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Biofilm formation enhances *Helicobacter pylori* survivability in vegetables

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ABSTRACT

To date, the exact route and mode of transmission of *Helicobacter pylori* remains elusive. The detection of *H. pylori* in food using molecular approaches has led us to postulate that the gastric pathogen may survive in the extragastric environment for an extended period. In this study, we show that *H. pylori* prolongs its survival by forming biofilm and micro-colonies on vegetables. The biofilm forming capability of *H. pylori* is both strain and vegetable dependent. *H. pylori* strains were classified into high and low biofilm formers based on their highest relative biofilm units (BU). High biofilm formers survived longer on vegetables compared to low biofilm formers. The bacteria survived better on cabbage compared to other vegetables tested. In addition, images captured on scanning electron and confocal laser scanning microscopes revealed that the bacteria were able to form biofilm and reside as micro-colonies on vegetable surfaces, strengthening the notion of possible survival of *H. pylori* on vegetables for an extended period of time. Taken together, the ability of *H. pylori* to form biofilm on vegetables (a common food source for human) potentially plays an important role in its survival, serving as a mode of transmission of *H. pylori* in the extragastric environment.

1. Introduction

Biofilms have been reported to harbour bacteria and assist in their survival under unfavourable growth environment (Costerton et al., 1999). A biofilm is an assemblage of dead and alive bacterial cells within a matrix of exopolysaccharides, proteins, and extracellular DNA and attach to inert or living surfaces (Carpentier and Cerf, 1993; Costerton et al., 1999). The structure of biofilm protects the bacterial cells and aids the cells to persist in the environment for extended periods of time (Marks et al., 2014). These bacterial cells in biofilm are highly resistant to killing and clearance by disinfectants and sanitizing agents, and are more apt in survival compared to the free-floating planktonic counterparts in environments (Costerton et al., 1999; Dunne, 2002; Jefferson, 2004; Pan et al., 2006). Owing to the unique properties of microbial biofilm, it is difficult to eliminate the biofilm-forming bacteria from food sources and food processes. This problem poses concerns in food safety (Kalmokoff et al., 2001; Valderrama and Cutter, 2013).

Helicobacter pylori is a Gram-negative microaerophilic bacterium that has been firmly established as an etiological agent for gastritis and peptic ulcer disease in humans (Warren and Marshall, 1983; Covacci et al., 1999). To date, more than 50% of the world population is infected by *H. pylori* (Pounder and Ng, 1995; Covacci et al., 1999; Bumann et al., 2001). Despite extensive investigation on the spread of *H. pylori*, the precise mode of transmission remains unclear. As *H. pylori* DNA has been detected in untreated well water, drinking water and treated water distribution systems in England using molecular approaches (Baker and Hegarty, 2001; Bunn et al., 2002; Park et al., 2001; Watson et al., 2004), it is highly possible that food can be contaminated through the use of contaminated water during its preparation (Vale and Vitor, 2010). Food may also be contaminated by *H. pylori* as a consequence of poor hygienic practices during the food processing. Therefore, it is postulated that ingestion of contaminated food could potentially be one of the transmission routes for *H. pylori*.

Several epidemiological studies have proposed that food may

Abbreviations: CM, complete medium; BU, biofilm unit.

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serve as a potential reservoir or vehicle in the transmission of *H. pylori* (Baker and Hegarty, 2001; Poms and Tatini, 2001; Quaglia et al., 2008). As early as 1993, Hopkins et al. reported higher prevalence of *H. pylori* infection among young Chilean subjects in association with the consumption of uncooked vegetables. Subsequently, Begue et al. (1998) reported that the increased prevalence of *H. pylori* infection in Lima, Peru, was significantly associated with the consumption of food from street vendors. Recently, Atapoor et al. (2014) and Yahaghi et al. (2014) not only detected *H. pylori* DNA but also isolated the bacterium from raw vegetables and salads. Thus, raw vegetables may potentially serve as transmission vehicle for *H. pylori*.

In 1999, Stark et al. demonstrated that the gastric pathogen, *H. pylori,* forms biofilm. This was followed closely by various other studies (Cole et al., 2004; Cellini et al., 2008; Giao et al., 2008; Yonezawa et al., 2009, 2010, 2011, 2013). Given its fastidious growth requirements, the formation of biofilm may potentially enhance the survival of *H. pylori* in vegetables for an extended period. Indeed, foodborne pathogens, such as *Listeria monocytogenes*, *Escherchia coli* O157 and *Salmonella enterica* serovar Typhimurium, are known to reside on vegetables in the form of biofilm, aiding in their survival (Carmichael et al., 1999; Lapidot et al., 2006; Niemira and Cooke, 2010). But, to date, the association of *H. pylori* biofilm with vegetables is still poorly documented. In order to elucidate this association, this study explores the ability of *H. pylori* to form biofilm on vegetables and the correlation between biofilm forming abilities of different *H. pylori* strains and their survival on different kinds of vegetables.

2. Materials and methods

2.1. Culturing of H. pylori

H. pylori strains used in this study comprised 3 laboratory strains and 10 clinical strains (Table 1). These 13 *H. pylori* strains were cultured on non-selective Blood Base agar (Oxoid, UK) supplemented with 5% horse blood (Quad Five, MT, USA) or in liquid complete medium (CM) comprising of brain heart infusion broth (Oxoid, UK) supplemented with 0.4% yeast extract and 1% β-cyclodextrin (Sigma Aldrich, USA). The cultures were incubated at 37 °C for 2–3 days in a humidified 10% CO_2 incubator (Forma Scientific, USA).

2.2. Evaluation of biofilm formation and evaluation

H. pylori cultures were suspended in CM and adjusted to 10^7 cfu/ml. An aliquot of 100 μl was then inoculated onto sterile 96-well non-treated plates (NEST Biotechnology, China) for the development of biofilm. The 96-well plates were incubated at 37 °C for 3, 6 and 12 days in a humidified 10% CO_2 incubator. A modified crystal violet staining method of Djordjevic et al. (2002) was used to quantify the biofilm formed. In brief, the bacterial suspensions (planktonic cultures) in the 96-well plates were aspirated and transferred onto new 96-well plates for the determination of cell turbidity at optical density at OD_{600}. The wells were then gently washed two times with 200 μl of phosphate-buffered saline (PBS), dried for 30 min and stained with 200 μl of 0.01% crystal violet (Merck, Germany) for 15 min. The stained wells were gently swirled to ensure uniform staining of the biofilm. After staining, the plates were washed thoroughly with distilled water before adding 100 μl of ethanol-acetic acid (95:5, v/v) to dissolve the crystal violet. Biofilm production was quantified by measuring the level of crystal violet present at OD_{600}. As control, uninoculated medium was used to determine the background OD. The mean OD_{600} value from the control wells were subtracted from the mean OD_{600} value of sample wells to determine the amount of crystal violet absorbed by the *H. pylori* biofilm. Cell turbidity of the planktonic cultures was determined by measuring the optical density at OD_{600}. The biofilm units (arbitrary units) were then calculated by dividing biofilm absorbance with the corresponding planktonic OD (Burmolle et al., 2006; Latimer et al., 2012). This study employs biofilm units (BU) in order to compare the biofilm development of different *H. pylori* strains. BU is the ratio of the absorbance values of the crystal violet stained biofilm to the OD600of the growth of corresponding planktonic form. Each BU was taken to be the amount of biofilm formed by the planktonic form of the particular strain measured.

Table 1
Bacterial strains used in the study.

Bacterial strains	Description	Reference
J99	Laboratory adapted strain	Alm et al., 1999
NCTC11637	Laboratory adapted strain	Akopyants et al., 1997
SS1	Laboratory adapted strain	Lee et al., 1997
UM032	Clinical isolate; PUD	Rehvathy et al., 2013
UM038	Clinical isolate; NUD	Rehvathy et al., 2013
UM045	Clinical isolate; NUD	This study
UM054	Clinical isolate; NUD	This study
UM066	Clinical isolate; PUD	Rehvathy et al., 2013
UM067	Clinical isolate; PUD	Rehvathy et al., 2013
UM085	Clinical isolate; NUD	Rehvathy et al., 2013
UM111	Clinical isolate; UNK	Rehvathy et al., 2013
UM114	Clinical isolate; PUD	Rehvathy et al., 2013
UM429	Clinical isolate; UNK	This study

UM032-UM429 were isolated by the Helicobacter Research Laboratory of the UM Marshall Centre, University of Malaya, Kuala Lumpur, Malaysia.
NUD, non-ulcer dyspepsia; PUD, peptic ulcer disease; UNK, clinical disease status unknown.

2.3. Types of vegetables

Four kinds of vegetables purchased directly from local supermarkets in Singapore were used in this study. The vegetables analyzed in the study were spring onion (*Allium chinense*), cabbage (*Brassica oleracea capitata*), lettuce (*Lactuca sativa longifolia*) and spinach (*Spinacia oleracea*). These vegetables are easily available at the local markets and are commonly consumed either raw or stirred fried.

2.4. Processing of vegetables for spiking study

The vegetables were first sanitized prior to the experiments. Briefly, the vegetables were washed under running tap before incubating in an anaerobic chamber (Don Whitley scientific, UK) for 30 min to reduce the load of obligate aerobic microorganisms. The vegetables were then soaked in sterile 5% sodium chloride (Sigma Aldrich, USA) for 3 min and rinsed 3 times with sterile water. Subsequently, the vegetables were soaked in 100 ppm hypochlorous acid (HOCl), pH 2.5, for 15 min followed by rinsing 3 times with sterile water. Finally, the vegetables were cut into smaller pieces of about 1 cm^2 using a sterile scalpel.

The sanitized vegetables were sampled to evaluate the effectiveness of bacterial removal. Three cut pieces of vegetables were suspended in 1 ml of CM and vortexed vigorously. Following which, the suspensions were plated onto nutrient agar, blood agar and chocolate blood agar plates. The plates were incubated under aerobic and microaerophilic conditions at 37 °C for 3 days. No growth was observed with up to 7 days of incubation indicating the absence of cultivable microbes on the vegetables.

2.5. Bacterial inoculation and sampling

Each piece of sanitized vegetable or 1 cm^2 sterile glass fibre filter