ORIGINAL ARTICLE

YEAST PROTECTS *HELICOBACTER PYLORI* AGAINST THE ENVIRONMENTAL STRESS

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Abstract

Background—*Helicobacter pylori* is a parasite which infects the gastric epithelium of a considerable proportion of the human populations worldwide. This study demonstrates that *H. pylori* establishes a symbiotic relationship with free-living yeast. Such association may explain the survival capacity of *H. pylori* in nature.

Methods—Yeasts and *H. pylori* isolates were obtained from antral biopsies and co-culture of the two microorganisms was prepared and their interactions studied by light and electron microscopy.

Results—The results of the study revealed that *H. pylori* employs its corkscrew-type motility to penetrate the capsule and cell wall of yeast and reach the plasma membrane. Adhesion of *H. pylori* to plasma membrane induced the formation of a vacuole within which the bacterium became sequestered. The presence of *H. pylori* inside the vacuole was microscopically evident by its fast, non-stop movement and its identity was confirmed by the polymerase chain reaction (PCR) method. In contrast to *H. pylori*, the yeast was found to tolerate environmental stress such as heat, desiccation, extreme pH values, and chlorination.

Conclusions—We propose that yeast commonly found in aquatic environments, foods and gastrointestinal tract of man, may play a crucial role in the survival of *H. pylori* during the environmental changes and its transmission to human.

Keywords • *Helicobacter pylori* • symbiosis • yeast • stress, environmental

Introduction

Helicobacter pylori has been viewed as a parasite which infects gastric epithelium of a considerable proportion of the human populations worldwide.[1] Infection of *H. pylori* is associated with gastritis and peptic ulcer disease[2,3] and the bacterium is categorized as a class I carcinogen.[4] Although antibacterial therapy has played an important role in the eradication of *H. pylori*, prophylaxis is also crucial in the challenge against *H. pylori* infection. Successful prophylaxis and even more effective therapy can be achieved when natural origin, possible intermediate host(s), and mode of transmission of *H. pylori* are determined. Failure to culture *H. pylori* from natural sources such as water, has made it a serious public health problem to uncover the mystery of *H. pylori* existence outside the human body.

H. pylori infection is very common in Iran and up to 85% of Iranian adults are seropositive for *H. pylori*.[5] The eradication rate of *H. pylori* with conventional triple therapy is suboptimal and recrudescence or reinfection occurs in up to 15% of patients after one year.[6,7] The high prevalence and relapse of *H. pylori* infection might be related to the persistent form of bacterium as an intracellular parasite of a higher organism. Intracellular existence[8,9] is a phenomenon the details of which

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are increasingly emerging by exploitation of new molecular diagnostic techniques, such as PCR.[10] It appears that several simple organisms choose this life style to resist environmental stress, including chlorination of potable water,[11] and immune system of the host.[12,13]

To eradicate the infection of *H. pylori*, it is very important to understand the mechanism (s) by which *H. pylori* persists in natural environments as well as in human gastrointestinal tract. Infection of human gastric epithelial cells by *H. pylori* is the only known example of association of this bacterium with other cellular organizations. However, in this report, we demonstrate that *H. pylori* is able to establish an endosymbiotic relationship with yeast. Yeasts are unicellular fungi that are isolated from a variety of environmental sources including soil,[14] fresh and salt water.[15] These microorganisms occur as free-living or in association with plants, fowl, animals, and insects.[14] Yeasts such as Candida species commonly colonize the mucocutaneous tissue of humans, particularly the oral cavity, gastrointestinal, genital, and urinary tracts.[16] These ubiquitous microorganisms may become pathogenic under certain circumstances such as immunosuppression of the host.[17] In this study co-culture of *H. pylori* and yeast was prepared and the mode of penetration of bacterium into the yeast cell was assessed. Also, the symbiotic relationship between the two microorganisms, regarding the protective role of yeast in the survival of *H. pylori* against the environmental stress, was investigated.

Materials and Methods

Bacterial isolates

H. pylori isolates were obtained from antral biopsy specimens using appropriate culture media and incubation conditions as described before.[18] The isolates were identified by colony morphology and biochemical tests.[19] *Campylobacter jejuni* and *Escherichia coli* strains were obtained from the Department of Microbiology Culture Collection, Faculty of Science, Tehran University.

Yeast isolates

Yeasts were isolated from antral biopsies that were cultured on solid media for the detection of *H. pylori*. Twenty five of the yeast isolates were identified according to the morphology of their vegetative cells on corn meal agar.[20]

Preparation of co-cultures

Co-cultures of yeast and *H. pylori* were prepared by inoculating 1×10^3 yeast cells per ml into 50 ml brucella broth containing 1×10^8 bacterial cells per ml. After 24 hours of microaerophilic incubation, yeasts were isolated on Sabouraud dextrose agar.

Light microscopy

Attachment of *H. pylori* to the yeast cell and its penetration was followed by light microscopy. Yeast cells were also examined for the presence of intracellular bacterium inside their vacuoles. Rapidly moving bacteria inside the vacuoles were scored positive for viability.

Electron microscopy

Attachment of *H. pylori* to yeast cells was further visualized by scanning electron microscopy.[21] The presence of *H. pylori* inside the yeast was further examined by transmission electron microscopy.[22]

PCR

Eighteen yeast isolates obtained from antral biopsies were analyzed by PCR for the presence of an intracellular *H. pylori*. Pure cultures of *C. jejuni* and *E. coli* were used as negative controls and pure culture of *H. pylori* as positive control. Bacterial genomic DNAs were extracted according to Wilson.[23] Extracted DNAs were amplified using primers CAM-2 and CAM-4.[24] The amplified PCR products were analyzed by agarose gel electrophoresis.

Resistance to environmental stress

Co-cultures of *H. pylori* and yeast were studied when heated from 40°C up to 100°C, kept desiccated for 3 months, exposed to different pH values of 1 to 9, chlorinated using 0.5 to 10 mg per liter of calcium hypochlorite, and treated with 0.3 μg per ml amoxicillin or 2 μg per ml cephalothin.[25]

Viability of *H. pylori* and co-cultures

Heat or antibiotic-treated *H. pylori* suspensions were cultured on brucella blood agar or brucella broth enriched with 5% fetal calf serum. Tetrazolium reduction method was used to assess the viability of *H. pylori* cultures exposed to different pH values.[26] Flow cytometry was used to confirm the viability of chlorinated cultures of *H. pylori*.[27] The viability of treated co-cultures was examined by growing them on Sabouraud dextrose or nutrient agar, or inoculating them into brucella broth. The viability of intracellular bacteria was determined by their active movement inside the vacuole as assessed by light microscopy.

Results

Bacterial isolates obtained from antral biopsies were identified as *H. pylori*. Of the 25 yeast isolates, 17 were identified as Candida species and 5 as *C. albicans*, and the remaining 3 were not identified. Light microscopic observations indicated that *H. pylori* passes through the mucoid polysaccharide capsule and the rigid cell wall of

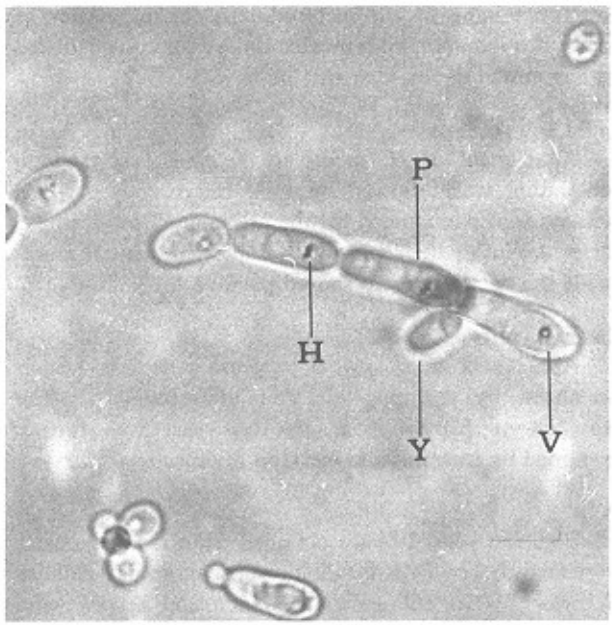

Figure 1: Light microscopy of yeast cells and resident *H. pylori*. Single yeast cell (Y) as well as cells forming pseudohyphae (p) are shown. Bacterial cells (H) inside the vacuoles (V) are evident (×1,250).

the yeast by its corkscrew-type motility. Attachment of bacterial cell to cytoplasmic membrane of yeast triggers the formation of a vacuole, inside which bacterium becomes sequestered and starts to multiply. It appears that after penetration and entrapment of *H. pylori*, resealing of the yeast's cell wall and cell membrane occurs, and other subcellular structures, including nucleus remain intact. The presence of a bacterium inside the yeast's vacuole is evident in the photographs prepared by light microscopy (Fig. 1). Despite the addition of formaldehyde, fast movement of bacteria inside the vacuoles disturbed the focusing of the image. Scanning electron micrographs show the attachment of spiral and coccoid forms of *H. pylori* to the yeast cell. Transmission electron micrographs show occurrence of the intracellular bacterium inside the yeast's vacuole (Fig. 2). Comparison of bands formed by the amplified PCR products on agarose gel revealed that the amplified bacterial DNAs extracted from the infected yeast cultures formed similar bands as did DNAs from pure culture of *H. pylori*. Of the 18 yeast isolates obtained from the biopsy specimens, 12 were positive for having the intracellular *H. pylori* (Fig. 3), 8 of which formed colonies along with *H. pylori* when the specimens were initially cultured on brucella blood agar. Biopsy cultures of the remaining 4 were negative for *H. pylori*.

H. pylori was inactivated at 40°C and was not recovered in enriched medium. Co-cultures heated at 40°C and 50°C were able to recover when transferred to favorable conditions. Both heated and recovered cultures contained actively moving intracellular bacteria. Co-cultures heated at 60-100°C were not recovered. Dried yeasts could produce turbidity in brucella broth after 24 h and actively moving bacteria were observed within the vacuoles. Bacterial cells at pH values 4, 5, 6, 7 and 8 remained viable and a large proportion retained the typical spiral morphology. Although bacterial cells at the extreme pH values of 1, 2, 3 and 9 became coccoid, they remained viable and reduced tetrazolium to formazan crystals. On the other hand, co-cultures could grow at pH values of 2 to 9 and intracellular bacteria were observed moving within the vacuoles.

Cultures of *H. pylori* could not tolerate chlorine residuals and became coccoid and nonculturable. Flow cytometry showed that 5.4% of the population remained viable at chlorine residuals of 0.5 mg per liter after the contact time of 30 min. At chlorine residuals of 1 and 5 mg per liter, after the contact time of 30 min, the viability of bac-

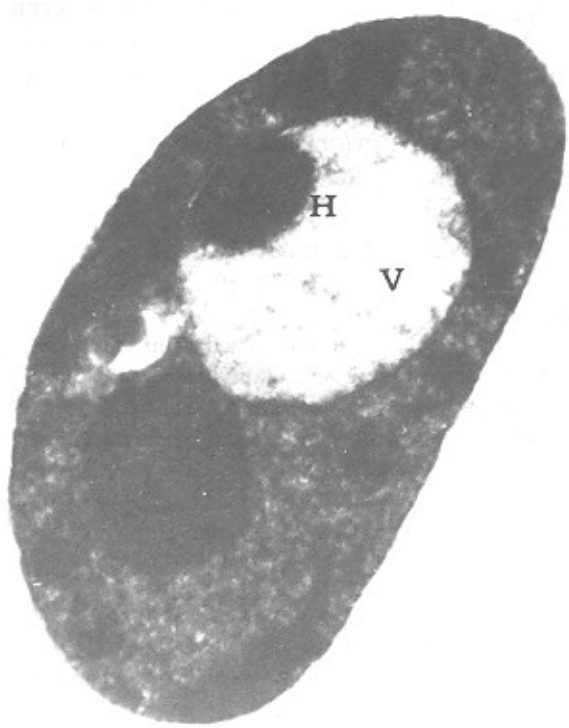

Figure 2: Transmission electron micrographs of the yeast cell, showing the intracellular *H. pylori*. *H. pylori* (H) sequestered inside the yeast's vacuole (V)

terial cells was reduced to 3.5% and 2% respectively. However, co-cultures resisted chlorine residuals up to 2 mg per liter after the contact time of 24 h., and yeast colonies grew when treated suspensions were cultured on nutrient agar. Intracellular bacteria were observed moving actively within the yeasts vacuoles. *H. pylori* cells treated with amoxicillin and cephalothin did not grow when inoculated into enriched brucella broth. However co-cultures survived antibiotics and produced turbidity when transferred to brucella broth. Actively moving bacteria were observed within the yeasts vacuoles.

Discussion

The results of this study show that yeast commonly found in nature, water reservoirs, foods, animals, and humans may act as a hospitable niche for *H. pylori*. It appears that *H. pylori* penetrates into the free-living yeast in a skillful fashion and establishes a symbiotic relationship with it. Entrance of *H. pylori* into the yeast occurs concomitant with the formation of a membrane-bound vacuole within which the bacterium becomes sequestered. Inside the vacuole, *H. pylori* can survive digestion, receive nutrients released from the cytoplasm and multiply. Residence of *H. pylori* inside the vacuole may protect the bacterium against the environmental stress including heat, desiccation, extremes of pH, and exposure to biocides.

Study of co-culture of *H. pylori* and yeast by light and scanning electron microscopy demonstrated the attachment of *H. pylori* to the yeast cells. Light microscopy revealed that *H. pylori* is able to penetrate into the thick mucoid capsule and the rigid cell wall of the yeast by exploiting its corkscrew-type motility.

Xenosomes, the bacterial symbionts of protozoa *Parauronema acutum* can enter the host's cytoplasm by direct contact to the protozoan's plasma membranes. Evidence suggests that motility plays an important role in the invasion process.[28] Results obtained by light and transmission electron microscopy suggest that adherence of *H. pylori* to yeast's plasma membrane triggers the internalization of bacterium by formation of a membrane-bound vacuole inside which the bacterium becomes entrapped. Thin sections prepared from *Legionella pneumophila-Tetrahymena pyriformis co-culture* have also shown cells containing bacteria within the vacuoles.[11] On the other hand, *Shigella flexneri* is trapped and efficiently killed in

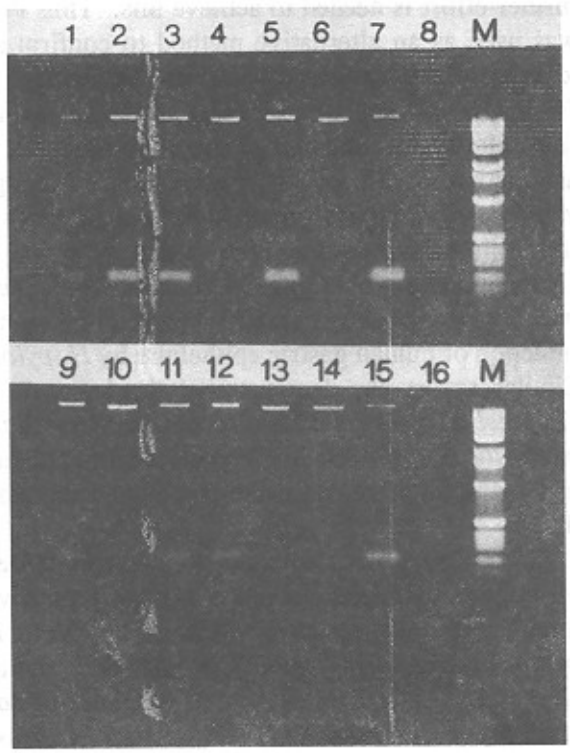

Figure 3: Amplified PCR products from either cultured *H. pylori* (lanes 7,15) or cultured yeast samples (lanes 1-6, 9-14) electrophoresed on 1.5% agarose gel. Lanes 1,2,3,5,9,11 and 12 are positive, proving the existence of intracellular *H. pylori*. lanes 4,6,10,13,14 are negative. Lanes 8 and 16 are negative controls for PCR and M is 1 Kb ladder DNA size marker.

polymorphonuclear leukocyte vacuoles.[29]

The survival of *H. pylori* inside the yeast might be a specialized and evolutionary phenomenon which has existed for a long time. While *H. pylori* is selected to establish itself as a symbiont, other bacteria may successfully enter the yeast cell but become destroyed by digestive mechanisms. The yeast's vacuolar sap is acidic (pH 5.5) and contains a number of hydrolytic enzymes, including proteases, nucleases, glycosidases and phosphatases.[30] It appears that *H. pylori* can survive and multiply at acidic pH,[31] and its enzyme machinery is functional under conditions inside the vacuole.

The mechanisms of resisting digestion of *H. pylori* within the yeast's vacuole must be elucidated, but it may be similar to those employed by pathogenic microorganisms that withstand the defense strategies of human phagocytes.[32-35]

Attempts to culture the intracellular *H. pylori* from broken yeast cells were not successful and

further effort is needed to achieve this. Thus PCR was used as an alternative method to confirm the identity of the symbiotic bacteria. Occurrence of *H. pylori* in 67% of the yeast isolates obtained from biopsy specimens confirmed the intracellular association of *H. pylori* with yeast. Amongst yeasts assayed by PCR method for the existence of intracellular *H. pylori*, 88% were identified as Candida species including *C.albicans*. Thus, these microorganisms might play an important role in the infection of human gastric epithelium by *H. pylori*. Similar report has demonstrated the key role of *Legionella pneumophila*-infected amoeba, *Hartmannella vermiformis* in the protection of bacteria in the environment and pathogenesis of pulmonary disease.[36]

Yeasts are more resistant to inhospitable environments than bacteria and if injured can recover after transfer to favorable conditions.[14,37] While *H. pylori* was inactivated at 40°C, yeast could tolerate heat up to 50°C. Studies on heat tolerance of *Saccharomyces cerevisiae* have shown that these yeasts can tolerate heat up to 50.4°C.[38] Yeast also tolerated desiccation for three months. It has been reported that yeasts, *C. albicans* and *Trichosporon beigelli* survive in dry sand for six months.[14] It has been revealed that occurrence of the thicker cell wall in yeasts makes them more resistant to heat and desiccation than bacteria.[39] Resistance of yeasts to heat and desiccation might also be due to high trehalose content of the cell wall.[38] Exposure of co-cultures and *H. pylori* to extreme pH values revealed that while cultures of *H. pylori* became coccoid and nonculturable at pH 3 and 9, co-cultures grew well at pH values of 2 to 9 and the actively moving bacteria could be seen within the vacuoles. Other studies have also revealed that yeasts, unlike most bacteria, can grow at extreme pH values such as 2 and 9.[14] It appears that inside the yeast, *H. pylori* is protected against heat, desiccation, and extremes of pH.

While *H. pylori* became coccoid and inactivated at chlorine residuals of 0.5 mg per liter, co-cultures survived chlorine residuals up to 2 mg per liter. Thus, it appears that *H. pylori* is protected against the chlorine disinfection inside the yeast. Resistance of endosymbiont *H. pylori* to antibiotics also shows that yeast cells protect the bacterium from the detrimental effects of such compounds. High biocide tolerance has been observed in yeast, notably Candida species.[39] It has been suggested that occurrence of a mannoprotein layer in the thick cell wall of yeasts can act as a molecular sieve, excluding larger molecules such as biocides. Furthermore, phosphate groups in the mannan side chains may bind biocides, restricting their entry to the cell.[40] Similar results have been reported on the survival of coliform and pathogenic bacteria when they were ingested by protozoa. *Citrobacter freundii*, *Enterobacter agglumerans*, and *Klebsiella pneumonia* were found resistant to free chlorine residuals of 2 to 10 mg per liter when ingested by amoeba, *Acanthamoeba castellanii*. Free-living isolates of pathogenic and environmental bacteria have been shown to be inactivated by free chlorine residuals of ≥ 1 mg per liter.[11] In the present study, survival of *H. pylori* within yeast against the environmental stress is in agreement with those concerning persistence of pathogenic and non-pathogenic bacteria inside protozoa. Thus, high resistance of protozoa and yeast to chlorine residuals may be one important reason for the survival of bacteria in chlorine-treated waters.

It is concluded that although *H. pylori* and other pathogenic bacteria differ in the way they enter yeast and protozoa or phagocytes, they might exploit similar mechanisms to survive and multiply within the vacuoles inside their hosts. Since yeast and protozoa are highly resistant to the environmental stress, including exposure to biocides, they might protect intracellular bacteria from such drastic conditions. Yeast and protozoa are ubiquitous in aquatic environments. Thus, they may act similarly as reservoirs of their respective pathogens in nature and water distribution systems. Accordingly, they may act as vehicles for transmission of bacteria. Based upon the above conclusion, we propose that endosymbiotic relationship between *H. pylori* and yeast may be used as a model to study the mechanisms employed by *H. pylori* to invade the human gastric epithelium and reside there as a successful parasite. Yeast is more resistant to the environmental stress than *H. pylori*, and has the ability to provide a safe biological niche for the bacterium and help it to survive in nature. Residence of *H. pylori* inside the yeast may also affect the bacterial resistance to chemotherapeutic agents. Since yeast is found in aquatic environments, potable water, and a variety of foods, it can play a very crucial role in the transmission of *H. pylori* to human. Yeast can be considered as a potent reservoir of *H. pylori* and its presence in water supplies, foods and even oral cavity might be an important issue for the public health authorities, food administrations, pharmacologists and gastroenterologists.

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