Acridine Orange Staining and Viability of the Coccoid form of *Campylobacter upsaliensis*

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Conversion of Campylobacter upsaliensis to the coccoid form during aerobic incubation at 37°C was not prevented by treatment with chloramphenicol and was accompanied by severe decreases in isocitrate dehydrogenase activity and oxygen uptake. Although the coccoid forms fluoresced orange-red by acridine orange staining, agarose gel electrophoresis indicated an extensive degradation of the ribosomal RNA. This suggests that acridine orange staining may not be a good indicator of viability and that the coccoid form of C. upsaliensis at 37°C is degenerative rather than part of the life cycle.

Keywords: *Acridine orange, Viable but nonculturable, Nonculturable form, VBNC, Campylobacter upsaliensis*

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Some bacteria enter an altered physiological state termed "viable but nonculturable" (VBNC). This is commonly defined as the inability to form colonies on a given solid medium yet giving indications of viability by other tests such as fluorescence staining with acridine orange[1]. Most *Campylobacter* species not only enter the VBNC state but also change their morphological appearance from spiral to a coccoid form. Attempts have been made to resuscitate campylobacters from this VBNC coccoid state in vivo by feeding preparations of the coccoid form to experimental animals[2-4]. Some of these attempts have seemingly resulted in resuscitation[2,4] but are inconclusive because the sample fed to the animal was not the same sample that was tested for the absence of culturable forms. If there were one or more spiral cells in a preparation of coccoid forms, these cells might multiply and lead to the false conclusion that resuscitation has occurred. Thus, questions have continued to be raised whether the VBNC forms of campylobacters are degenerate forms or merely dormant resting forms. "Vital" staining, reduction of tetrazolium salts, RNA content, and ATP levels have been proposed for demonstrating that the coccoid forms are in a viable, dormant state[5,6]. Acridine orange is used widely for direct epifluorescence enumeration of viable bacteria[7]. The dye fluoresces green when attached to double-stranded DNA and orange-red when attached as a dimer to single-stranded RNA or denatured DNA. Viable cells should fluoresce orange-red because they contain much more RNA than DNA, whereas nonviable cells contain little RNA and should fluoresce green. The ability of VBNC forms to fluoresce orange-red suggests that they are still viable although they cannot be cultured on laboratory media.

Campylobacter upsaliensis is a microaerophilic, vibrioid bacterium found in the normal and diarrheic feces of dogs and cats[4,8] and in humans it can cause bacteremia[9-12], miscarriage[13], abscesses[14], and diarrhea[15,16]. It is closely related to *C. coli* and *C. jejuni* in genotype, phenotype, and ecology[17], and changes to a coccoid morphology

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when exposed to air[18]. The coccoid forms of *Campylobacter jejuni*[2,4,18,19] and *Campylobacter coli*[20] have been reported to be viable but not culturable (VBNC) but this is not known for *C. upsaliensis*. The present report assesses for the first time the culturability of the coccoid forms of this species and compares the oxygen uptake, enzyme activity, and condition of the ribosomal RNA (rRNA) in the vibrioid vs the coccoid form.

Material and Method

Bacterial strains and growth conditions

Campylobacter upsaliensis VPI strain CG-1 was obtained from C. Gebhart, Veterinary Diagnostic Laboratory, University of Minnesota, St.Paul, MN and maintained in semisolid Brucella agar (Difco). Log phase cultures were obtained by inoculating 2L of Brucella broth with 20 ml of a 48-h-old culture grown in Brucella broth and incubating at 37^{0}C under an atmosphere of 6% O_2, 3% CO_2 and 91% N_2 in a reciprocal shaking waterbath at 40-45 oscillations per min. Mid-log phase occurred in 22-24 h at 80-84 nephelometer turbidity units as measured with a ratio turbidimeter (Hach Co., Loveland, Colorado, USA).

Scanning electron microscopy

A 3-d-old colony grown on Brucella agar at 37^{0}C under microaerobic conditions was fixed for 3 h in 100 mM HEPES buffer (pH 6.8) containing 2.5% glutaraldehyde and 2mM $MgCl_2$ at 25^{0}C. The colony was washed twice in HEPES buffer, suspended in OsO_4 for 3h, washed in HEPES buffer, and dehydrated in an alcohol series. After preparation by critical point drying, it was sputter-coated with gold and examined with a scanning electron microscope model 505 (Philips Electronic Co., Mahwah, New Jersy, USA).

Determination of the optimum temperature of conversion to the coccoid form

Fifty-ml portions of a mid-log phase culture grown at 37^{0}C in Brucella broth were transferred to four sterile 250-ml flasks for static incubation aerobically at 4, 25, 30, and 37^{0}C, respectively. The cultures were examined at 24-h intervals until complete coccoid form development occurred.

Effect of chloramphenicol on conversion

The minimum bactericidal concentration (MBC) of chloramphenicol for strain CG-1 in Brucella broth was 16 mg/ml. A mid-log culture in Brucella broth was divided into five 50-ml portions, each contained in a 250-ml sterile flask. Chloramphenicol was added to the flasks at a concentration of 16, 32, 64, and 128 mg/ ml. The fifth flask was a control that lacked chloramphenicol. The cultures were aerobically incubated at 37^{0}C and examined for conversion to the coccoid form.

Viability measurements

Viability measurements were made by colony counts and acridine orange staining. The spread plate method was used for enumeration of CFU on Brucella agar plates (Difco) incubated an atmospheres of 6% O_2, 3% CO_2, and 91% N_2. For acridine orange staining, 20-ml portions of cultures were added to 2 ml of 0.1% aqueous acridine orange for 2 min. The mixture was filtered through a black membrane filter (0.22 mm pore size; Micron Separations Inc., Westborough, Massachusetts) and the cells were observed with a Zeiss epifluorescence microscope and filters appropriate for differentiating orange-red cells from green cells.

Isocitrate dehydrogenase activity

Preliminary studies indicated that mid-log phase cells contained cytoplasmic isocitrate dehydrogenase (ICDH;EC1.1.1.42) of high specific activity compared to other citric acid cycle enzymes. To compare the activity in the vibrioid form vs the coccoid form, 2-ml samples were removed from cultures at various times of exposure to air of batch culture, centrifuged at 16,000 x g, washed once with phosphate-buffered saline (pH 7.4), and disrupted by sonic oscillation. Whole cells and large particles were removed at 16,000 x g and the supernate was assayed for ICDH activity by the method of Daron[21] modified by decreasing the concentration of the $MnCl_2$ to l mM to prevent turbidity and by increasing the concentration of the FL-sodium isocitrate to 50 mM to obtain maximal activity. The increase in absorbance at 340 was linear with time and protein concentration. Total protein was determined by the Bio-Rad assay method.

Oxygen uptake

A sample of predominantly (>95%) spiral cells from a mid-log phase culture and a sample of predominantly (> 95%) coccoid forms from a 4-day-old culture were washed twice with 40 mM Tris-HCl buffer (pH 7.0) and suspended in 20 ml of this

buffer. Protein concentration was determined by the Bio-Rad assay method. Oxygen consumption was measured with an oxygen monitor calibrated by the method of Robinson and Cooper[22]. The oxygen electrode was inserted into a water-jacketed Clark cell-type chamber (Gilson Medical Electronics, Middleton, Wisconsin) maintained at 37^{0}C. The Electron donors were injected into the cell suspension to a final concentration of 10 mM (formate was used at 0.05 mM).

Isolation and characterization of RNA

For characterization of rRNA, *C. upsaliensis* was cultured in six 200-ml volumes of Brucella broth at 37^{0}C, with a gas mixture of 6%O_2,3%CO_2, and 94% N_2 bubbled continuously through the medium. At mid-log phase on culture was centrifuged at 7500 x g for 10 min and the cells were suspended in 2 ml of suspending buffer containing dithiothreitol[23]. Total RNA was isolated by the guanidine isothiocyanate procedure and subjected to agarose gel electrophoresis using the method of Johnson[23]. The remaining cultures were incubated statically under an air atmosphere for various periods before extracting the RNA. Mean and standard deviation were applied to describe the experimental results.

Results

The remarkable contrast in shape between the spiral and coccoid forms in a colony of strain CG-l is shown in Fig. 1. In determining the optimum temperature for conversion to the coccoid form in aerobically incubated broth cultures, > 95% conversion occurred in 4 d at 30 and 37^{0}C, whereas at 25^{0}C it took 7 d. During the first and second days of air exposure, intermediate forms exhibiting a bulb at one pole of the spiral cell were occasionally observed. Maximum conversion to the coccoid forms converted in 3 d and no further increase occurred with prolonged incubation. Two repetitions of the experiment gave similar results and 37^{0}C was chosen for all conversion experiments.

A chloramphenicol concentration of 128 mg/ml, which is 8 times the minimum bactericidal concentration, failed to prevent conversion of mid-log phase cultures to the coccoid form.

Measurement of the culturability of strain CG-1 in mid-log phase cultures and in cultures

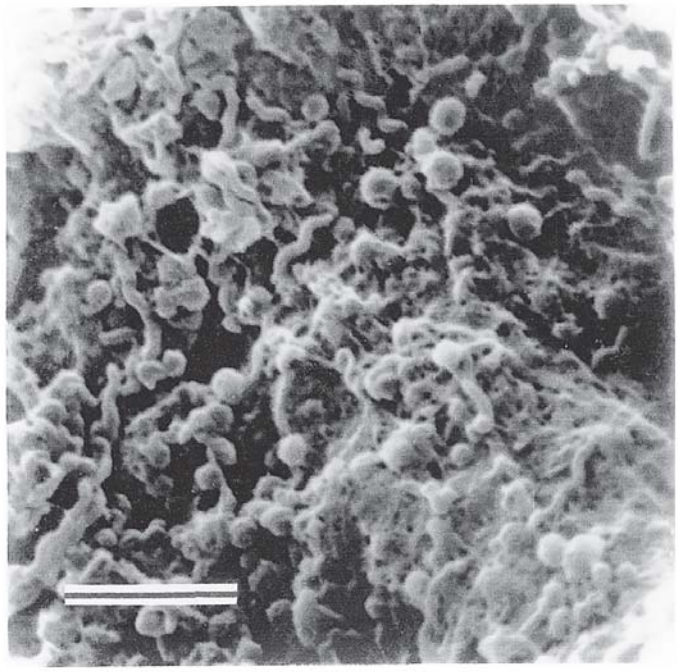

Fig. 1. Scanning electron micrograph of *Campylobacter upsaliensis* strain CG-1 from a colony grown on Brucella agar at 37^{0}C and subsequently exposed to air for 3 days. The bar represents 5 m

subsequently exposed to air indicated that on the first day of air exposure colony counts decreased to about 99% compared to the mid-log phase (Table1). From the second day to the fifth day of air exposure no colonies developed, even when 0.1 ml of undiluted culture was spread onto the Brucella agar. Measurement of viability by acridine orange staining revealed that all of the cultures, > 95% of the cells-both vibrioid and coccoid-exhibited orange-red fluorescence (Table1). A few cells (< 5%) did exhibit a green fluorescence.

The specific activity of ICDH decreased by 40% on the first day of air exposure compared to exponential growth phase cells, by 85% on the second day, and by 95% on the third day to the fifth day (Table 2).

Oxygen uptake by the vibrioid form with succinate, malate, and formate was 126, 3200, and 3600 nanomoles O_2/min, respectively, whereas no detectable oxygen uptake occurred with the coccoid form. Neither form exhibited detectable oxygen uptake with fumarate, isocitrate, glutamate, aspartate, alanine, glucose, fructose, galactose, and ribose.

Cells in the exponential growth phase showed intact 23S, 16S, and 5S rRNA but on the first day of air exposure the cells began showing apparent degradation of 23S and 16S (Fig. 2). One fragment occurred below the 23S band and two fragments below the 16S band, suggesting preferential sites of attack. From the second to the fifth day of air exposure, the RNA progressively showed more extensive degradation with the nearly complete disappearance of the 23S band (Fig.2). Two repetitions of the experiment yielded similar results. Despite the degradation of the RNA, no apparent decrease in the amount of RNA per ml of culture occurred after the log phase. This was presumably due to the fact that degraded RNA still gives similar or even higher optical density values when estimating RNA concentration by ultraviolet spectrophotometry.

Table 1. Viability measurements of *C. upsaliensis* CG-1 by colony counts and acridine orange Staining

Time after exposure (hours)	CFU per ml[a] to air	% Fluorescing orange-redby acridine orange stain
0[b]	$1.8\pm0.3 \times 10^{10}$	100
24	$1.5\pm0.2 \times 10^{5}$	99
48	NG[c]	98
72	NG	95
96	NG	95
120	NG	95

[a] Values represent the mean and standard deviation from three different experiments
[b] Cell were from the exponential phase of growth
[c] NG = no growth

Table 2. Isocitrate dehydrogenase (ICDH) activity in *C. upsaliensis* CG-1 after various periods of air exposure

Time after exposure to air (hours)	ICDH activity, mmole per min per mg protein
0[a]	514 ± 8[b]
24	290 ± 4
48	77 ± 4
72	27 ± 2
96	18 ± 2
120	3 ± 1

[a] Cells were from the exponential phase of growth
[b] Values represent the mean and standard deviation from three different experiments

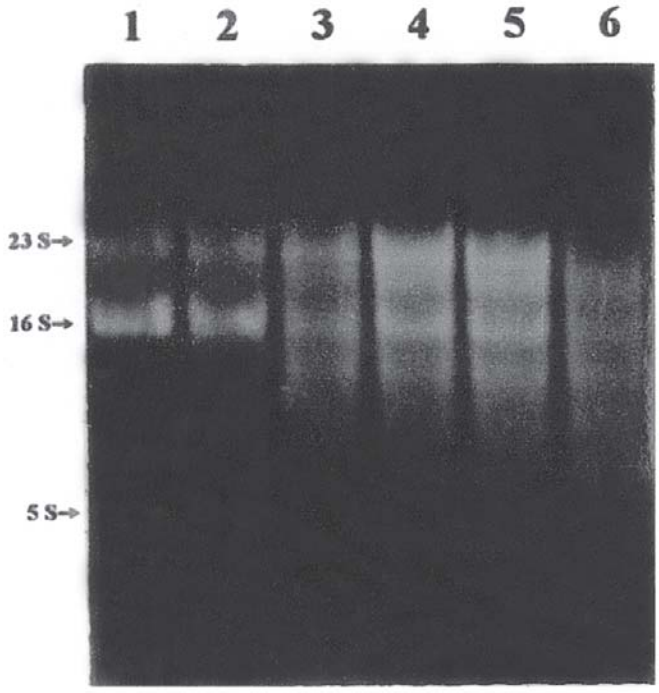

Fig. 2 Agarose gel electrophoresis of rRNA isolated from *C. upsaliensis* during exponential growth (lane 1) and at 1, 2, 3, 4, and 5 days of exposure of the culture to air (lanes 2-6, respectively). The locations of 23S, 16S, and 5S RNA are shown

Discussion

Whether or not the vegetative form of *C. upsaliensis* can be resuscitated from the coccoid form cannot be determined definitively from these experiments. Some investigators[2,4] have reported success in resuscitating the viable-but-nonculturable coccoid form of *C. jejuni* by animal feeding experiments but others have not[3]. Such experiments are open to the criticism that a few vegetative forms may have been presented in the portion fed to the animal and that these may have multiplied, leading to the erroneous conclusion that resuscitation had occurred. In the case of *C. upsaliensis* strain CG-1, although no growth occurred on Brucella agar by the second day of air exposure, vital staining with AO suggested that most of the coccoid cells might still be viable. Since most of the RNA in bacterial cells is rRNA, this suggests that the coccoid cells, although nonculturable on Brucella agar, might still possess the ability to synthesize proteins and thus might be able to be resuscitated under suitable conditions. However, degenerative forms of rRNA, as indicated by a smear on agarose gel electrophoresis, also can bind to fluorochrome vital stains[24]. Yamamoto et al[24] reported that electrophoresis of nucleic acids extracted from nonculturable cells of Legionella pneumophila revealed degradation of the 23S,16S, and 5S rRNA. Tolker- Nielsen and Molin[25] have suggested that the degradation of rRNA is a direct cause of bacterial cell death.

This suggests that acridine orange staining may not be a good indicator of viability and that the coccoid form of *C. upsaliensis* at 37^0C is degenerative rather than part of the life cycle of the organism. Other evidence to support a lack of viability in *C. upsaliensis* includes the lack of isocitrate dehydrogenase (ICDH) activity and lack of detectable oxygen uptake.

It is possible that at temperatures lower than 37^0C the characteristics of the coccoid form might more closely resemble to those of the spiral form. For instance, Hazeleger et al[26] reported that coccoid forms of *Campylobacter jejuni* produced at 4^0C and 12^0C showed two-dimensional protein profiles and fatty acid patterns similar to those of the spiral forms, whereas the coccoid forms produced at 25^0C seemed to be degenerative. With *C. upsaliensis*, the conversion of only 10% of the cells to the coccoid form at 4^0C makes it difficult to assess their physiological condition, since there is no way yet available

to separate the two forms physically. Nevertheless, research with additional strains of *C. upsaliensis* under varying conditions of temperature and other physical parameters may provide additional data of the VBNC state of this organism.

The failure of chloramphenicol to inhibit the conversion from spiral to coccoid form by preventing the synthesis of enzymes that might cause the spiral change to coccoid form is in accordance with results obtained with *Campylobacter jejuni*[6,26]. This suggests that the specific enzyme(s) for conversion may already exist in the spiral form before adding chloramphenicol. Therefore, the conversion to the coccoid form may involve preexisting autolysins that destroy the peptidoglycan of the cell wall.

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การย้อมด้วยสีแอ็คคริดินออรินจ์และการมีชีวิตอยู่ของcoccoid formของเชื้อ Campylobacter upsaliensis

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การเปลี่ยนแปลงเป็น coccoid form ของเชื้อ Campylobacter upsaliensis ใน ระหว่าง การ อบเพาะ ต่อ ในสภาวะแอโรบส์ที่ 37°C พบว่าไม่ถูกยับยั้งด้วย chloramphenicol ที่เติมลงไปและยังพบว่ามีระดับของ isocitrate dehydrogenase และการใช้ ออกซิเจนลดลงอย่างมาก ถึงแม้ว่า coccoid form ที่ ถูกย้อมด้วยสี แอ็คคริดินออรินจ์ จะสามารถเรืองแสงเป็นสีส้มแดงได้ แต่จากการทำ agarose gel electrophoresis บ่ง ชี้ว่ามีการสลายของ ribosomal RNA อย่างมาก ซึ่งแสดงให้เห็นว่าการย้อมด้วยสีแอ็คคริดินออรินจ์อาจจะไม่ดีนักกับการดูการมีชีวิตอยู่ของเชื้อ ดังนั้น coccoid form ของเชื้อ C. upsaliensis ที่เพาะต่อที่ 37°C จึงน่าจะเป็นรูปแบบของการเสื่อมสภาพของเชื้อ มากกว่าที่จะเป็นส่วนหนึ่งของเซลล์ที่มีชีวิตในวงจรชีวิตของเชื้อ
