 0.025% each of sodium metabisulfite, sodium pyruvate and ferrous sulfate

Calculation of lag time (λ), for the purpose of this study, the lag phase was defined as the time for the initial count to double and was calculated using Graphpad Prim version 4 (GraphPad Software, Inc.,San Diego, CA).

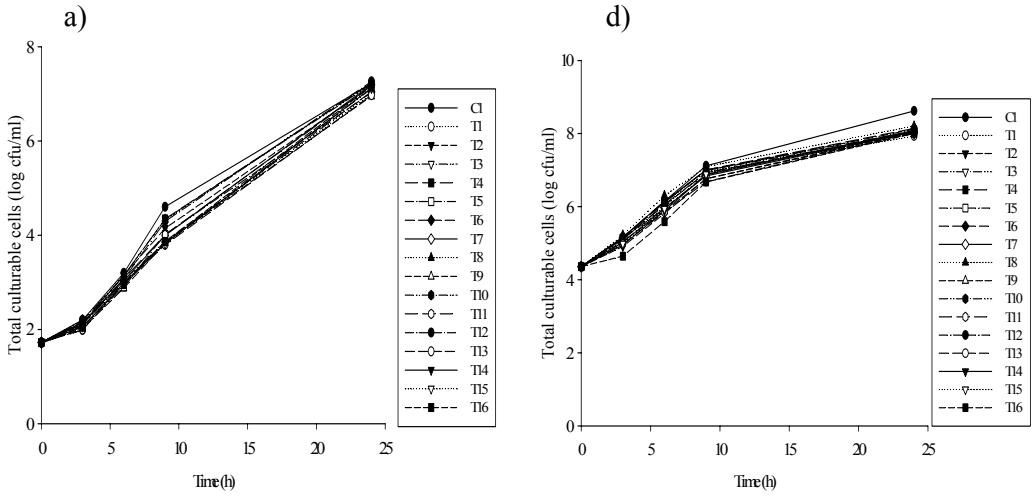
Statistical analysis, for the initial experiments, data were subjected to analysis of variance (ANOVA) and differences between means were determined using Duncan's multiple range test ($P < 0.05$). For experiments involving incorporation of supplements in broth, a 4 X 4 factorial completely randomized design was used and statistical analyses were performed using the response surface design of Sigmaplot version 10 (Systat Software, Inc., Point Richmond, CA, USA). Data were subjected to analysis of variance (ANOVA) and differences between means were determined using pdiff that provides the p-values for the tests of equality of all pairs of treatment means ($P \leq 0.05$).

Results and Discussion

Effect of supplements on recovery of *C. jejuni*, previous work has shown that Nutrient Broth No. 2 incorporating FBP and 5% laked horse blood was the best medium for the recovery of stressed and low numbers of late exponential phase *C. jejuni* ATCC 35921 when compared with Nutrient Broth No. 2 incorporating oxyrase, haemin or activated charcoal. In addition, NB2 incorporating ranges of 0 - 15 mM formate solution or 20 - 30 mM fumarate solution were the best media for the recovery of 10^3 CFU /ml of late exponential phase *C. jejuni* (Tangwatcharin, Chanthachum, Khopaibool and Griffiths, unpublished results).When *C. jejuni* ATCC 35291 was inoculated at levels of 10^1 CFU /ml into NB2 with or without formate and fumarate (treatment T1 to T16), growth was rapid during the first 24 h of aerobic or microaerobic incubation (Figs. 1a & 1b). Similar results were achieved at inoculum levels of 10^2 and 10^3 CFU/ml (data not shown).

From results, the cells in each treatment were recovered rapidly during the first 24 h of aerobic of microaerobic incubation that the percentage of oxygen in each treatment was approximately 8% after 24 h. The specific acitivity of fumarate reductase (Frd) was higher in culture lysates of *C. jejuni* grown under 5 and 10% O_2 than of those grown under 17% O_2; whereas fumarase (Fum) activity was the same in the lysates of all the cultures [14]. The growth rate of *C. jejuni* was significantly reduced in brain heart infusion broth supplemented with 5% fetal calf serum under oxygen-limiting conditions. The effect of the addition of 20 mM fumarate to such cultures was dramatic. Strict anaerobiosis can thus be viewed as a stressor for *C. jejuni* [13]. In the present study, when low numbers of late exponential phase cells of *C. jejuni* were inoculated into Nutrient Broth No. 2 that did not contain supplements and incubated aerobically, cell numbers decreased rapidly within 3 h and it was not possible to recover cells after 6 h of incubation in this medium (data not shown). Previous studies demonstrated that campylobacters are more sensitive than aerobic bacteria to toxic forms of oxygen, such as superoxide anions and hydrogen peroxide, that are formed in culture media incubated aerobically. Compounds which enhance the aerotolerance of microaerophilic bacteria do so by quenching these toxic forms of oxygen [17]. *Campylobacter* spp. are unable to utilize sugars or glycolytic intermediates and obtain energy through electron transport-coupled phosphorylation through a metabolic pathway that includes reactions catalyzed by Frd and formate dehydrogenase. Thus, the presence of both anaerobic and aerobic metabolic pathways appears to be a characteristic of the microaerophily of these organisms [14].

When cold-shocked cells were used as the inoculum, the ratio of non-stressed to stressed cells increased rapidly within the first 5 h, especially in the medium containing 5 mM formate + 30 mM fumarate (T8) (Fig. 1f). The ratio of non-stressed to stressed cells also increased in this medium when inoculated with late exponential cells (Fig. 1c). A higher ratio of non-stressed to stressed cells was observed in this medium than any other. Table 2 and 3 show the calculated lag time (λ) for *C. jejuni* ATCC 35921 observed in media containing varying concentrations of formate and fumarate, respectively, following inoculation with 10^1, 10^2 and 10^3 CFU/ml. There did not appear to be a significant interaction ($P > 0.05$) between formate and fumarate on recovery of cells. These tables demonstrated the addition of formate or fumarate to NB2 increased the rate of growth of *C. jejuni*. The presence of formate in Isosensitest agar plates increased *C. jejuni* growth in a concentration-dependent manner up to 10 mM concentration. At higher concentrations cell growth decreased in proportion to the amount of formate added and was half of the control values at approximately 35 mM concentration [12]. At 10 mM initial fumarate concentration, the Fum and Frd activities in *C. jejuni* culture lysates were 53 ± 3 and 3.1 ± 0.2 nmol/min/mg, respectively. The addition of formate to cell suspensions increased the rates of both enzyme activities [14]. The genes encoding Frd, the hydrogenase complex and the proteins involved in the formation of hydrogenase complex (*hyp*FBCDEA) occur as clusters in *Wolinella succinogenes*. The Frd of this bacterium comprises three subunits; a catalytic subunit (FrdA) which contains FAD^+ as a prosthetic group, an iron-sulfur protein (FrdB) and a cytochrome *b* (FrdC) which mediates electron transport from menaquinone [29]. The order of the genes encoding the subunits of Frd in *C. jejuni* is *frd*CAB, similar to that found in *Helicobacter pylori* and *W. succinogenes* [14].



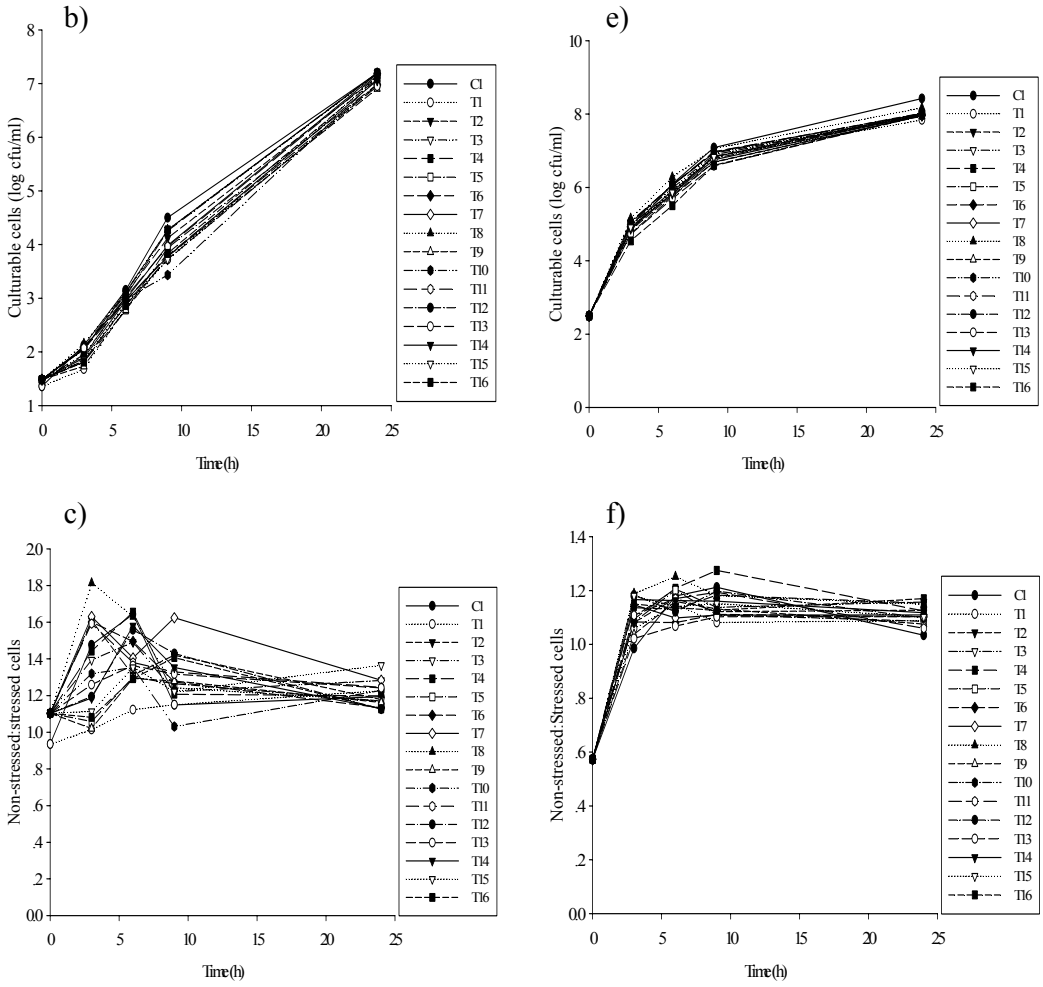


Figure 1. Resuscitation of *C. jejuni* in various media, NB2 incorporation ranges of 0-15 mM formate solution or 20-30 mM fumarate solution, following inoculation with 10^1 CFU/ml of cells in late exponential phase or cold stressed cells; a) or d) total culturable cells, b) or e) culturable cells or non-stressed cells and c) or f) ratio nonstressed and stressed cells, respectively.

Table 2. Lag time (calculated as the time for the initial cell number to double) of *C. jejuni* in media containing varying concentrations of formate following inoculation with 10^1, 10^2 and 10^3 CFU/ml of cells in late exponential phase

Formate (mM)	Lag time (h)[1]		
	Inoculum levels		
	10^1	10^2	10^3
0	2.59 ± 0.43 [a]	3.31 ± 0.34 [a]	3.30 ± 0.40 [a]
5	2.42 ± 0.33 [a]	3.17 ± 0.42 [a]	3.18 ± 0.42 [a]
10	2.63 ± 0.35 [a]	3.37 ± 0.35 [a]	3.43 ± 0.39 [ab]
15	2.89 ± 0.33 [b]	3.67 ± 0.32 [b]	3.67 ± 0.36 [b]

[1] Different letters within each inculum level are significantly different ($P \leq 0.05$).

Table 3. Lag time (calculated as the time for the initial cell number to double) of *C. jejuni* in media containing varying concentrations of fumarate following inoculation with 10^1, 10^2 and 10^3 CFU/ml of cells in late exponential phase

Fumarate (mM)	Lag time (h)[1]		
	Inoculum levels		
	10^1	10^2	10^3
0	3.00 ± 0.31 [b]	3.75 ± 0.34 [b]	3.76 ± 0.38 [b]
20	2.64 ± 0.25 [a]	3.30 ± 0.27 [a]	3.35 ± 0.30 [a]
25	2.46 ± 0.33 [a]	3.25 ± 0.33 [a]	3.29 ± 0.36 [a]
30	2.43 ± 0.40 [a]	3.21 ± 0.39 [a]	3.19 ± 0.44 [a]

[1] Different letters within each inoculum level are significantly different ($P \leq 0.05$).

The addition of formate to media also increased the rate of growth of *Campylobacter* spp. [7, 12, 30, 31]. The energy required for reduction of fumarate to succinate can be supplied by a number of hydrogen donors through their corresponding dehydrogenases. The order of potential donors with increasing free energy change upon fumarate reduction is: hydrogen > formate > NADH > lactate > glycerol-3-phosphate > malate. Electrons are passed from the dehydrogenases to menaquinone (or dimethyl-menaquinone), which, in turn, is oxidized by Frd (menaquinone: fumarate oxidoreductase), with fumarate as the terminal electron acceptor [32]. The respiratory activity of *C. jejuni* membrane vesicles was 50- and 100-fold higher in the presence of formate or molecular hydrogen than with other proton donors. This was attributed to formate dehydrogenase and hydrogenase being located on the periplasmic side of the cytoplasmic membrane [33]. *C. jejuni* is able to use oxygen as the terminal electron acceptor for hydrogen and formate oxidation, via two oxidases which have different oxygen affinities. In addition, *C. jejuni* possesses hydrogen-fumarate oxidoreductase activity, which is mediated via cytochrome b [7].

While significant differences in the λ of low numbers (approximately 1, 2 and 3 log CFU/ml) of late exponential phase *C. jejuni* cells were observed in NB2 incorporating formate or fumarate ($P \leq 0.05$). In the present study, the shortest λs during aerobic incubation were obtained in T8, T4, T7, T3, T11, T12, T6 and T2 (2.11 ± 0.07, 2.27 ± 0.31, 2.33 ± 0.28, 2.37 ± 0.31, 2.41 ± 0.32, 2.45 ± 0.29, 2.50 ± 0.27 and 2.60 ± 0.22 h, respectively, for media inoculated with 10^1 CFU/ml) (Fig. 2). This was the case irrespective of the number of cells in the inoculum (Fig. 2). However, none of these times were significantly different ($P > 0.05$). A significant decrease ($P \leq 0.05$) in λ was observed when growth in T8 (λ = 2.11 ± 0.07 h) was compared with that in T1 (without formate and fumarate solution; λ = 3.12 ± 0.36 h).

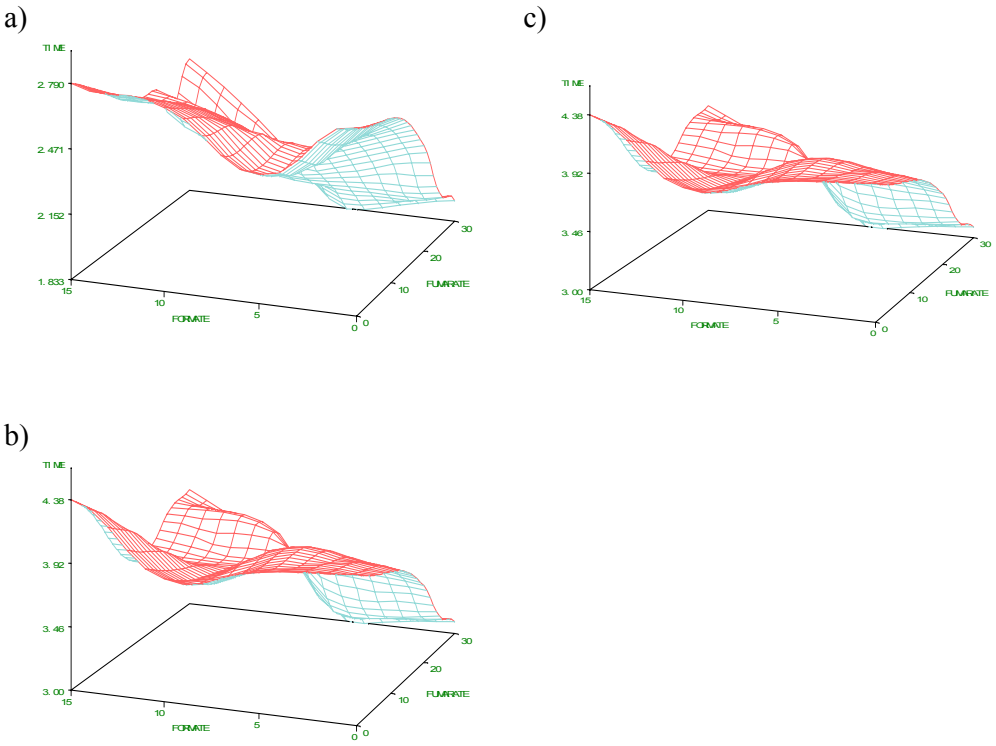


Figure 2. Response surface plots for lag time (calculated as the time for the initial cell number to double) of *C. jejuni* in response to varting concentrations of formate and fumarate following inoculation with a) 10^1 CFU/ml, b) 10^2 CFU/ml and c) 10^3 CFU/ml of cells in late exponential phase.

Resuscitation of stressed *C. jejuni*, when the resuscitation of cold, heat, starvation and acid stressed cells of *C. jejuni* ATCC 35291 was investigated, there were slight differences in the way these cells responded. In the media that had been supplemented with formate or fumarate, populations of culturable cells of *C. jejuni* increased more rapidly than numbers of total culturable cells within 3 h of incubation following inoculation with cold-stressed bacteria, regardless of incubation atmosphere (Fig. 1e and 1d). Furthermore, the ratio of non-stressed to stressed cells increased rapidly within the same time period, indicating that there was recovery from the stressed to non-stressed state (Fig. 1f). The highest recovery was observed in medium T8, and the recovery of cold-stressed cells in this medium was greater than that observed when late exponential phase cells were used as the inoculum. In media that did not contain the supplements, the culturability decreased rapidly within 3 h of aerobic incubation and did not recover (data not shown). In supplemented media, the population of the initially stressed cells increased by approximately 8 log cycles after 24 h at 42^{o}C. Similar results were obtained with cells of *C. jejuni* that had been subjected to heat, starvation and acid induced stress (data not shown), but recovery of cold-shocked cells appeared to occur more rapidly.

The lag times (λ) of *C. jejuni* ATCC 35921 cells that had been cold, heat, acid and starvation stressed then grown subsequently in NB2 with added supplements are shown in Tables 4, 5, 6 and 7, respectively. The interaction between formate and fumarate solution had a significant effect ($P \leq 0.05$) on λ of cold, heat and acid stressed cells; while, there did not appear to be a significant interaction ($P > 0.05$) between formate and fumarate

solutions on resuscitation of starved cells. Nevertheless, the shortest λs achieved during aerobic growth were obtained in media T8, T11, T5, T6, T3, T4, T12, T10 and T9 for cold-shocked cells, T8 for heat-shocked cells, T8, T7, T6, T3, T11, T4, T5, T2, T10 and T12 for acid-stressed cells and T8, T12, T7, T11, T4, T3, T10, T6, T14, T2, T15, T9 and T16 for starved cells. However, these times were not significantly different ($P > 0.05$) for any of the treatments. The λs were approximately 1 h for cold-shocked, 4 h for heat-shocked, 1 h for acid-stressed cells and 0.8 h for starved cells. In addition, the λ observed during growth in T8 was shorter than that in T1 (without formate and fumarate solution) ($P \leq 0.05$). Response surface plots of lag time for cold, heat, acid and starvation stressed cells demonstrated that NB2 incorporating 5 mM formate and 30 mM fumarate solution (T8) resulted in the lowest λ.

Table 4. Lag time (calculated as the time for the initial cell number to double) of *C. jejuni* in media containing varying concentrations of formate and fumarate following inoculation with cold stressed cells

Types and concentrations of supplement	Lag time (h)[1]			
	Fumarate (mM)			
Formate (mM)	0	20	25	30
0	1.49 ± 0.11^{bcde}	1.83 ± 0.29^{f}	1.19 ± 0.22^{a}	1.22 ± 0.02^{ab}
5	1.19 ± 0.02^{a}	1.19 ± 0.07^{a}	1.49 ± 0.27^{bcde}	1.12 ± 0.03^{a}
10	1.32 ± 0.07^{abcd}	1.30 ± 0.03^{abc}	1.16 ± 0.04^{a}	1.23 ± 0.00^{ab}
15	1.58 ± 0.01^{cdef}	1.71 ± 0.22^{ef}	1.60 ± 0.16^{def}	3.05 ± 0.39^{g}

[1] Different letters indicate significant differences ($P \leq 0.05$).
Lag time in control medium (C1) was 1.16 ± 0.05 h.

Table 5. Lag time (calculated as the time for the initial cell number to double) of *C. jejuni* in media containing varying concentrations of formate and fumarate following inoculation with heat stressed cells

Types and concentrations of supplement	Lag time (h)[1]			
	Fumarate (mM)			
Formate (mM)	0	20	25	30
0	5.07 ± 0.01^{g}	4.59 ± 0.01^{cde}	4.45 ± 0.06^{bc}	4.46 ± 0.08^{bc}
5	4.80 ± 0.02^{f}	4.50 ± 0.00^{bcde}	4.37 ± 0.09^{b}	4.08 ± 0.14^{a}
10	4.85 ± 0.01^{f}	4.59 ± 0.02^{cde}	4.46 ± 0.10^{bcd}	4.60 ± 0.17^{de}
15	5.03 ± 0.06^{g}	4.81 ± 0.07^{f}	4.63 ± 0.09^{e}	5.21 ± 0.16^{h}

[1] Different letters indicate significant differences ($P \leq 0.05$).
Lag time in control medium (C1) was 4.15 ± 0.20 h.

The addition of formate and fumarate to NB2 also resuscitated and increased growth rate of cold stressed *C. jejuni*. Chilling is known to promote the survival of *C. jejuni*, but, nevertheless, this process will generate stress and the organism must be able to respond to this in order to survive [34]. The ability of many bacteria to replicate at temperatures far below that required for optimum growth is associated with the production of characteristic cold shock proteins, some of which are thought to act as RNA chaperones which block the formation of secondary structures in mRNA [35]. A previous study demonstrated that the percentage of stressed cells increased from 7.3 ± 1.7 to 29.6 ± 5.2% after incubation for 15

d at 4°C. There was some loss of outer membrane by the cells of the bacterium following exposure to cold [36]. Furthermore, previous work has also shown that the level of Frd activity varied according to the age of cultures after 24 h of incubation, with lower enzyme activities measured in older cultures [14]. Also, when cells enter the stationary phase of growth, the percentage of vibrioids and total culturable cells of *C. jejuni* decrease according to the age of cultures [3].

Table 6. Lag time (calculated as the time for the initial cell number to double) of *C. jejuni* in media containing varying concentrations of formate and fumarate following inoculation with acid stressed cells

Types and concentrations of supplement	Lag time (h)[1]			
	Fumarate (mM)			
Formate (mM)	0	20	25	30
0	1.42 ± 0.13^{de}	1.19 ± 0.10^{abcd}	1.13 ± 0.14^{abc}	1.15 ± 0.16^{abc}
5	1.18 ± 0.13^{abcd}	1.11 ± 0.19^{abc}	1.07 ± 0.18^{ab}	0.99 ± 0.12^{a}
10	1.30 ± 0.14^{bcde}	1.19 ± 0.21^{abcd}	1.13 ± 0.18^{abc}	1.24 ± 0.14^{abcd}
15	1.35 ± 0.07^{cde}	1.34 ± 0.15^{cde}	1.25 ± 0.13^{bcd}	1.52 ± 0.06^{e}

[1] Different letters indicate significant differences ($P \leq 0.05$).
Lag time in control medium (C1) was 0.99 ± 0.09 h.

Table 7. Lag time (calculated as the time for the initial cell number to double) response surfaces of *C. jejuni* in various supplements following inoculation with starvation stressed cells

Types and concentrations of supplement	Lag time (h)[1]			
	Fumarate (mM)			
Formate (mM)	0	20	25	30
0	1.04 ± 0.15	0.90 ± 0.12	0.86 ± 0.13	0.84 ± 0.09
5	1.04 ± 0.10	0.89 ± 0.11	0.81 ± 0.08	0.77 ± 0.03
10	0.95 ± 0.21	0.88 ± 0.13	0.82 ± 0.10	0.79 ± 0.05
15	1.07 ± 0.25	0.89 ± 0.16	0.94 ± 0.24	0.98 ± 0.26

[1] Interaction between formate and fumarate solution was non-significantly different ($P > 0.05$).
Lag time in control medium (C1) was 0.83 ± 0.01 h.

Reduction of fumarate can serve biosynthetic and bioenergetic roles [37]. The free energy change occurring during fumarate reduction can be used for phosphorylation via the generation of proton gradients [38]. Again, no cells could be recovered from Nutrient Broth No. 2 in the absence of fumarate or formate and incubated aerobically for 24 h (data not shown). Furthermore, the λs of stressed cells were shorter than those obtained when a low number of late exponential phase cells was used as the inoculum. This may be due to transformation of VBNC cells to the culturable state. Some strains of *C. jejuni* cells can recover from the VBNC state after passage through egg, chick and mouse [22, 24].

Conclusion

Several advantages of T8 (NB2 incorporating 5 mM formate and 30 mM fumarate solution) for the resuscitation of stressed *Campylobacter* cells under aerobic atmosphere

were observed in this study. Nutrient Broth No. 2 incorporating 0.025% FBP, 5% blood, 5 mM formate and 30 mM fumarate solution is a simple, convenient and time-saving method for the isolation of non-stressed and stressed cells of *C. jejuni*, and can replace the more cumbersome, inconvenient and expensive gassing procedure.

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