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Survival of spinach-associated *Helicobacter pylori* in the viable but nonculturable state

Alan Buck, James D. Oliver *

Department of Biology, University of North Carolina at Charlotte, Charlotte, NC 28223, USA

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ABSTRACT

The environmental reservoir and mode of transmission of *Helicobacter pylori* is currently unknown due to difficulties in isolating *H. pylori* from non-human sources. The purpose of this study was to determine the ability of *H. pylori* to survive in a viable but nonculturable (VBNC) state when in association with spinach. *H. pylori* cells rapidly became non-detectable by plating, however mRNA transcripts were detected 6 days after the cells were introduced to the spinach. It was found that exposure to white light rapidly induced the VBNC state in *H. pylori*, suggesting sunlight may be a factor in loss of culturability of this pathogen. Our study indicates that spinach-associated *H. pylori* cells can remain viable and virulent despite their lack of culturability, which may help explain the lack of culturability from environmental sources.

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1. Introduction

Helicobacter pylori is a fastidious, gram-negative, microaerophilic bacterium that is the causative agent of chronic gastritis, peptic ulcers, gastric adenocarcinoma, and lymphoma of the stomach (Cover & Blaser, 1995). Despite its relatively recent discovery, *H. pylori* is considered to be the most prevalent human pathogen world-wide, having an infection rate as high as 80% in developing countries and 40% in developed countries (Cover & Blaser, 1995; Nayak & Rose, 2007). It is believed that *H. pylori* is transmitted via the fecal/oral and oral/oral routes, based on the isolation of *H. pylori* cells from human stool (Gramley, Asghar, Frierson, & Powell, 1999; Nayak & Rose, 2007; Queralt & Araujo, 2007; Thomas, Gibson, Darboe, Dale, & Weaver, 1992) and saliva samples (Ferguson et al., 1993), however the exact method of transmission from person-to-person continues to elude investigators. This is primarily due to the inability to recover culturable *H. pylori* cells from the environment. If the fecal/oral route is to adequately account as the means of transmission between humans, *H. pylori* must remain viable in the environment (e.g. in water or on food surfaces) for a sufficient period of time to allow for infection of a new host.

Despite many studies that have implicated foodstuffs as a means of transmission of various enteric pathogens (Ingham et al., 2004; Islam et al., 2004; Karenlampi & Hanninen, 2004; Kroupitski, Pinto, Bradl, Belausov, & Sela, 2009; Lang, Harris, & Beuchat, 2004; Tholozan, Cappelier, Tissier, Delattre, & Federighi, 1999), the possible involvement of foodstuffs in the transmission of *H. pylori* has not been studied in any great detail. Poms and Tatini (2000) reported culturable cells of *H. pylori* could be recovered from spiked skim milk and tofu through 5 days of storage at 4 °C. That study also reported recovery from spiked lettuce and raw chicken for up to 2 days at this temperature. The isolation of culturable cells from such samples indicates that this bacterium does indeed have the ability to survive on the surfaces of produce and in other foods for at least some length of time.

Drinking and irrigation water contaminated with *H. pylori* may also help explain the high prevalence of this pathogen in human populations (Gião, Azevedo, Wilks, Vieira, & Keevil, 2008). A study by Lang et al. (2004) indicated that pathogens found in irrigation waters may adhere to the surfaces of vegetables that eventually make their way to consumers. Thus, *H. pylori*-contaminated produce may act as an environmental reservoir for *H. pylori*, lending support for the fecal–oral mode of transmission.

Several studies have suggested that *H. pylori*, like many human pathogens, enters a viable but nonculturable (VBNC) state outside the human host (Adams, Bates, & Oliver, 2003; Can et al., 2008; Nilsson et al., 2002). In this state, bacteria remain metabolically active but fail to develop into colonies when cultured on routine media (Oliver, 2005a). Cells typically enter the VBNC state in

* Corresponding author. Tel.: +1 704 687 8516; fax: +1 206 600 7948.
E-mail address: jdoliver@uncc.edu (J.D. Oliver).

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response to such natural stresses as temperature extremes, starvation, osmotic shock, and even some intensities of white light (Oliver, 2005b). Detection of VBNC cells has required innovative techniques and a re-evaluation of the definition of "viability" in bacteria, due to the inability of routine culturability assays to detect such cells. Techniques employed include PCR and reverse transcriptase-PCR (RT-PCR) technology to detect the DNA of, and active gene expression by, VBNC cells. Use of PCR in the identification of pathogens is routine and has been used to detect *H. pylori* DNA in drinking water samples (Hulten et al., 1996). However, detection of DNA alone is not sufficient to claim viable cells are present, as DNA can persist in the environment and could have originated from dead cells (Dupray, Caprais, Derrien, & Fach, 1997). In a study by Nilsson et al. (2002), mRNAs of known virulence factors (*vacA* and *ureA*) were detected in VBNC *H. pylori* cells using RT-PCR. This technique utilizes the inherently unstable nature of bacterial mRNAs to infer viability when culturability is below the limit of detection (Lahtinen et al., 2008; Sheridan, Masters, Shallcross, & Mackey, 1998; Smith & Oliver, 2006). Furthermore, detection of transcripts from known virulence genes may concurrently infer continued virulence of these organisms when present in the VBNC state.

In the present study, we found that while *H. pylori* became nonculturable within 24 h on spinach leaves, the cells could be shown to retain viability for 6 days or more. We also found that white light, such as sunlit plants would be exposed to, appears to be a major factor in inducing these cells into the VBNC state. Finally, we found that expression of *vacA*, a known virulence factor in this pathogen, continued in these VBNC cells, suggesting *H. pylori* remains virulent after entry into this physiological state. These results may help explain the epidemiology of this pathogen, despite its lack of culturability from environmental sources.

2. Materials and methods

2.1. Bacterial strain and culture conditions

H. pylori strain ATCC 43504 was cultured in vented tissue culture flasks in Brucella broth (Becton Dickinson and Co., Sparks, Md.) containing 5% fetal bovine serum (Quad Five, Ryegate, MT), 10 mg/l vancomycin, 5 mg/l trimethoprim, and 2500 IU/l polymyxin B sulfate (Sigma, St. Louis, MO, USA). Cells were maintained on either of two solid media: (1) Brucella blood agar containing 5% defibrinated donor sheep blood (Quad Five, Ryegate, MT), the same concentration of antibiotics as the broth, in addition to 7.5 mg/l amphotericin B, and 5 mg/l cefsulodin or (2) Wilkins-Chalgren blood agar (Acumedia Manufacturers, Inc., Lansing, MI, USA) containing 5% sheep blood, 30 mg/l colistin methanesulfonate, 100 mg/l cycloheximide, 30 mg/l nalidixic acid, 30 mg/l trimethoprim, and 10 mg/l vancomycin (all from Sigma, St. Louis, MO, USA). These media reduce contamination by the normal flora typically present on produce surfaces. Liquid cultures and plated cells were incubated in a CO_2 incubator at 37 °C under conditions of 100% humidity and a 7.5% CO_2 atmosphere.

2.2. Treatment conditions

Baby spinach (Earthbound Farms Organic, washed, bagged and "Ready-to-Eat") was acquired from a local grocery. Spinach leaves of approximately the same size (ca. 75–100 mm in length and 50–75 mm wide) and which were not otherwise washed or treated, were dip inoculated into *H. pylori* cells which had been diluted in phosphate buffered saline (PBS) to achieve a final concentration of 10^7 cells/mL. Leaves were incubated in the inoculum in sterile Whirl-Pak bags (Nasco, Ft. Atkinson, WI, USA) at room temperature under normal air conditions for 24 h. Because we found light to be a major factor in culturability of this bacterium, bags were incubated in the dark. Leaves were then removed and sealed in separate, sterile Whirl-Pak bags held at room temperature in the dark. Samples were selected and cells removed from both sides of each leaf using sterile swabs at hourly intervals up to 24 h then at daily intervals up to 7 days. Swab heads were agitated into dilution tubes containing 1 mL of PBS to remove cells from the swabs. It must be noted that cells recovered during this process may have come not only from the inoculated leaves, but also from the small amount of inoculum present within the bags but not spinach associated. For this reason, we consider our results to represent spinach-associated *H. pylori* cells. These studies were repeated three times.

2.3. Determination of culturability

Bacterial samples recovered via swabbing were serially diluted into PBS and plated onto either Brucella blood agar or Wilkins-Chalgren blood agar, as described above. Plate counts were recorded after incubation for 7–10 days at 37 °C and CO_2 incubation (Adams et al., 2003). Bacterial samples were considered to be nonculturable when less than 10 CFU/mL could be detected. At each time point, the three remaining sample volumes were combined, centrifuged, and resuspended in 500 μL of PBS prior to RNA stabilization (described below).

2.4. Direct microscopic determination of viability

When no culturable cells were detected, viability was determined using the *Bac*Light LIVE/DEAD® bacterial viability kit (Molecular Probes, Eugene, OR) as previously described (Adams et al., 2003). This kit utilizes SYTO 9 and propidium iodide to discriminate between live cells with intact membranes (green fluorescence) and dead cells with compromised membranes (red fluorescence). Stained samples were filtered onto polycarbonate filters and viewed using epifluorescence microscopy. A minimum of 300 cells or 30 fields was counted.

2.5. Determination of viability and virulence by RT-PCR

At each time point, 500 μL of the removed bacterial cells was treated with 1 mL of RNAprotect (Qiagen) and stored at −80 °C. RNA was extracted using the TRIzol® Max™ Bacterial RNA Isolation Kit (Invitrogen) and suspended in RNA Storage Solution (Ambion). Extracted RNA was then treated with Turbo DNase (Ambion) to remove contaminating DNA. PCR using *Taq* polymerase (Takara) and the *vacA* primer sequences described below were used to confirm the absence of contaminating DNA. RT-PCR was performed using the Qiagen One-Step RT-PCR Kit (Qiagen) to detect the presence of mRNA containing the *vacA* gene sequence. Up (*vacA*1: 5′ATGGAAATACAACAAACACA 3′) and down (*vacA*2: 5′CTCCAG AACCCACACGATT 3′) primers for amplification of the *vacA* gene (658 bp product) were created using Vector NTI software (Invitrogen) and purchased through Bio Synthesis, Inc. (Lewisville, TX, USA). The cycling profile for PCR included an initial denaturation step of 94 °C for 3 min; followed by 35 cycles of 94 °C denaturation temperature (30 s), 48 °C annealing temperature (30 s), and 72 °C extension temperature (1 min); and a final extension of 72 °C for 10 min. The RT-PCR profile was the same as PCR with the exception of a reverse-transcription step of 50 °C for 30 min followed by an initial denaturation of 95 °C for 15 min. PCR and RT-PCR products were visualized on a 1% agarose TAE gel with 0.1% ethidium bromide.

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