Infection, Genetics and Evolution 69 (2019) 127–133

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Sequestration inside the yeast vacuole may enhance *Helicobacter pylori* survival against stressful condition

Farideh Siavoshi[a,*], Samira Heydari[a], Mahsa Shafiee[a], Somayeh Ahmadi[a], Parastoo Saniee[b], Abdolfattah Sarrafnejad[c], Shadi Kolahdoozan[d]

[a] Department of Microbiology, School of Biology, University College of Sciences, Tehran University, Tehran, Iran
[b] Department of Microbiology and Microbial Biotechnology, Faculty of Life Science and Biotechnology, Shahid Beheshti University G.C, Tehran, Iran
[c] Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[d] Digestive Disease Research Institute, Tehran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Keywords:
Helicobacter pylori
Intracellular bacteria
Sequestration
Vacuole
Yeast

ABSTRACT

Vacuole of eukaryotic cells, beyond intracellular digestion plays additional roles such as storage of nutrients that provide favorable conditions for bacterial survival. In this study, occurrence of *H. pylori* inside the vacuole of *Candida* yeast was studied and the role of vacuolating cytotoxin A (VacA) in constructing the vacuole was discussed. One gastric *Candida* yeast was used for Live/Dead stain and fluorescence *in situ* hybridization (FISH) with universal bacterial probe. Yeast total DNA was used for amplification of full-length bacterial *16S rDNA* as well as *H. pylori*-specific *16S rDNA* and *vacA* alleles. Vacuoles were isolated from yeast cells and stained with fluorescent yeast vacuole membrane marker MDY-64. DNA extracted from vacuoles was used for amplification of *H. pylori*-specific *16S rDNA*. Fluorescent microscopy showed occurrence of viable bacteria inside the vacuole of intact *Candida* yeast cells. FISH showed intracellular bacteria as fluorescent spots inside the vacuole of mother and daughter yeast cells, suggesting bacterial transmission to next generations of yeast. Sequencing of amplified products of bacterial *16S rDNA* and amplification of *H. pylori 16S rDNA* and *vacA* confirmed the identity of intracellular bacteria as *H. pylori*. Isolated vacuoles were stained with membrane-specific marker and *H. pylori 16S rDNA* was amplified from their DNA content. Results of this study suggest yeast vacuole as a specialized niche for *H. pylori*. It appears that sequestration inside the vacuole may enhance bacterial survival.

1. Introduction

Development of intracellular membrane-bound organelles with specialized functions was a critical event in the evolution of eukaryotic cells. One important consequence of organelle development was transition from extracellular digestion of food that occurred in prokaryotes to intracellular digestion in primitive eukaryotic cells (Cavalier-Smith, 2002). Among intracellular organelles, lysosome the digestive vacuole became specialized for breaking down the self-cellular contents at times of starvation, autophagy, and digestion of the internalized microorganisms, xenophagy. Accordingly, vacuole biogenesis in primitive eukaryotic cells happened to ensure the achievement of two vital goals; survival against nutritional stress and protection against the invading microorganisms. These properties conferred to eukaryotes the ability to establish in a variety of newly explored niches and the advantages of adaptation and diversity (Levine, 2005).

Although several reports indicate that unicellular eukaryotes were first using prokaryotes for nutrients (Dyall and Johnson, 2000; Levine, 2005), other reports describe that intracellular bacteria learned to induce their own internalization and sequestration in a membrane-bound vacuole to escape from environmental stress (Mauel, 1983) and antimicrobial defense strategies of the host cytoplasm (Kumar and Valdivia, 2009). Furthermore, internalized bacteria became equipped with mechanisms that allowed their survival in the host as parasites or mutualists (Dyall and Johnson, 2000). In the other words, by establishing in the membrane-bound vacuoles within eukaryotic cells, intracellular bacteria created an appropriate niche for survival and proliferation (García-del Portillo, 1999). Consistent with this is a large body of evidence that shows many bacteria with intracellular life, becoming permanent or transient endosymbionts of eukaryotic cells (McFall-Ngai, 2008; Ruiz-Lozano and Bonfante, 1999). Details of the biological significance of bacterial invasion into the eukaryotic host and its

* Corresponding author at: Department of Microbiology, School of Biology, University College of Sciences, Tehran University, Enghelab avenue, Tehran 14176-14411, Iran.
E-mail address: siavoshi@khayam.ut.ac.ir (F. Siavoshi).

https://doi.org/10.1016/j.meegid.2019.01.029
Received 27 September 2018; Received in revised form 24 December 2018; Accepted 22 January 2019
Available online 23 January 2019
1567-1348/

establishment in the vacuole, await to be elucidated. However, it is suggested that intracellular replication of bacteria happens with low rate and limited gene expression not destroying the host cell (Corsaro et al., 1999). The overall benefit of endosymbiotic establishment inside the vacuole of eukaryotic cell would be maintenance of the vacuole and survival of both partners (Bianciotto et al., 1996).

Studies on the vacuole of different yeasts have revealed that this organelle in addition to digestion of molecules has other important functions (Armstrong, 2010; Klionsky et al., 1990) such as sorting, transporting and redistribution of nutrients. It is also the site for storage of Ca^{2+}, phosphate and a high concentration of amino acids (Sato et al., 1984). Moreover, pH and osmotic regulation, ion homeostasis and cytoplasmic detoxification take place in the vacuole (Weisman, 2003). Since vacuoles occupy a significant proportion (10–20%) of yeast cell's volume, by changing their number, size and position yeast cell can respond to environmental stresses (Armstrong, 2010). It has been demonstrated that compared with autophagosomes, bacteria-containing vacuoles are significantly larger and mainly contain bacteria rather than cytoplasmic materials (Nakagawa et al., 2004). Accordingly, yeast vacuole with all these favorable properties might serve as a sophisticated shelter for the internalized bacteria, enhancing their survival and growth instead of degradation (Kirkegaard et al., 2004; Shintani and Klionsky, 2004).

According to reports, intracellular bacteria typically show low abundance in the host cell and are difficult to detect by culture, probably due to high dependence to their host (Görtz and Brigge, 1998) or lack of knowledge about their optimum culturing conditions in microbiology lab. Nevertheless, non-culturability of intracellular bacteria has hampered the elucidation of details of their relationship with eukaryotic host (McFall-Ngai, 2008; Ruiz-Lozano and Bonfante, 1999). However, culture-independent methods including light, fluorescent and electron microscopy as well as polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH) have been recruited to reveal the identity and subcellular localization of intracellular bacteria (Amann et al., 1991; Bianciotto et al., 1996; Bianciotto et al., 2000; Fritsche et al., 1999).

It has been revealed that *H. pylori* is a facultative intracellular bacterium. Microscopic examination of gastric biopsies showed occurrence of *H. pylori* cells in gastric and duodenal epithelial cells (Shousha et al., 1984), parietal cells (Chen et al., 1986) and immunocytes (Bode et al., 1988). Furthermore, examination of *H. pylori*-infected cultured cells with immunohistochemistry, FISH (Semino-Mora et al., 2003) and ultrastructural methods (Necchi et al., 2007) revealed the identity of *H. pylori* and its intracellular localization. The intracellular *H. pylori* expressed mRNA and antigens and showed intact cell morphology (Necchi et al., 2007; Semino-Mora et al., 2003). These results suggest that intracellular niche of *H. pylori* in human stomach could provide nutrients for the slow growing bacterium and protect it from being destroyed by acidic pH, immune system and antibiotics (Chu et al., 2010; Dubois and Borén, 2007). In our previous studies, microscopic observations as well as detection of *H. pylori*-specific genes and immunodetection of *H. pylori* proteins in *Candida* yeasts showed the intracellular occurrence of *H. pylori* inside the yeast. These results suggested that *Candida* yeast could serve as a reservoir for *H. pylori* outside human stomach (Salmanian et al., 2008; Salmanian et al., 2012; Saniee et al., 2013a, 2013b; Siavoshi et al., 1998; Siavoshi et al., 2005). It is not known when and how *H. pylori* established inside the yeast cells, it probably followed similar evolutionary principles that other bacteria did. In this study, one yeast isolate from a gastric biopsy was sub-cultured on antibacterial-containing medium, > 10 times and studied for the occurrence of intracellular *H. pylori*. The aim was to demonstrate the occurrence of intracellular *H. pylori* inside the intact and isolated vacuole of *C. albicans,* proposing that yeast vacuole is the specialized intracellular niche of *H. pylori* that promotes bacterial survival. Light microscopic observation on a wet mount of one gastric yeast showed the occurrence of bacterium-like bodies (BLBs) inside the yeast's vacuole. To reveal the viability of BLBs, yeast cells were stained with the LIVE/DEAD bacterial viability kit and examined by the fluorescent microscope. Furthermore, FISH was performed with *16S rRNA*-specific oligonucleotide probe to confirm the bacterial nature of BLBs and their intracellular localization. Specific primers were used for amplification of full-length bacterial *16S rDNA, H. pylori-16S rDNA* and vacuolating cytotoxin A (VacA) from the whole DNA of yeast. To demonstrate that yeast vacuole can serve as an intracellular niche for bacteria, yeast's vacuoles were isolated and stained with fluorescent vacuole membrane marker MDY-64. Moreover, DNA extracted from the isolated vacuoles was used for amplification of *H. pylori-16S rDNA*.

2. Materials and methods

2.1. Yeast

One gastric yeast was recruited in this study. The yeast was isolated from a gastric biopsy which was cultured on brucella blood agar for isolation of *H. pylori*. The yeast produced green colonies on CHROMagar (CHROMagar, France) and identified as *Candida albicans*. Single colonies of yeast were sub-cultured on in house yeast extract glucose chloramphenicol (YGC) agar > 10 times to eliminate any extracellular bacterial contamination.

2.2. Staining the intracellular bacteria with LIVE/DEAD BacLight Bacterial Viability kit

A wet mount was prepared from fresh and pure culture of *C. albicans* on in house yeast extract glucose agar and examined for the presence of intracellular bacteria, by light microscopy (data not shown). A wet mount was also prepared from fresh culture of yeast and stained with LIVE/DEAD *Bac*Light Bacterial Viability Kit to confirm viability of intracellular bacteria (Molecular Probes, Eugene, OR, USA). Fluorescent microscope (Olympus, Tokyo, Japan) was used for observing live intracellular bacteria inside yeast's vacuole and record their movement.

2.3. Detection of intracellular bacteria using fluorescence in situ hybridization (FISH)

FISH was performed using bacterial *16S rRNA*-specific probe (EUB338) (5′-GCTGCCTCCCGTAGGAGT-3′) (Amann et al., 1990), to confirm the bacterial nature of bacteria-like bodies inside yeast's vacuole. We performed fixation-free FISH experiment according to Yilmaz et al. (Yilmaz et al., 2010). Briefly, yeast cells were grown in YG broth at 37 °C in shaking incubator (200 rpm). After 24 h, yeast culture was washed twice in 0.01 M phosphate-buffered saline (PBS) and resuspended in 100 μL of FISH hybridization buffer (5 M NaCl, 1 M Tris/HCl, 10% Formamide in distilled water) (Pernthaler et al., 2001) containing 50 ng tetramethylrhodamine isothiocyanate (TRITC)-labeled EUB338 probe (Bioneer Co., korea) and 0.01% evans blue (46160; Fluka) solution (for colour contrast). The mixture was incubated at 37 °C for 1.5 h while shaking at 120 rpm. After hybridization, cells were washed with PBS three times, 5 min each time. A 10-μL volume of the yeast culture was smeared on a glass slide and air-dried. Samples were covered with mounting medium (Invitrogen, USA) and observed with the fluorescent microscope (Olympus, Tokyo, Japan). Similarly, FISH was conducted with FAM-labeled non-EUB338 probe, the reverse complement of EUB338, to detect any nonspecific binding.

2.4. Detection of full-length bacterial 16S rDNA, H. pylori- specific 16S rDNA and vacA alleles in yeasts using polymerase chain reaction (PCR)

A fresh culture of *C. albicans* was used for extraction of DNA, using glass beads (0.5 mm, Glasperlen, Germany) and phenol–chloroform method (Sambrook, 2001). DNA extracted from yeast was first used for amplification of the full-length bacterial *16S rDNA* with universal

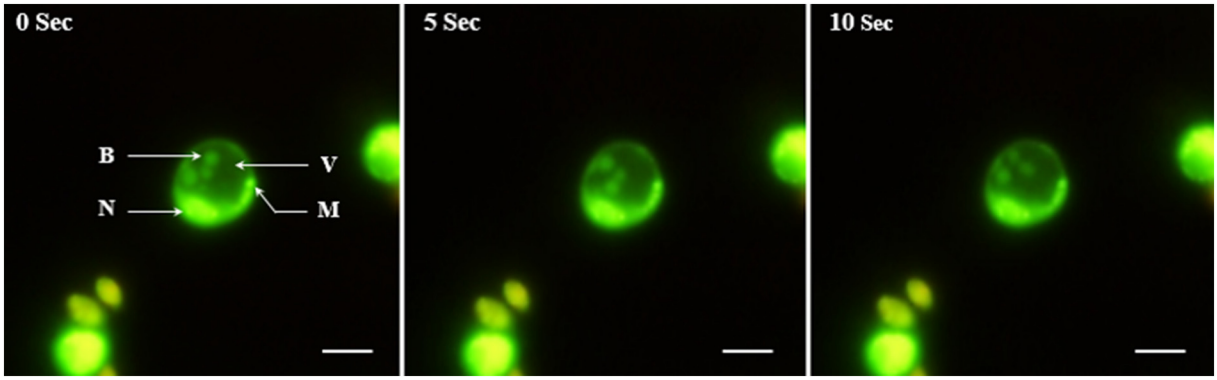

Fig. 1. Fluorescent microscopy of *C. albicans* stained with live/dead viability kit. Live and green bacteria (B) are demonstrated in the vacuole (V) of a yeast cell. Arrow points to one of the three bacteria. Photographs taken at 3 time intervals, 0, 5 and 10 s show the movement of bacteria. Nucleus (N), mitochondria (M). Scale bars: 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTT ACCTTGTTACGACTT-3′) (Lane, 1991). The PCR conditions were one cycle at 95 °C for 5 min, 35 cycles at 94 °C for 30 s, 50 °C for 45 s and 72 °C for 2 min and a final extension step at 72 °C for 10 min. *16S rDNA* amplicon with the size of 1500 bp was purified, sequenced and matched with published sequences in GenBank by the Basic Local Alignment Search Tool (BLAST). Bacterial identification to species level was defined as > 99% sequence similarity with the sequence of the type strain of only one species and having the higher score of similarity. Results of data analysis showed 99% homology with *H. pylori*. Next step was detection of *H. pylori*-specific genes in the yeast total DNA; *16S rRNA* and *vacA* (*s* and *m* regions). Amplification of all the genes was initiated with 94 °C for 3 min and 33 cycles of 94 °C for 45 s and 72 °C for 1 min, followed by 72 °C for 5 min. The primer pair of *H. pylori-16S rDNA* was HP1: 5′-GCAATCAGCGTCAGTAATGTTC-3′ and HP2: 5′-GCTAAGAGA TCAGCCTATGTCC-3′ (Lu et al., 2002) with the annealing temperature of 55 °C. The primer pair of *H. pylori-vacA s1/s2* was VA1-F: 5′- ATGG AAATACAACAAACACAC -3′ and VA1-R: 5′- CTGCTTGAATGCGCCA AAC -3′ (Atherton et al., 1995) with the annealing temperature of 54 °C. The primer pair of *H. pylori-vacA m1/m2* was VAG–F: 5′-CAATCTGTC CAATCAAGCGAG-3′ and VAG-R: 3′-GCGTCAAAATAATTCCAAGG-3′ (Yamaoka et al., 1998) with annealing temperature of 52 °C. Two clinical *H. pylori* isolates which were previously identified by amplification and sequencing of *H. pylori*-specific *16S rDNA* were used as positive controls. These two controls were genotyped for *vacA* alleles as s1/m1 and s2/m2. PCR reaction mixture without template was used as a negative control.

2.5. Isolation of yeast's vacuoles

Yeast vacuoles were isolated using the protocol described by Cabrera et al. (Cabrera and Ungermann, 2008). Briefly, a single colony of yeast was inoculated into 100 mL of YPD broth (1% yeast extract, 1% peptone and 2% glucose) and incubated in shaker incubator (220 rpm) at 33 °C, until turbidity of yeast culture reached 1.1 at 600 nm. Yeast cells were separated from the liquid culture by centrifugation at 4000 rpm for 10 min. The pellet was resuspended in wash buffer (10 mL of 1 M Tris-HCl pH 9.4, 1 mL of β-mercaptoethanol and 89 mL of distilled H_2O) and incubated in 40 °C water bath for 30 min, with occasional mixing. Yeast cells were harvested and resuspended in 2 mL of spheroplasting buffer (80 mL of 0.2% YPD, 15 mL of 4 M sorbitol and 5 mL of 1 M potassium phosphate, pH 7.5) containing 0.5 mg/mL lyticase (Sigma L4025) and incubated at 30 °C for 30 min. The spheroplasts were *pelleted* and resuspended in 2 mL of 15% ficoll in PS buffer (10 mM PIPES/KOH, pH 6.8, 200 mM sorbitol). Lysis of spheroplasts was induced by the addition of DEAE (diethylaminoethyl)-dextran (10 mg/mL of 15% ficoll). The vacuolar fraction was then recovered by ficoll density gradient at 30,000 rpm for 90 min at 4 °C in a Beckman SW40 Ti rotor.

2.6. Staining of yeast vacuoles and detection of intravacuolar H. pylori

Intact yeast cells and isolated vacuoles were stained with vacuole marker fluorescent probes (Yeast Vacuole Marker Sampler Kit, Molecular Probes Y-7531). MDY-64 and CellTracker Blue CMAC were used for vacuole membrane and lumen staining, respectively. Dual staining of intact yeast cell vacuoles with MDY-64 and blue CMAC was carried out. Briefly, yeasts were first washed 3 times with PBS, then incubated in 10 mM HEPES buffer containing both 100 μM CMAC and 10 μM MDY-64 for 15 min. After being washed with fresh buffer, stained preparation was examined with the fluorescent microscope. The membrane of purified vacuoles was stained with 10 mM HEPES buffer pH 7.4, containing 5% glucose and MDY-64 with final concentration of 10 μM, for 5 min. Furthermore, DNA extracted from isolated vacuoles, was used for detection of *H. pylori 16S rDNA*.

3. Results

3.1. Observation of green and live bacteria inside the yeasts' vacuole with fluorescent microscopy

Examination of wet mount of yeast cells with the light microscope showed the presence of actively moving BLBs (data not shown). Fluorescent microscopic observation of the wet mount of yeast cells stained with LIVE/DEAD *Bac*Light bacterial-viability kit, showed green and live bacteria inside the yeasts' vacuole. Photographs taken at 0, 5 and 10 s time intervals showed the movement of bacteria inside the yeast vacuole, confirming the viability of intracellular bacteria (Fig.1). A video record also showed live and actively moving bacteria inside the dark vacuoles of yeasts (Supplementary Video 1).

3.2. Detection of intracellular bacteria using FISH

To further validate bacterial nature of BLBs, we performed FISH experiment using universal bacterial probe that target bacterial-specific *16S rRNA*. Observation of yellow fluorescent spots inside the vacuoles of yeast cells showed hybridization of labeled oligonucleotide probe with bacterial *16S rRNA* (Fig.2). These results confirmed the occurrence of intracellular bacteria inside the yeast vacuole. Presence of fluorescent spots in mother and daughter yeast cells indicated bacterial transmission to next generation. Absence of non-specific fluorescent signals, showed lack of non-specific interaction of antisense probe (Fig.2).

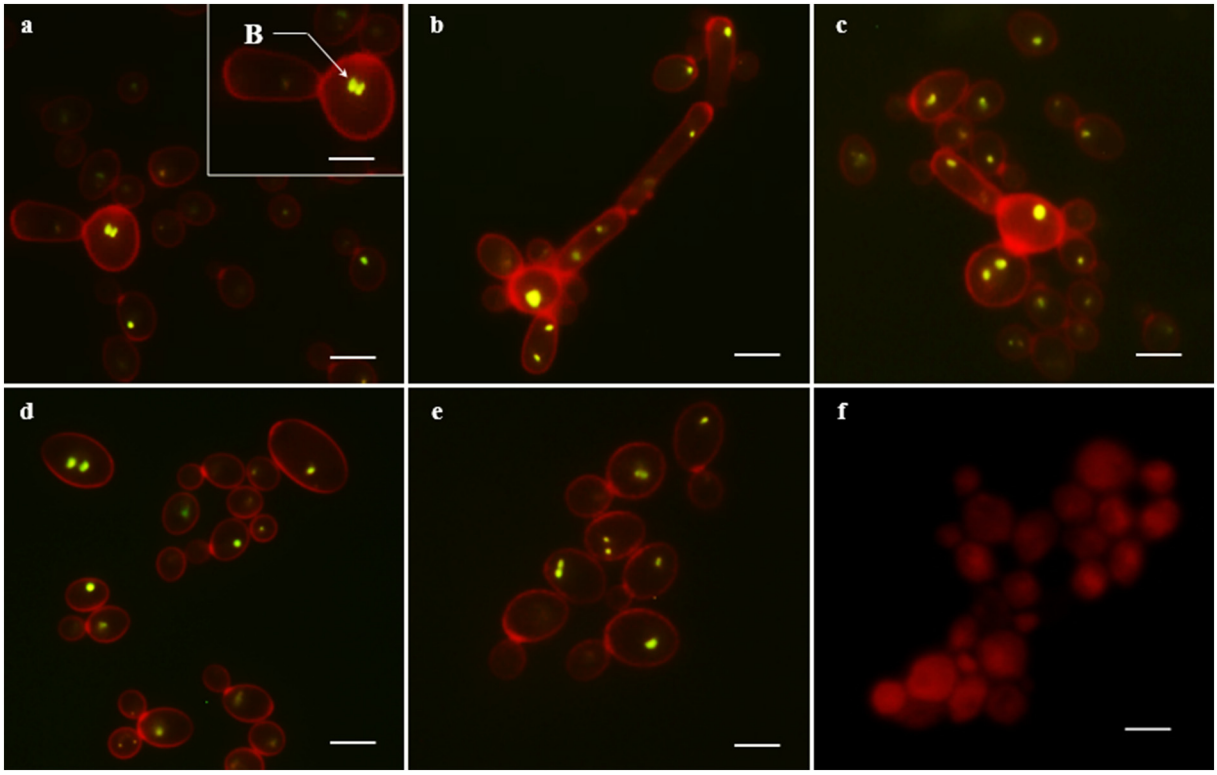

Fig. 2. Fluorescence *in situ* hybridization of yeast with bacterial *16S rRNA*-targeted oligonucleotide probe. All 5 photographs (a, b, c, d and e) show intracellular bacteria (B) as fluorescent spots (arrow). Presence of fluorescent spots in mother and daughter yeast cells indicates bacterial transmission to next generation. Absence of non-specific fluorescent signal using non-EUB338 probe, showed lack of non-specific interaction (f). The inset in the panel a, shows magnification of bacterial cells inside the yeast cell. Scale bars in photographs represent: 5 µm, and in inset: 4 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Amplification of full-length bacterial 16S rDNA and H. pylori-specific genes (16S rDNA and vacA alleles) from the yeast total DNA using PCR

Sequencing of amplified product of full-length bacterial *16S rDNA* with the size of 1500 bp, showed 99% homology with *H. pylori*. Amplified products of *H. pylori*-specific *16S rDNA* with the size of the 521-bp, *vacA* s1 with the size of the 259-bp and *vacA* m1 with the size of 570 bp showed similarity to the size of amplified products from control *H. pylori* (Fig.3). Comparison of *vacA* amplified products from yeast with those of two control *H. pylori* showed similarity between *vacA* alleles of intracellular *H. pylori* detected in yeast and *vacA* s1/m1 of control *H. pylori*.

3.4. Staining of yeast vacuoles and detection of intravacuolar H. pylori

Yeast vacuolar membrane was labeled with MDY-64, appearing green. Intracellular bacteria were also stained green due to interaction of MDY-64 with their membrane (Fig.4). Yeast vacuole lumen was also labeled with CellTracker Blue CMAC appearing blue. MDY-64 and CMAC dual staining confirmed the bacterial localization in the lumen of large vacuoles. Staining of isolated vacuoles membrane with MDY-64 showed green vacuoles with stained intracellular bacteria. PCR product

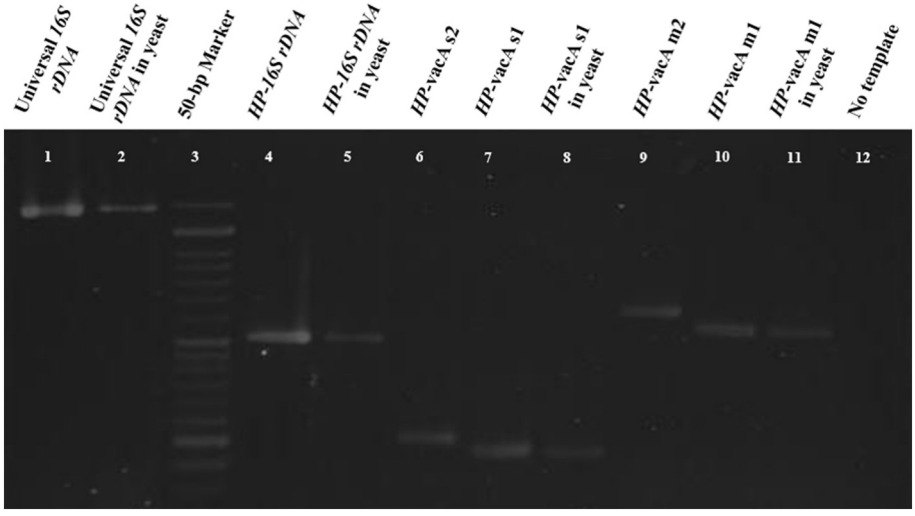

Fig. 3. Amplification of full-length bacterial *16S rDNA* and *H. pylori*-specific genes (*16S rDNA* and *vacA* alleles) from the yeast total DNA. Size of products (bp) from left to right: lane 1 and 2 (1500), 3 (Marker), 4 and 5 (521), 6 (286), 7 and 8 (259), 9 (645), 10 and 11 (570) and 12 (no template). The *vacA* s1/m1 amplified products of intracellular *H. pylori* detected in yeast (lanes 8 and 11) showed silmilarity to those of control *H. pylori* (lanes 7 and 10).

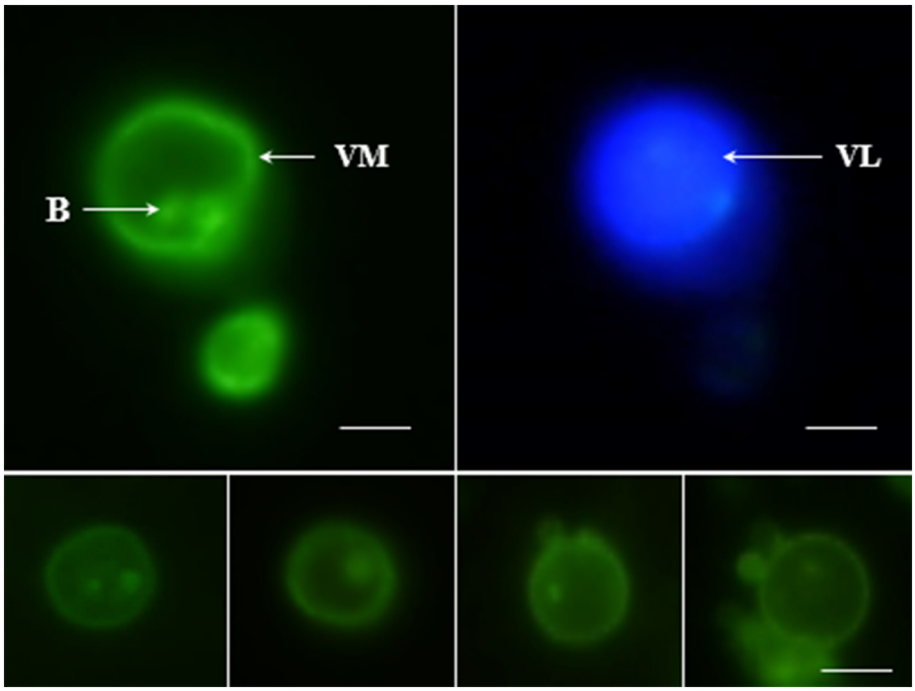

Fig. 4. Fluorescence microscopy of yeast cells and isolated vacuoles stained with fluorescent lumen and membrane markers. Top left: Intact yeast vacuole membrane (VM) stained with MDY-64, showing intracellular bacteria (B). Top right: Intact yeast vacuole lumen (VL) stained with blue CMAC. Scale bars: 2 µm. Four bottom: Isolated vacuoles with membranes and intracellular bacteria appearing green after staining with MDY-64. Scale bar: 2.5 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of *H. pylori*-specific *16S rDNA* with the size of 521 bp was amplified from DNA of isolated vacuoles (data not shown). Sequencing of amplified product showed 99% similarity with *H. pylori*. These results indicate the subcellular localization of *H. pylori* in the vacuoles.

4. Discussion

In this study, microscopic observation of BLBs that were stained with bacterial viability kit showed the occurrence of viable bacteria inside the vacuole of *C. albicans*. The positive interaction of fluorescent universal bacterial probe, with the intracellular bacterial RNA resulted in the appearance of fluorescent spots observable only in the vacuole of yeast cells. These fluorescent spots were observed in the mother as well as daughter yeast cells, suggesting the bacterial transmission to the next generations of *C. albicans*. According to reports, only bacteria containing enough ribosomes produce detectable fluorescence in FISH method. In the other words, dead bacteria lacking RNA and starved or dormant bacteria, usually with very low rRNA levels cannot be detected by FISH (Amann et al., 1995; Christensen et al., 1999). FISH has been used as a successful method for detection of *H. pylori* inside the epithelial cells in gastric biopsies (Rüssmann et al., 2001) and in *H. pylori*-infected cultured cells (Semino-Mora et al., 2003). FISH has also been used for detection and localization of intracellular bacteria, *Paenibacillus* spp., inside the mycelium of a mycorrhizal fungus, *Laccaria bicolor*, showing that intracellular bacteria were clearly localized inside the fungal hyphae. Furthermore, detection of these intracellular bacteria in next generations of the fungus showed that they were physiologically active and able to multiply (Bertaux et al., 2003). FISH was used for demonstrating the coexistence of multiple bacterial endosymbionts in arbuscular mycorrhizal fungus (Desiro et al., 2014). Amplification and sequencing of universal *16S rDNA* and *H. pylori*-specific *16S rDNA* and *vacA* from the yeast total DNA demonstrated the occurrence of *H. pylori* inside the yeast vacuole. In the present study, fluorescent microscopic observations showed that isolated yeast vacuoles that were stained with vacuole-specific membrane stain contained bacteria. Furthermore, amplification of *H. pylori*-specific *16S rDNA* from the DNA extracted from isolated vacuoles, confirmed that yeast vacuole serves as a specific niche for the intracellular bacteria.

In our previous study, light microscopy observation of BLBs inside the yeast vacuole and amplification of genes related to *H. pylori 16S rDNA*, *cagA* (cytotoxin-associated gene A), urease (ureAB) and vacuolating cytotoxin (vacA) and peroxyredoxin (aphC) from total DNA extracted from yeasts, showed the occurrence of intracellular *H. pylori* in oral and gastric yeasts (Salmanian et al., 2008; Saniee et al., 2013b; Siavoshi et al., 2005). Furthermore, using *H. pylori*-specific immunoglobulins raised in hens (IgY-Hp) and in mice (IgG1-Hp) and western blotting, *H. pylori* proteins; VacA large and small subunits, urease-A subunit, thiol peroxidase and peroxyredoxin were detected in the protein pool of oral and gastric *Candida* yeasts (Saniee et al., 2013b). These results indicated that inside the yeast vacuole, *H. pylori* is viable and expresses proteins for its survival, peroxiredoxin and thiol peroxidase to detoxify oxygen metabolites formed during respiratory burst in eukaryotic cells (Mauel, 1983) and urease and vacA to disrupt phagolysosome fusion in macrophages (Schwartz and Allen, 2006). Accordingly, it was suggested that *H. pylori* is well-equipped with powerful tools to establish and survive in the vacuole of eukaryotic cells. These results were confirmed by microscopic observation of *H. pylori* cells and detection of bacterial genes in the consecutive generations of yeasts (Saniee et al., 2013a, 2013b). Reports indicate that many intracellular bacteria have evolved to recruit certain proteins for protecting their membrane-bound vacuole and promoting intracellular partnership (Mauel, 1983; McFall-Ngai, 2008; Ruiz-Lozano and Bonfante, 1999).

Interestingly, reports suggest that the most prominent function of *H. pylori* VacA is induction of vacuolation in mammalian cells (Cover and Blanke, 2005; Figura, 1996). Upon insertion into mammalian cell membrane, VacA produces ion channels, internalizes and induces formation of large vacuoles that occupy a considerable portion of the cell (Torres et al., 2005). As reports indicated, *H. pylori*-containing autophagosome was non-acidic or non-degradative and did not show fusion with the lysosome (Raju et al., 2012). It has been proposed that VacA traffics to late endosomal-lysosomal compartments where it induces vacuolation through a mechanism dependent on the small GTPase Rab 7 (Johansson et al., 2005). VacA also disrupts the degradative capacity of lysosomes by affecting lysosomal hydrolases such as cathepsin (Cover and Blanke, 2005) It appears that *H. pylori* recruits VacA to induce autophagy (Cover and Blanke, 2005; Molinari et al., 1997; Satin et al., 1997; Terebiznik et al., 2009) and similar to a diverse range of

intracellular bacteria subverts autophagic pathway, inhibits their maturation and provides a shelter for bacterial survival and replication (Dorn et al., 2002; Kirkegaard et al., 2004; Niu et al., 2012; Steele et al., 2013). This could be the fundamental step in development of symbiotic relationship between intracellular bacteria and its eukaryotic host (Downs et al., 2009). Accordingly, VacA-induced large vacuoles have been regarded as an intracellular replicative niche for *H. pylori* (Cover and Blanke, 2005; Molinari et al., 1997; Satin et al., 1997; Terebiznik et al., 2009).

Investigators believe that the intracellular survival and replication of bacteria in eukaryotic host cells dates back to > 150 million of years ago, long before the emergence of mammalian hosts about 60 million years ago (Moran and Baumann, 2000; Sauer et al., 2000). One example of intracellular relationship of prokaryotes with eukaryotic hosts is between bacteria and fungi which are old and evolutionary partners since 3–5 billion years ago, before that time bacteria were the only inhabitants of earth. Eukaryotic cells such as fungi appeared later and acted as predators of bacteria as sources of energy and essential nutrients. However, among ingested bacteria, some persisted digestion in eukaryotic phagosome and turned into permanent endosymbiotic partners (Cavalier-Smith, 1975, 2002). An important consequence of this relationship was the evolution of modern cellular organelles, mitochondria and chloroplast in the majority of eukaryotic cells existing today (Dyall and Johnson, 2000).

It is concluded that vacuole a principal organelle for yeast's viability, with major roles in degradation of intracellular molecules, storage of nutrients and stress tolerance (Klionsky et al., 1990) could have been selected through evolution to serve as a sophisticated niche for intracellular bacteria. It appears that intracellular bacteria induced formation of their own vacuolar niche in which they were protected, and could reach nutrients and replicate. Moreover, they served their eukaryotic host by digesting food and producing essential nutrients such as amino acids and vitamins. It seems plausible to suggest that mutual relationship between intracellular bacteria and eukaryotic host provides conditions that facilitate replication of endobacteria in a balanced way that both partners survive the entire life span of the eukaryotic host (Goebel and Gross, 2001). This mutual beneficiary relationship does not end here, occurrence of endosymbiotic bacteria in new generations of eukaryotic cells indicates that the endosymbiotic bacteria are transmitted to next generation (Bianciotto et al., 2004). This indicates that eukaryotic host cells, such as yeast, could serve as important and endless reservoirs of intracellular bacteria (Corsaro et al., 1999). Accordingly, the endosymbiotic relationship of bacteria with eukaryotic host is a conserved evolutionary phenomenon with a great impact on eukaryotes' evolution (McFall-Ngai et al., 2013). Results of this study suggest that sequestration inside the yeast vacuole as a specialized intracellular niche may enhance *H. pylori* survival against stressful condition.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.01.029.

Declarations of interest

None.

Acknowledgment

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.
Amann, R., Springer, N., Ludwig, W., Görtz, H.-D., Schleifer, K.-H., 1991. Identification in situ and phylogeny of uncultured bacterial endosymbionts. Nature 351, 161.
Amann, R.I., Ludwig, W., Schleifer, K.-H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.
Armstrong, J., 2010. Yeast vacuoles: more than a model lysosome. Trends Cell Biol. 20, 580–585.
Atherton, J.C., Cao, P., Peek, R.M., Tummuru, M.K., Blaser, M.J., Cover, T.L., 1995. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori association of specific vacA types with cytotoxin production and peptic ulceration. J. Biol. Chem. 270, 17771–17777.
Bertaux, J., Schmid, M., Prevost-Boure, N.C., Churin, J., Hartmann, A., Garbaye, J., Frey-Klett, P., 2003. In situ identification of intracellular bacteria related to Paenibacillus spp. in the mycelium of the ectomycorrhizal fungus Laccaria bicolor S238N. Appl. Environ. Microbiol. 69, 4243–4248.
Bianciotto, V., Bandi, C., Minerdi, D., Sironi, M., Tichy, H.V., Bonfante, P., 1996. An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. Appl. Environ. Microbiol. 62, 3005–3010.
Bianciotto, V., Lumini, E., Lanfranco, L., Minerdi, D., Bonfante, P., Perotto, S., 2000. Detection and identification of bacterial endosymbionts in arbuscular mycorrhizal fungi belonging to the family Gigasporaceae. Appl. Environ. Microbiol. 66, 4503–4509.
Bianciotto, V., Genre, A., Jargeat, P., Lumini, E., Becard, G., Bonfante, P., 2004. Vertical transmission of endobacteria in the arbuscular mycorrhizal fungus Gigaspora margarita through generation of vegetative spores. Appl. Environ. Microbiol. 70, 3600–3608.
Bode, G., Malfertheiner, P., Ditschuneit, H., 1988. Pathogenetic implications of ultrastructural findings in Campylobacter pylori related gastroduodenal disease. Scand. J. Gastroenterol. 23, 25–39.
Cabrera, M., Ungermann, C., 2008. Purification and in vitro analysis of yeast vacuoles. Methods Enzymol. 451, 177–196.
Cavalier-Smith, T., 1975. The origin of nuclei and of eukaryotic cells. Nature 256, 463.
Cavalier-Smith, T., 2002. The phagotrophic origin of eukaryotes and phylogenetic classification of Protozoa. Int. J. Syst. Evol. Microbiol. 52, 297–354.
Chen, X.G., Correa, P., Offerhaus, J., Rodriguez, E., Janney, F., Hoffmann, E., Fox, J., Hunter, F., Diavolitsis, S., 1986. Ultrastructure of the gastric mucosa harboring Campylobacter-like organisms. Am. J. Clin. Pathol. 86, 575–582.
Christensen, H., Hansen, M., Sørensen, J., 1999. Counting and size classification of active soil bacteria by fluorescence in situ hybridization with an rRNA oligonucleotide probe. Appl. Environ. Microbiol. 65, 1753–1761.
Chu, Y.-T., Wang, Y.-H., Wu, J.-J., Lei, H.-Y., 2010. Invasion and multiplication of Helicobacter pylori in gastric epithelial cells and implications for antibiotic resistance. Infect. Immun. 78, 4157–4165.
Corsaro, D., Venditti, D., Padula, M., Valassina, M., 1999. Intracellular life. Crit. Rev. Microbiol. 25, 39–79.
Cover, T.L., Blanke, S.R., 2005. Helicobacter pylori VacA, a paradigm for toxin multifunctionality. Nat. Rev. Microbiol. 3, 320.
Desiro, A., Salvioli, A., Ngonkeu, E.L., Mondo, S.J., Epis, S., Faccio, A., Kaech, A., Pawlowska, T.E., Bonfante, P., 2014. Detection of a novel intracellular microbiome hosted in arbuscular mycorrhizal fungi. ISME J. 8, 257.
Dorn, B.R., Dunn, W.A., Progulske-Fox, A., 2002. Bacterial interactions with the autophagic pathway. Cell. Microbiol. 4, 1–10.
Downs, C.A., Kramarsky-Winter, E., Martinez, J., Kushmaro, A., Woodley, C.M., Loya, Y., Ostrander, G.K., 2009. Symbiophagy as a cellular mechanism for coral bleaching. Autophagy 5, 211–216.
Dubois, A., Borén, T., 2007. Helicobacter pylori is invasive and it may be a facultative intracellular organism. Cell. Microbiol. 9, 1108–1116.
Dyall, S.D., Johnson, P.J., 2000. Origins of hydrogenosomes and mitochondria: evolution and organelle biogenesis. Curr. Opin. Microbiol. 3, 404–411.
Figura, N., 1996. Helicobacter pylori exotoxins and gastroduodenal diseases associated with cytotoxic strain infection. Aliment. Pharmacol. Ther. 10, 79–96.
Fritsche, T.R., Horn, M., Seyedirashti, S., Gautom, R.K., Schleifer, K.-H., Wagner, M., 1999. In situ detection of novel bacterial endosymbionts of Acanthamoeba spp. phylogenetically related to members of the order Rickettsiales. Appl. Environ. Microbiol. 65, 206–212.
García-del Portillo, F., 1999. Pathogenic interference with host vacuolar trafficking. Trends Microbiol. 7, 467–469.
Goebel, W., Gross, R., 2001. Intracellular survival strategies of mutualistic and parasitic prokaryotes. Trends Microbiol. 9, 267–273.
Görtz, H.-D., Brigge, T., 1998. Intracellular bacteria in protozoa. Sci. Nat. 85, 359–368.
Johansson, M., Lehto, M., Tanhuanpää, K., Cover, T.L., Olkkonen, V.M., 2005. The oxysterol-binding protein homologue ORP1L interacts with Rab7 and alters functional properties of late endocytic compartments. Mol. Biol. Cell 16, 5480–5492.
Kirkegaard, K., Taylor, M.P., Jackson, W.T., 2004. Cellular autophagy: surrender, avoidance and subversion by microorganisms. Nat. Rev. Microbiol. 2, 301.
Klionsky, D., Herman, P.K., Emr, S., 1990. The fungal vacuole: composition, function, and biogenesis. Microbiol. Rev. 54, 266–292.
Kumar, Y., Valdivia, R.H., 2009. Leading a sheltered life: intracellular pathogens and maintenance of vacuolar compartments. Cell Host Microbe 5, 593–601.
Lane, D., 1991. 16S/23S rRNA Sequencing. Nucleic Acid Techniques in Bacterial Systematics. pp. 115–175.
Levine, B., 2005. Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. Cell 120, 159–162.
Lu, Y., Redlinger, T.E., Avitia, R., Galindo, A., Goodman, K., 2002. Isolation and genotyping of Helicobacter pylori from untreated municipal wastewater. Appl. Environ. Microbiol. 68, 1436–1439.
Mauel, J., 1983. Mechanisms of intracellular microbicide. Bull. Eur. Physiopathol. Respir.

19, 115–122.
McFall-Ngai, M., 2008. Are biologists in'future shock'? Symbiosis integrates biology across domains. Nat. Rev. Microbiol. 6, 789.
McFall-Ngai, M., Hadfield, M.G., Bosch, T.C., Carey, H.V., Domazet-Lošo, T., Douglas, A.E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S.F., 2013. Animals in a bacterial world, a new imperative for the life sciences. Proc. Natl. Acad. Sci. 110, 3229–3236.
Molinari, M., Galli, C., Norais, N., Telford, J.L., Rappuoli, R., Luzio, J.P., Montecucco, C., 1997. Vacuoles induced by Helicobacter pylori toxin contain both late endosomal and lysosomal markers. J. Biol. Chem. 272, 25339–25344.
Moran, N.A., Baumann, P., 2000. Bacterial endosymbionts in animals. Curr. Opin. Microbiol. 3, 270–275.
Nakagawa, I., Amano, A., Mizushima, N., Yamamoto, A., Yamaguchi, H., Kamimoto, T., Nara, A., Funao, J., Nakata, M., Tsuda, K., 2004. Autophagy defends cells against invading group A Streptococcus. Science 306, 1037–1040.
Necchi, V., Candusso, M.E., Tava, F., Luinetti, O., Ventura, U., Fiocca, R., Ricci, V., Solcia, E., 2007. Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by Helicobacter pylori. Gastroenterology 132, 1009–1023.
Niu, H., Xiong, Q., Yamamoto, A., Hayashi-Nishino, M., Rikihisa, Y., 2012. Autophagosomes induced by a bacterial Beclin 1 binding protein facilitate obligatory intracellular infection. Proc. Natl. Acad. Sci. 109, 20800–20807.
Pernthaler, J., Glöckner, F.-O., Schönhuber, W., Amann, R., 2001. Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes. Methods Microbiol. 30, 207–226.
Raju, D., Hussey, S., Ang, M., Terebiznik, M.R., Sibony, M., Galindo–Mata, E., Gupta, V., Blanke, S.R., Delgado, A., Romero–Gallo, J., 2012. Vacuolating cytotoxin and variants in Atg16L1 that disrupt autophagy promote Helicobacter pylori infection in humans. Gastroenterology 142, 1160–1171.
Ruiz-Lozano, J., Bonfante, P., 1999. Identification of a putative P-transporter operon in the genome of a Burkholderia strain living inside the arbuscular mycorrhizal fungus Gigaspora margarita. J. Bacteriol. 181, 4106–4109.
Rüssmann, H., Kempf, V.A., Koletzko, S., Heesemann, J., Autenrieth, I.B., 2001. Comparison of fluorescent in situ hybridization and conventional culturing for detection of Helicobacter pylori in gastric biopsy specimens. J. Clin. Microbiol. 39, 304–308.
Salmanian, A.H., Siavoshi, F., Akbari, F., Afshari, A., Malekzadeh, R., 2008. Yeast of the oral cavity is the reservoir of Heliobacter pylori. J. Oral Pathol. Med. 37, 324–328.
Salmanian, A.H., Siavoshi, F., Beyrami, Z., LATIFI-NAVID, S., Tavakolian, A., Sadjadi, A., 2012. Foodborne yeasts serve as reservoirs of Helicobacter pylori. J. Food Saf. 32, 152–160.
Sambrook, J., Russell, D.W., 2001. Molecular Clonning. A Laboratory Mannual. Cold Spring Harbor Laboratory Press. Cold Spring Halbor, New York.
Saniee, P., Siavoshi, F., Nikbakht, B.G., Khormali, M., Sarrafnejad, A., Malekzadeh, R., 2013a. Immunodetection of Helicobacter pylori-specific proteins in oral and gastric Candida yeasts. Arch. Iran. Med. 16, 624–630.
Saniee, P., Siavoshi, F., Nikbakht, B.G., Khormali, M., Sarrafnejad, A., Malekzadeh, R., 2013b. Localization of H. pylori within the vacuole of Candida yeast by direct immunofluorescence technique. Arch. Iran. Med. 16, 705–710.
Satin, B., Norais, N., Rappuoli, R., Telford, J., Murgia, M., Montecucco, C., Papini, E., 1997. Effect of Helicobacter pylori vacuolating toxin on maturation and extracellular release of procathepsin D and on epidermal growth factor degradation. J. Biol. Chem. 272, 25022–25028.
Sato, T., Ohsumi, Y., Anraku, Y., 1984. Substrate specificities of active transport systems for amino acids in vacuolar-membrane vesicles of Saccharomyces cerevisiae. evidence of seven independent proton/amino acid antiport systems. J. Biol. Chem. 259, 11505–11508.
Sauer, C., Stackebrandt, E., Gadau, J., Hölldobler, B., Gross, R., 2000. Systematic relationships and cospeciation of bacterial endosymbionts and their carpenter ant host species: proposal of the new taxon Candidatus Blochmannia gen. nov. Int. J. Syst. Evol. Microbiol. 50, 1877–1886.
Schwartz, J.T., Allen, L.A.H., 2006. Role of urease in megasome formation and Helicobacter pylori survival in macrophages. J. Leukoc. Biol. 79, 1214–1225.
Semino-Mora, C., Doi, S.Q., Marty, A., Simko, V., Carlstedt, I., Dubois, A., 2003. Intracellular and interstitial expression of Helicobacter pylori virulence genes in gastric precancerous intestinal metaplasia and adenocarcinoma. J. Infect. Dis. 187, 1165–1177.
Shintani, T., Klionsky, D.J., 2004. Autophagy in health and disease: a double-edged sword. Science 306, 990–995.
Shousha, S., Bull, T., Parkins, R., 1984. Gastric spiral bacteria. Lancet 2, 101.
Siavoshi, F., NouraliAhari, F., Zeinali, S., HashemiDogahed, M., Malekzadeh, R., Massarrat, S., 1998. Yeast protects Helicobacter Pylori against the environmental stress. Arch. Iran. Med. 1.
Siavoshi, F., Salmanian, A.H., Kbari, F.A., Malekzadeh, R., Massarrat, S., 2005. Detection of helicobacter pylori-specific genes in the oral yeast. Helicobacter 10, 318–322.
Steele, S., Brunton, J., Ziehr, B., Taft-Benz, S., Moorman, N., Kawula, T., 2013. Francisella tularensis harvests nutrients derived via ATG5-independent autophagy to support intracellular growth. PLoS Pathog. 9, e1003562.
Terebiznik, M.R., Raju, D., Vázquez, C.L., Torbricki, K., Kulkarni, R., Blanke, S.R., Yoshimori, T., Colombo, M.I., Jones, N.L., 2009. Effect of Helicobacter pylori's vacuolating cytotoxin on the autophagy pathway in gastric epithelial cells. Autophagy 5, 370–379.
Torres, V.J., Ivie, S.E., McClain, M.S., Cover, T.L., 2005. Functional properties of the p33 and p55 domains of the Helicobacter pylori vacuolating cytotoxin. J. Biol. Chem. 280, 21107–21114.
Weisman, L.S., 2003. Yeast vacuole inheritance and dynamics. Annu. Rev. Genet. 37, 435–460.
Yamaoka, Y., Kodama, T., Kita, M., Imanishi, J., Kashima, K., Graham, D.Y., 1998. Relationship of vacA genotypes of Helicobacter pylori to cagA status, cytotoxin production, and clinical outcome. Helicobacter 3, 241–253.
Yilmaz, S., Haroon, M.F., Rabkin, B.A., Tyson, G.W., Hugenholtz, P., 2010. Fixation-free fluorescence in situ hybridization for targeted enrichment of microbial populations. ISME J. 4, 1352.