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ORIGINAL ARTICLE

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Helicobacter pylori release from yeast as a vesicle-encased or free bacterium

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Abstract

Background: Yeast has been suggested as a potent reservoir of *H pylori* that facilitates bacterial spread within human populations. What mechanism ensures effective *H pylori* release from yeast? Here, *H pylori* release from yeast as a vesicle-encased or free bacterium was studied.

Materials and methods: Liquid culture of *Candida* yeast was examined by light, fluorescence and transmission electron microscopy methods to observe the released vesicles. Vesicles were isolated and examined by TEM. Immunogold labeling was used for detection of *H pylori*-specific proteins in vesicles' membrane. Free bacterial cells, released from yeast, were separated by immunomagnetic separation and observed by field emission scanning electron microscopy (FESEM). DNA of bead-bound bacteria was used for amplification of *H pylori*-16S rDNA. Viability of bead-bound bacteria was examined by live/dead stain and cultivation on Brucella blood agar.

Results: Microscopic observations showed that vesicles contained bacterium-like structures. Thin sections showed release of vesicle-encased or free bacterium from yeast. Immunogold labeling revealed occurrence of *H pylori* proteins in vesicles' membrane. FESEM showed attachment of *H pylori* cells to magnetic beads. Sequencing of 521 bp PCR product confirmed the identity of bead-bound *H pylori*. Live/dead staining showed viability of bead-bound *H pylori* but the result of culture was negative.

Conclusions: Escape of intracellular *H pylori* from yeast as a membrane-bound or free bacterium indicates that *H pylori* uses safe exit mechanisms that do not damage the host which is the principle of symbiotic associations. In human stomach, certain conditions may stimulate yeast cells to release *H pylori* as a vesicle-encased or free bacterium.

KEYWORDS
free *H pylori*, release, vesicle-encased *H pylori*, yeast

1 | INTRODUCTION

Extracellular vesicles are membrane-bound structures released to the environment by all living cells.[1] Release of vesicles has been a conserved phenomenon in the evolution of all types of cells,[2] playing crucial roles in determining their physiological and pathological activities.[3] These vesicles by carrying hundreds of different cargos mediate intercellular communication.[4] Extracellular vesicles may contain proteins [4] and nucleic acids such as miRNA, mRNAs,[5] and DNA.[6] It has been proposed that vesicles can be involved in transfer of genetic information between cells.[7] On the other hand, observations of bacterial cells inside the vesicles released

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from protozoa demonstrated that vesicles may carry intracellular bacteria.[8]

According to published reports, escape of intracellular bacteria from host cell may occur through lytic and nonlytic strategies.[9] Lytic escape involves bacterial release from host cell through destroying cell and vacuolar membrane by lipases, proteases, and pore-forming proteins.[10] However, in nonlytic strategy intracellular bacteria escape from the host cell without destroying it, either as free bacteria or encased by host membranes.[10,11]

Results of our previous studies indicated that *Candida* yeast can serve as a host for *Helicobacter pylori*.[12,13] These studies started when yeast colonies were frequently observed along with *H pylori* colonies on cultures of gastric biopsies. When light and fluorescence microscopy observations revealed occurrence of live bacteria inside the vacuole of yeast's cell, our studies were designed to demonstrate the intracellular occurrence of *H pylori* in yeast. Although attempts to culture *H pylori* from mechanically disrupted yeasts were not successful, detection of *H pylori*-specific genes and proteins in several generations of oral and gastric *Candida* yeasts showed viability of intracellular *H pylori*.[14] Furthermore, *H pylori* was localized inside the vacuole of yeast's cell by direct immunofluorescence.[15] With these results, yeast was suggested as a potent reservoir of *H pylori* that facilitates bacterial spread in the environment and within human hosts.[16] All of these results suggested that there should be an effective mechanism for release of intracellular *H pylori* from yeast. In this study, release of *H pylori* from yeast as a vesicle-encased or free bacterium was assessed.

A *Candida albicans* isolate from gastric biopsy of a *H pylori*-positive patient was used. Intracellular occurrence of *H pylori* inside the yeast was previously demonstrated by molecular and microscopy methods.[17] Liquid culture of yeast was used to observe the released vesicles by light microscopy. Vesicles were stained with lipophilic dye, and their ultrastructure was examined by transmission electron microscopy (TEM). Furthermore, vesicles were isolated and examined for the presence of *H pylori*-specific proteins in their membrane, using polyclonal anti-*H pylori* antibody conjugated with gold particles and TEM. Finally, magnetic beads conjugated with anti-*H pylori* antibody were used to separate the free *H pylori* released from yeast in liquid culture. Field emission scanning electron microscopy (FESEM) was used to visualize the bead-bound bacterial cells. The identity of bead-bound *H pylori* was confirmed by amplification of *H pylori*-specific 16S rDNA and sequencing. Viability of bead-bound *H pylori* was examined by live/dead staining and culture.

2 | MATERIALS AND METHODS

2.1 | Yeast strain and culture condition

In this study, a *C albicans* isolate from gastric biopsy of a *H pylori*-positive patient was used. Growth medium for yeast cultivation was yeast extract peptone dextrose (YPD: 1% yeast extract, 2% peptone and 2% dextrose) broth, supplemented with 10% horse serum. Cultures were incubated in shaker incubator (150 rpm) at 37°C for 48 hours.

2.2 | Observing release of vesicles from yeast's cells by light, fluorescence, and transmission electron microscopy

Fresh culture of yeast was used for preparation of a wet mount which was examined by light microscopy for the release of vesicles from yeast's cells. For showing that released vesicles are membrane-enclosed, MDY-64 (Molecular Probes), a green lipophilic dye that stains membranes, was added to fresh culture of yeast with final concentration of 5 μM. A wet mount of stained preparation was covered by mounting oil (Invitrogen) and examined with fluorescence microscope (BX51; Olympus, Tokyo, Japan). To visualize the released vesicles with more structural detail, thin sections were prepared from yeast culture. A 200-μL volume of fixative solution containing 3% glutaraldehyde in sodium cacodylate buffer was added to 200 μL of yeast culture. After 30 minutes, yeast cells were washed with 0.1 M cacodylate buffer and resuspended in 2% glutaraldehyde for 2 hours. Fixed sample was post-fixed in 2% osmium for 90 minutes, sequentially dehydrated in ethanol, and finally embedded in resin. Thin sections were obtained and stained with uranyl acetate and lead citrate. Electron micrographs were taken by TEM (ZEISS, LEO 906E), operated at 100 kV.

2.3 | Isolation of extracellular vesicles

A 200-mL volume of yeast's fresh culture in YPD broth was used for isolation of extracellular vesicles according to the protocol described by Rodrigues et al,[18] with minor modifications. Briefly, yeast cells were separated from liquid medium by centrifugation at 4,000 × *g* for 10 minutes at 4°C. The supernatant was collected and centrifuged at 10,000 × *g* for 10 minutes at 4°C. Vesicles were separated by ultracentrifugation at 100,000 × *g* for 70 minutes at 4°C. The pellet was washed with phosphate-buffered saline (PBS; 0.15 M) and harvested at 100,000 × *g* for 1 hour at 4°C. Isolated vesicles were used for thin sectioning and immunogold labeling.

2.4 | Observation of isolated vesicles by TEM

For thin sectioning, vesicle pellet was resuspended in fixative solution containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 hours and incubated overnight in 4% formaldehyde and 1% glutaraldehyde. Fixed vesicles were dehydrated, embedded, and thin-sectioned for TEM as described above.

2.5 | Localization of H. pylori-specific proteins in vesicle membrane by immunogold labeling

Gold nanoparticles with the size of 15 nm were synthesized according to Frens method [19] with citrate reduction reaction of HAuCl4.[20] The synthesized gold nanoparticles were directly conjugated with anti-*H pylori* antibody in the presence of carbodiimide/N-hydroxysuccinimide (EDC/NHS) cross-linking agents as described before.[21] For immunogold labeling, a drop of isolated vesicles was mounted on a carbon-coated formvar grid for 1 minute and washed 3 × 5 minutes in PBS. Subsequently, the grid was blocked in one drop of 5% bovine serum albumin in PBS for 10 minutes. After blockage, the grid was incubated in one drop of gold-conjugated antibody for 30 minutes at 37°C. Grid was then washed 3 × 5 minutes in PBS, negative stained with one drop of 2% uranyl acetate, and blotted dry for a few seconds. Electron micrographs were taken by TEM, operated at 100 kV.

2.6 | Detection of released H. pylori from yeast by immunomagnetic separation (IMS)

2.6.1 | Coating of magnetic beads with anti-*H pylori* antibody

Anti-*H pylori* magnetic beads were made by direct coating of Tosyl activated magnetic beads (Dynabeads M-280, 2.8 μm in diameter, Dynal, Invitrogen, 14 203) with polyclonal rabbit anti-*H pylori* antibody according to manufacturer's instructions. Clinical isolates of *H pylori* and *Escherichia coli* were used for evaluating the capture efficiency and specificity of magnetic beads. Two 200-μL volumes of each bacterial suspension, with the turbidity of 0.5 McFarland standard, were added to 50 μL of anti-*H pylori* coated beads and 50 μL of control noncoated beads. Tubes were incubated at room temperature for 1 hour with gentle shaking. Beads were then separated with a magnet (Dynal DynaMag-2, Invitrogen, 123-21D) and washed twice with PBS. Collected beads were fixed in 2% glutaraldehyde, dehydrated, coated with gold, and examined with Hitachi S4160 FESEM (Tokyo, Japan), operated at 20 kV.

2.6.2 | IMS and characterization of released *H pylori* from yeast

A 48-h culture of *C albicans* in serum-enriched YPD broth was used for detection of released *H pylori* from yeast's cells. Five mg of coated beads was added to 5 mL of yeast culture and incubated for 3 hours with gentle shaking. Beads were collected with magnet and resuspended in 500 μL of PBS. A 100-μL volume of bead-bound bacteria was prepared for FESEM as described above. The remaining 400 μL of bead-bound bacteria was used for molecular identification as well as examination of viability and culturability of isolated bacteria. DNA was extracted with phenol-chloroform method and used for amplification of *H pylori*-specific 16S rDNA with HP1: 5′-GCAATCAGCGTCAGTAATGTTC-3′ and HP2: 5′-GCTAAGAGATCAGCCTATGTCC-3′ primers.[22] PCR was performed as previously described.[17] PCR product with the size of 521 bp was sequenced and matched with sequences in GenBank by the Basic Local Alignment Search Tool (BLAST). Bead-bound bacteria were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes) and examined with fluorescence microscope. A 100-μL volume of bead-bound bacteria was inoculated into 100 μL of brain heart infusion broth enriched with 10% horse serum and incubated under microaerophilic conditions at 37°C for 72 hours. The enriched culture was then surface inoculated onto Brucella blood agar, incubated under microaerophilic conditions at 37°C and inspected for *H pylori* growth up to three weeks.

3 | RESULTS

3.1 | Observation of vesicle release from yeast cells by light, fluorescence, and transmission electron microscopy

Examination of wet mounts by light microscopy showed several vesicle-like structures outside yeast's cells (Figure 1 Ia). Fluorescence microscopy showed that yeasts' cell membrane and released vesicles stained green with lipophilic dye, revealing that they were membrane-bound structures (Figure 1 Ib). Furthermore, some of the vesicles contained one or more bacterium-like structures (Figure 1 Ic-Ih) that showed nonstop moving (data not shown). Examination of thin sections showed the presence of vesicular structures inside the yeast's cell and close to the surface or outside and around the yeast's cell (Figure 1 IIa- IIc). The released vesicles were either empty or contained bacterium-like structures (Figure 1 IId). Interestingly, free bacterium-like structures were also observed outside the yeast's cell (Figure 1 IIe).

3.2 | TEM of isolated vesicles-Thin sectioning and immunogold labeling

Electron micrographs of purified vesicles showed a heterogeneous population of rounded, oval, or tubular vesicles (size range: 50-800 nm) surrounded by 1-3 layered membrane (Figure 2A), and some vesicles contained bacterium-like structures (Figure 2B,C). Examination of immunogold-labeled vesicles revealed occurrence of *H pylori* proteins in the membrane of vesicles released from yeast's cell (Figure 2D,E).

3.3 | Detection of released H. pylori from yeast by IMS

FESEM observations of bead-bound *H pylori* showed efficiency of coated beads to capture *H pylori* (Figure 3A). No interaction was

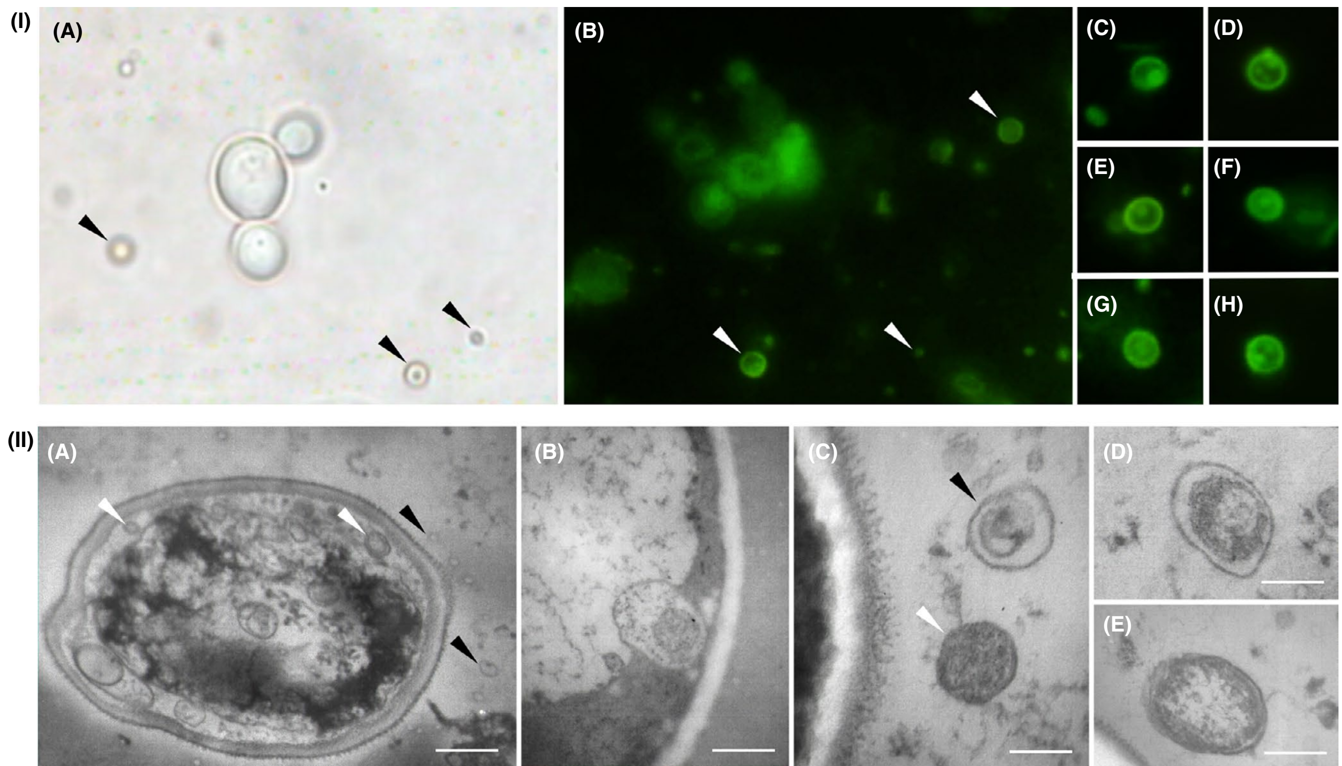

FIGURE 1 Light, fluorescence, and transmission electron microscopy of vesicle-encased and free *H pylori* released from yeast's cell. Top: (A) Light microscopy: Released vesicles (arrowhead) outside the yeast's cells. (B) Fluorescence microscopy: Yeast cell and vesicle membranes stained green with lipophilic dye. (C-H) Some of the released vesicles contained one or more bacterium-like structures. Bottom: (A) Thin section of a yeast cell showed the presence of vesicular structures inside (white arrowheads) or outside (black arrowheads) the yeast's cell. (B) Some vesicles were observed inside the yeast's cell and close to the surface. (C) Release of double-membrane vesicle (black arrowhead) and dense bacterium-like cell (white arrowhead) from yeast. (D) A released vesicle containing a bacterium-like structure. (E) A free bacterium-like cell. Bars, 500 nm (A) and 300 nm (B to E)

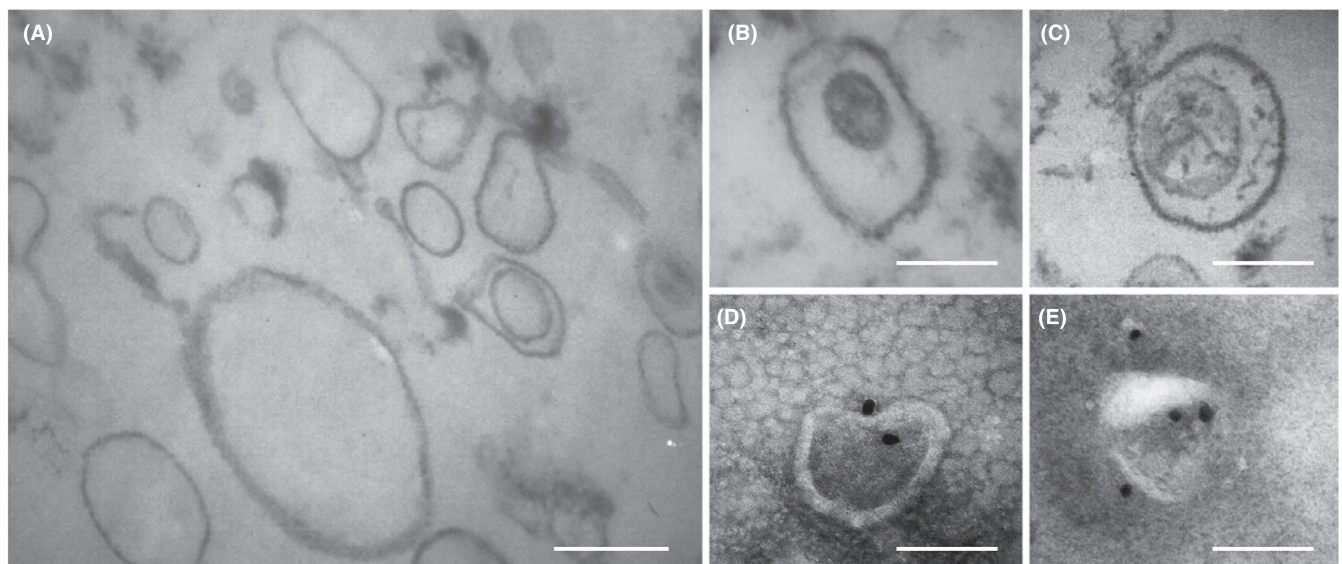

FIGURE 2 Transmission electron microscopy of isolated vesicles from yeast. (A) Thin section preparations of isolated vesicles showed a heterogeneous population with different size and shape. Bar, 400 nm. (B and C) Some vesicles contained dense bacterium-like structures. Bars, 300 nm. (D and E) Examination of negatively stained and immunogold-labeled vesicles revealed occurrence of *H pylori* proteins in the membrane of vesicles. Bars, 200 nm

observed between coated beads and *E coli* and between noncoated beads and *H pylori* (photographs not shown). FESEM observation of immunomagnetic beads recovered from liquid culture of yeast showed binding of beads with free bacterial cells (Figure 3B-D). BLAST analysis showed 99% sequence similarity between the amplified product of *H pylori*-specific 16S rDNA and that of several strains of *H pylori*, confirming the identity of bead-bound bacteria as *H pylori*. Bead-bound *H pylori* cells appeared green when stained with live/dead kit showing the viability of bacterial cells (photograph not shown). However, bead-bound *H pylori* showed no growth on Brucella blood agar.

4 | DISCUSSION

Considerable worldwide prevalence of *H pylori* infection, estimated as 44.3% [23] indicates its successful transmission within human populations. In the other words, there should be an effective way for *H pylori* spread. In our previous studies, occurrence of *H pylori* was reported in yeasts isolated from several gastric biopsies, oral cavity of adult and newborn, vaginal mucosa, and different foods,[16] also natural flowers [24] and even feces (unpublished data). These results suggested that yeasts from different origins may serve as reservoirs of *H pylori*, facilitating bacterial transmission within human hosts. It is noteworthy that dense populations of yeasts in soil are transferred to plants by ants,[25] also bees and birds.[26] These yeasts can enter food cycle of animals and humans through consumption of plant-derived foods. With high potential to resist against stresses, yeasts survive in human gastrointestinal tract and return to natural soils when excreted. Accordingly, populations of yeasts in soil, plants, animals, and humans are more or less similar.[27]

Results of the present study indicated that *Candida* yeast, as a potent environmental reservoir of *H pylori*, releases *H pylori* as a vesicle-encased or free bacterium. Examination of a gastric *C albicans* in liquid culture by light, fluorescence and transmission electron microscopy showed that yeast cells release vesicles with various morphology and size. Release of vesicles has been recognized as a new secretion system in fungi with crucial role in their pathogenesis.[28] Reports indicate that fungi including *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, *Saccharomyces cerevisiae*, and *C albicans* release vesicles with RNA inside that by mimicking endogenous miRNA may regulate expression of different genes in target cell.[29] According to reports, heterogeneity in ultrastructural morphology of released vesicles might indicate that they carry different cargo [30] and originate from distinct cellular compartments.[31] The size of released vesicles examined in this study was estimated as 50-800 nm. The size of vesicles that can pass through the cell wall of *S cerevisiae* and *C neoformans* has been estimated as 50-500 nm [31] and 60-300 nm,[18] respectively. Reports suggest fungal cell wall as a dynamic structure with flexible viscoelastic properties [32] that allows its pores to enlarge when exposed to stressful conditions.[33] These data show that flexibility of cell wall pores in *C albicans* may let the large vesicles to pass through.

Observation by TEM also showed that some of the vesicles released from yeast cell carry bacterium-like structures. Packaging and release of vesicle-encased bacteria such as *Escherichia coli* O157,[34] *Listeria monocytogenes*, [35] and *Salmonella enterica* [36] has been reported in protozoa. Vesicle-encased *L monocytogenes* released from protozoa was resistant to gentamicin and sodium hypochlorite,[35] and *S enterica* sequestered in protozoa vesicles was protected against low concentrations of calcium hypochlorite.[36] Furthermore, bacterial release

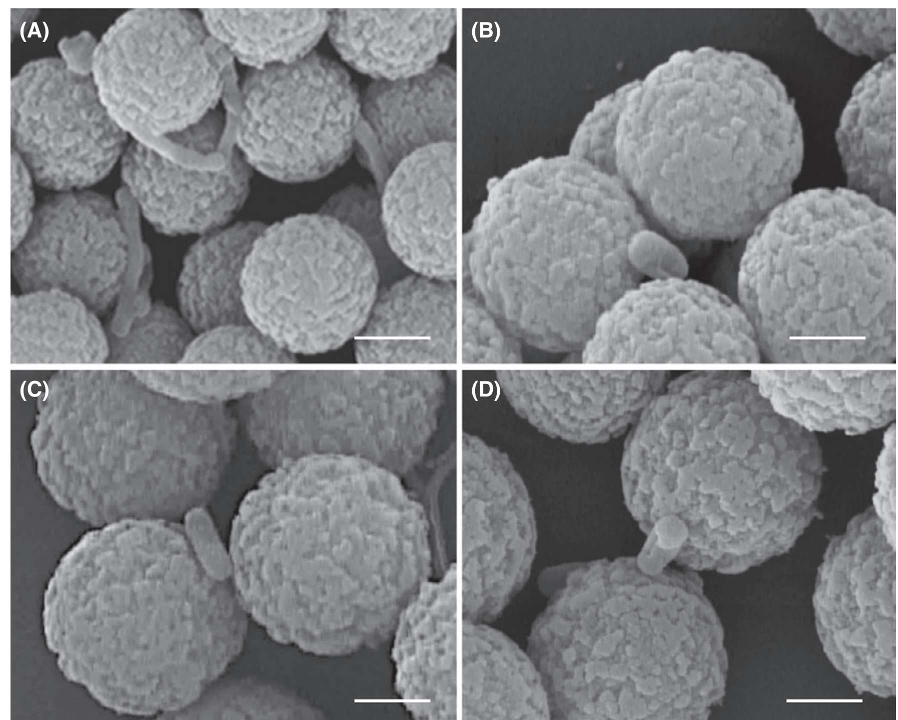

FIGURE 3 Electron micrographs of bead-bound free *H pylori*. (A) Control *H pylori* cells bound to antibody-coated magnetic beads. (B-D) Binding of free *H pylori* cells, released from yeast, to antibody-coated magnetic beads. Bars, 2 μm (A) and 1 μm (B to D)

within a membranous compartment has been reported in *Chlamydia*-infected epithelial cells [37] and *Orientia*-infected endothelial cells.[38] It has been proposed that membrane-encased exit could protect the intracellular bacterium against stresses such as host immune system and antimicrobial factors.[9] TEM observation also revealed release of free bacterial cells from intact yeast. Similarly, release of free *Legionella pneumophila* [39] and mycobacteria [40] from unharmed ameba host has been reported. In addition to packaging of bacteria in a membranous compartment, free exit appears to be another peaceful strategy that enables bacteria to leave the host cell without destroying it.[11]

Observation of immunogold-labeled vesicles showed localization of *H pylori*-specific proteins in vesicles' membrane, indicating that these vesicles might have been originated from *H pylori* containing vacuole. Similarly, lipopolysaccharide (LPS) of intravacuolar *Salmonella* was detected in membrane of vesicles released from host epithelial cell.[41] According to reports, intracellular bacteria secrete bacterial-derived components, such as proteins and lipids, to different subcellular compartments of eukaryotic host cell. For example, bacterial protein and LPS were detected in the membrane of ameba vacuole that contained bacteria,[42] *Mycobacterium tuberculosis* lipoarabinomannan in vesicles dispersed in host cytosol [43] and *Chlamydia* protein in the membrane of bacteria-containing vacuole of epithelial cells.[44]

FESEM observation showed attachment of magnetic beads to released *H pylori* cells in yeast culture and thus the presence of *H pylori*. Amplification of *H pylori*-specific 16S rDNA from bead-bound bacterial cells and sequencing confirmed their identity as *H pylori*. IMS has been used for separation and concentration of target bacterium from clinical and environmental samples, enhancing the results of other methods such as culture, PCR, and microscopy.[45] IMS was applied as an effective method for detection of *H pylori* in samples from oral cavity [46] and water and stool.[47,48] Live/dead staining showed that bead-bound *H pylori* cells were viable; however, failure in their culturing could be due to low density of released intracellular bacteria and our lack of knowledge about their optimized culture conditions.

All living cells secrete vesicles as their housekeeping activity. Environmental factors such as the presence of serum, lack of amino acids, occurrence of antibiotics, antimicrobial peptides and pH and temperature changes may stimulate secretion of vesicles from microbial cells. Moreover, signals exchanged within host cells and tissues may stimulate vesicle formation and release.[2] To escape from host cell and remain protected from lysis and stresses, intracellular bacteria redirect themselves into this dynamic process of vesicle secretion. Majority of intracellular bacteria utilize more than one strategy to escape from the host cell, depending on life cycle stage, environmental factors, or type of the host cell.[49] Compared with bacterial release upon lysis of the host, free escape or being carried in membrane-bound vesicles are the current mechanisms for bacterial release without damaging the host cell. It has been indicated that lipid bilayer structure of extracellular vesicle provides stability to the vesicle and protects its cargo against degradation.[50,51] Release of bacterial cells encased by host membranes seems to be the safest and most effective way to exit from the old host and reach the new one. It is concluded that to exit from yeast cell, intracellular *H pylori* uses safe mechanisms that do not damage the host which is the principle of symbiotic associations. Vesicles that carry *H pylori*, while protecting thebacterium, may facilitate bacterial entry into a new target cell. In human stomach, certain conditions may stimulate yeast cells to produce and release vesicles that contain *H pylori*. Accordingly, vesicles released from yeast cell can serve as safe carriers that facilitate effective transmission and spread of *H pylori*. Would this vesicle encapsulation of *H pylori* change the current eradication strategies? Considering that *C albicans* vesicles could serve as a barrier against penetration of antibiotics and subsequent failure in therapeutic measurements, how can the eradication efficacy be optimized by destroying the stable structure of vesicle membrane without damaging the gastric epithelium? Further studies are warranted for answering these important questions.

CONFLICT OF INTEREST

There is no conflict of interest to declare.

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