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Research Paper

Localization of *Staphylococcus* inside the vacuole of *Candida albicans* by immunodetection and FISH

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ABSTRACT

In our previous study, two bacteria B1 and B2 were excised from two amphotericin B-treated *Candida albicans* Y1 and Y2, respectively. Bacteria were identified as B1: *Staphylococcus hominis* and B2: *Staphylococcus haemolyticus* according to their biochemical characteristics and detection and sequencing of *Staphylococcus*-specific genes. In this study the intracellular origin of staphylococci inside the vacuole of yeast was examined. Polyclonal antibodies against *S. hominis* and *S. haemolyticus* were raised in rabbit and used for detection of staphylococcal proteins in protein pool of yeasts by western blotting (WB). Fluorescein-isothiocyanate (FITC)-conjugated antibodies were used for bacterial localization inside yeast's vacuole by direct immunofluorescence (DIF). Fluorescent *in situ* hybridization (FISH) with *Staphylococcaceae* –specific probe was performed for validation of immunodetection results. WB results showed occurrence of several proteins in protein pool of yeasts that were similar to staphylococcal proteins such as those with molecular weight of 57.5 and 66 kDa. Fluorescent microscopy showed interactions of FITC-antibodies with intracellular staphylococci which appeared as green spots. Hybridization of staphylococcal- specific probe with bacteria inside yeasts' vacuole confirmed immunodetection results. Detection of staphylococcal proteins and genes inside *Candida albicans* yeast indicates existence of intracellular bacteria inside the vacuole of yeast. These results suggest *C. albicans* as the potential reservoir of medically important bacteria.

1. Introduction

Endosymbiotic association of bacteria with eukaryotic cells has greatly influenced the evolution of eukaryotes (Corsaro et al., 1999; Dyall and Johnson, 2000). Reports show endosymbiotic association of bacteria with a wide range of eukaryotic hosts such as insects (Baumann et al., 1995), bivalves (Distel et al., 1991), plants (Rai et al., 2000), protozoa (Douglas, 1994), sponges (Erwin et al., 2012) and fungi (Scannerini and Bonfante, 1991). Many endosymbiotic bacteria have evolved to establish in the membrane-bound vacuole of eukaryotic cells (Garcia-del Portillo and Finlay, 1995), where they can maintain their vital activities (Greub et al., 2003) and structural integrity (Corsaro et al., 1999). The consequences of bacterial endosymbiosis with eukaryotic cell would be maintenance of the vacuole and bacterial replication in a balanced way that both partners survive the entire life span of eukaryotic host (Bianciotto et al., 1996; Goebel and Gross, 2001). This relationship has been regarded as a mutual relationship with many unknown details that have remained elusive due to non-culturability of endobacteria. In several studies, molecular biology methods such as polymerase chain reaction (PCR) and microscopy have been used for identification of nonculturable intracellular bacteria and their localization inside the host cell (Barbieri et al., 2000; Bertaux et al., 2003; Bianciotto et al., 1996; Schüßler and Kluge, 2001). Fluorescence *in situ* hybridization (FISH) (Barbieri et al., 2000; Bianciotto et al., 2000) as well as direct immunofluorescence (DIF) and western blotting (WB) techniques (Saniee et al., 2013a, 2013b) can also serve as powerful tools for identification and localization of the target intracellular bacteria.

It is not clear why intracellular bacteria are generally nonculturable with conventional microbiological methods. However, it is rationally

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possible that under certain conditions the intracellular bacteria become culturable. It appears that replication of endosymbiotic bacteria is under a tight control by eukaryotic host, however, they can replicate normally when escaped from the host control (Corsaro et al., 1999; Ratzka et al., 2012). In our previous study, among 50 clinical isolates of *Candida* spp. that were used for determination of minimum fungicidal concentration of amphotericin B, two yeasts one gastric (Y1) and one oral (Y2) that were identified as *Candida albicans*, released culturable bacteria, B1 and B2, respectively. The released bacteria were identified as B1: *S. hominis* and B2: *S. haemolyticus* according to their biochemical characteristics and amplification and sequencing of *Staphylococcus*-specific genes, *16S rRNA* and *tuf*. Intracellular origin of the two released staphylococci was demonstrated by microscopic observation of moving bacterium-like bodies inside the yeasts' vacuole followed by amplification and sequencing of *Staphylococcus- 16S rRNA* and - *tuf* genes from the total DNA extracted from yeasts. It was suggested that treatment of yeasts with amphotericin B that affects fungal cell and vacuolar membranes (Kerridge, 1986; Obara et al., 2001) could have led to bacterial release (Tavakolian et al., 2018). This was the first report demonstrating the release of intracellular bacterial cells from yeast which were culturable. In our previous studies, the intracellular occurrence of *H. pylori* inside *Candida* yeast was determined by microscopic observations and amplification of *H. pylori*-specific genes from the total DNA of yeast. However, attempts to culture *H. pylori* or other bacteria from mechanically-disrupted yeasts were never successful (Salmanian et al., 2008; Siavoshi et al., 2005).

In this study, two staphylococci, *S. hominis* and *S. haemolyticus* which were previously released from two *C. albicans*, were used for raising polyclonal antibodies in rabbits. These antibodies were used for detection of staphylococcal proteins in protein pool of yeasts by WB method. Furthermore, antibodies were labeled with fluorescein-isothiocyanate (FITC) and used for localization of staphylococci inside the vacuole of yeasts by DIF. Finally, FISH was used to confirm the identity of intracellular staphylococci and their localization inside the yeasts' vacuole.

2. Materials and methods

2.1. Yeasts and bacterial strains

Two clinical isolates of *C. albicans* yeasts, one gastric (Y1) and one oral (Y2), and their corresponding released bacteria *S. hominis* and *S. haemolyticus*, respectively were used in this study. Single colonies of Y1 and Y2 were subcultured on YGC agar composed of yeast extract-glucose (YG) agar (Pronadisa, Spain) containing 0.1 g/L chloramphenicol (Merck, Germany), more than 10 times to eliminate any possible bacterial contamination. In every subculturing, only a single isolated yeast colony was used to ensure the absence of bacterial contamination. It is worthy to note that staphylococci were totally inhibited when cultured on the medium containing chloramphenicol.

2.2. Production of polyclonal antibodies

Four rabbits were used for raising polyclonal antibodies against *S. hominis* and *S. haemolyticus*. This part of the study was approved by the ethics committee of research council of university of Tehran. A suspension of each *Staphylococcus* species in physiological saline (8×10^9 cells/mL) was treated with 2% formalin and heat killed by boiling for 2 h. Volumes of 0.5 mL of each bacterial suspension were used for 10 intravascular injections, with 3-day intervals, to ear lobes of two rabbits: *S. hominis* to rabbit H (RH-I) and *S. haemolyticus* to rabbit L (RL-I) (Dresser 1986). The same bacterial suspensions were used for 4 intramuscular injections, with 2-week intervals, to leg muscles of another two rabbits: *S. hominis* to rabbit H (RH-II) and *S. haemolyticus* to rabbit L (RL-II). Ten days after the last injections, all 4 rabbits were bled and the obtained sera were evaluated for production of IgG-H-I, IgG-H-II, IgG-L-I and IgG-L-II by enzyme-linked immunosorbent assay (ELISA).

To perform ELISA, suspensions of *S. hominis*, *S. haemolyticus* and a clinical isolate of *S. aureus* (positive control) were prepared in bicarbonate buffer at a density of 1.5×10^9 cells/mL and heat-killed for 15 min. A 100-μL volume of each bacterial suspension (antigen) was dispensed into four rows of a 96-well microtiter plate with five different dilutions. Negative control wells contained bicarbonate buffer only. Plates were incubated at 4 °C overnight, then at 37 °C for one h for antigen coating to take place. After antigen coating, drying and washing, blocking was performed with a 10% skim milk solution in washing buffer. After the wells were washed with washing buffer, 100-μL volumes of diluted (10^{-1}- 10^{-5}) rabbit sera, in 0.2% skim milk solution, were added to each well. Rabbit sera with 10^{-1} dilution were added to positive control well (*S. aureus*) and with 10^{-2} dilution to negative control well. Plates were washed after one h incubation at 37 °C, and treated with 100 μL of 10^{-5} diluted secondary goat anti rabbit-horseradish peroxidase (Thermo-USA). After one h incubation at 37 °C, plates were washed and treated with 100 μL of 3,3′,5,5′-tetramethylbenzidine for 15 min. The last step was addition of distilled water to stop the reaction and measurement of absorbance at 450 nm.

2.3. Detection of staphylococcal proteins in protein pool of yeasts by western blotting

The two *C. albicans* yeasts Y1 and Y2 and three additional *C. albicans* yeasts; two gastric (G1, G2) and one fecal (F) were examined for detection of staphylococcal proteins in yeast. Fresh cultures of yeasts, *S. hominis*, *S. haemolyticus* and the control *S. aureus* were suspended in 1 mL of normal saline and the turbidities were adjusted to McFarland standard No. 5. A 50-μL volume was taken from each yeast and bacterial suspension and mixed with equal volume of the sample buffer (0.06 M Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 4% 2-mercaptoethanol, and 0.0025% bromophenol blue). After boiling for 15 min, suspensions were centrifuged and supernatants were used for western blotting analysis. Two sets of primary antibodies were used; one set included rabbit polyclonal anti-*Staphylococcus aureus* antibody, SA-IgG (ABIN165528, Gemacbio, France) and the other set included the mixture of 1:250 diluted IgG-H-I and IgG-L-I. The secondary antibody was goat anti rabbit-horseradish peroxidase. A 15-μL volume of extracted proteins from yeasts and bacteria were subjected to SDS-PAGE under reducing condition, using 7% stacking gel and 15% resolving gel. The proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane at 100 V for 50 min. The membrane was exposed to primary antibodies at 37 °C for 2 h. After the washing steps, the membrane was incubated with 1:5000 diluted goat anti rabbit-horseradish peroxidase at 37 °C, overnight. Colorimetric detection was performed by adding 10 mL of 0.03% 3, 3′-diaminobanzidine and 15 μL of H2O2.

2.4. Conjugation of antibodies with fluorescein-isothiocyanate (FITC)

Antibodies (IgG-H-I and IgG-L-I) were conjugated with FITC as

described previously (Wisdom, 2005). Briefly, serum antibodies were dialyzed against carbonate/bicarbonate buffer, overnight. After the protein content of antibodies was measured, it was diluted to 10 mg/mL in 0.5 M carbonate buffer (pH 9.1). A 100-μL volume of FITC solution (1 mg/mL in dimethyl sulfoxide) was added and the mixture was incubated in dark at room temperature for 2 h. To remove the excess FITC, antibodies were passed through a Sephadex G-25 column and equilibrated with phosphate-buffered saline (PBS).

2.5. Interaction of FITC-conjugated IgG-H-I and IgG-L-I with bacteria and yeasts

Fresh cultures of *S. hominis*, *S. haemolyticus*, *S. aureus* (positive control), *Escherichia coli* and *Helicobacter pylori* (negative controls) on Brucella blood agar and those of Y1 and Y2 yeasts on YGC agar were used for preparing the suspensions in distilled water with a turbidity of McFarland unit No 3. A 10-μL volume of the five bacterial and two yeast suspensions was spotted on glass slides, in duplicate. After slides were air-dried and heat fixed, 50 μL of FITC-conjugated IgG-H-I or IgG-L-I were added. Slides were incubated in the dark at room temperature for 30–45 min while enclosed in a small chamber to avoid drying. Slides were washed three times with PBS and then distilled water, dried in dark and observed by fluorescence microscope (Olympus, Tokyo, Japan), using mounting medium (Invitrogen, USA).

2.6. Detection of staphylococci inside yeasts by direct immunofluorescence (DIF) assay

Since both IgG-H-I and IgG-L-I showed strong reaction with staphylococci, one of them (IgG-L-I) was selected for detection of staphylococci inside yeasts by DIF. Fresh cultures of Y1 and Y2 as well as control (negative for amplification of *Staphylococcus*-specific *16S rDNA*) yeasts on YGC agar were used for DIF. A 100-μL volume of each yeast suspension was mixed with 20 μL of FITC-labeled IgG-L-I and vortexed. A 20-μL volume of 5% evans blue solution in PBS was added to create the color contrast. The mixtures were incubated in the dark at 37 °C for 10 min while shaking at 200 rpm. To remove unbound antibodies, suspensions were washed twice with PBS and re-suspended in distilled water. A 10-μL volume of each yeast suspension was spotted onto a glass slide and air-dried in the dark. Dried spots were covered with mounting medium and observed by fluorescence microscopy.

2.7. Detection of intracellular staphyloccoci using fluorescence in situ hybridization (FISH)

To confirm the staphylococci existence inside yeast's cell, oligonucleotide probe specific for *Staphylococcaceae* (Trebesius et al., 2000) was used and FISH was performed according to Yilmaz et al. (Yilmaz et al., 2010). Briefly, yeast cells were cultured overnight in a shaking incubator (200 rpm at 37 °C), harvested and washed twice in 0.01 M phosphate-buffered saline (PBS). Hybridization was performed by adding 200 μL of FISH hybridization buffer (5 M NaCl, 1 M Tris/HCl, 10% Formamide in distilled water) containing Cy3-labeled oligonucleotide probe (Bioneer Co., korea) to yeast pellet and incubating at 37 °C for 2 h while shaking (150 rpm). Following hybridization, cells were washed three times with washing buffer (20 mM Tris–HCl, 100 mM NaCl) and finally resuspended in 200 μL of PBS. A 10-μL volume of the yeast suspension was smeared onto a pre-cleaned microscope slide, air-dried, covered with mounting medium and observed with the fluorescent microscope. Non-target control probe, FAM-labeled non-EUB338 probe (Amann et al., 1990), was used to detect any non-specific binding.

3. Results

3.1. Determination of antibody titer in the sera of immunized rabbits

The titer of polyclonal antibodies against *S. hominis* and *S. haemolyticus* increased remarkably in immunized rabbits compared with that before immunization. The titer of antibodies was found to be higher in intravascularly-immunized rabbits than those immunized intramuscularly. Titers of antibodies that reacted with *S. hominis* and *S. haemolyticus* measured in 10^{-5} diluted sera were 2.84 for IgG-H-I and 1.83 for IgG-L-I, respectively, showing high sensitivity of the antibodies. The polyclonal antibodies also reacted with the control *S. aureus*, due to shared antigens among the *Staphylococcus* genus.

3.2. Detection of staphylococcal proteins in protein pool of yeasts by western blotting

The SDS-PAGE of extracted proteins from yeasts and bacteria and subsequent western blotting with SA-IgG and mixture of IgG-H-I and IgG-L-I revealed several different and strongly reactive proteins (about 10 to >100 kDa) in three staphylococci. Interestingly, some of the *Staphylococcus*- specific proteins were also detected in yeasts (Table 1). WB with SA-IgG showed occurrence of 57.5 and 66 kDa staphylococcal proteins in all the five yeasts (Fig. 1). However, WB with mixture of IgG-H-I and IgG-L-I showed occurrence of a 66 KDa staphylococcal protein in all the five yeasts and 29.5 and 75 kDa proteins in Y2 only (Fig. 2).

Table 1
Detection of staphylococcal proteins in the protein pool of yeasts by western blotting.

Bacterial/yeasts isolates	*Staphylococcus*-specific proteins (kDa)							
	66	57.5	50.1	44.7	38	33.1	29.5	20.4
S. aureus	+	+	+	+	+	+	+	+
S. hominis	+	+	+	+	+	+	+	+
S. haemolyticus	+	+	+	+	+	+	+	+
C. albicans (Y1)	+	+	−	+	+	−	+	−
C. albicans (Y2)	+	+	−	+	+	−	+	+
C. albicans (G1)	+	+	−	−	−	−	−	+
C. albicans (G2)	+	+	+	−	−	−	−	−
C. albicans (F)	+	+	+	+	−	+	−	−

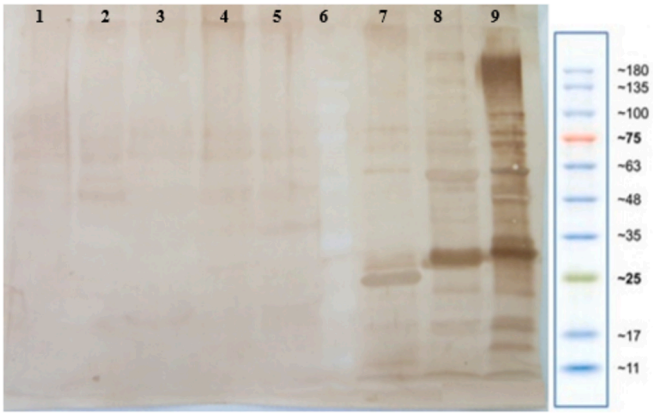

Fig. 1. Western blotting analysis reaction with SA-IgG. Lane 1: yeast G2 which contained 3 *Staphylococcus*-specific proteins, lane 2, 4 and 5: yeast F, Y1 and Y2 which contained 5 *Staphylococcus*-specific proteins, lane 3: yeast G1 which contained 3 *Staphylococcus*-specific proteins, lane 6: molecular weight marker (11–180 kDa), lane 7: *S. hominis* (B1), lane 8: *S.haemolyticus* (B2), lane 9: *S. aureus*.

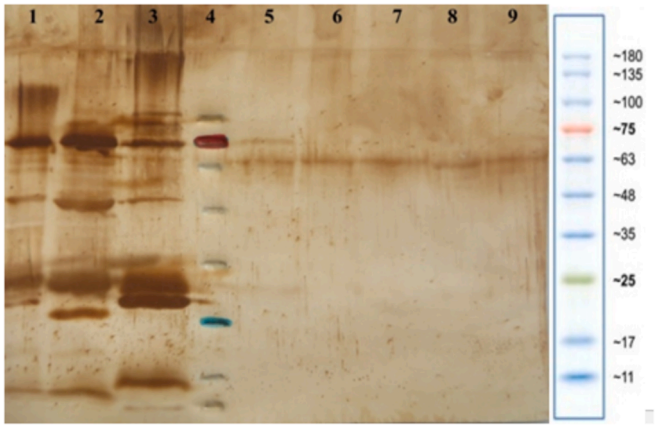

Fig. 2. Western blotting analysis reaction with mixed of IgG-H-I and IgG-L-I. Lane 1: *S. aureus,* lane 2: *S. haemolyticus* (B2), lane 3: *S. hominis* (B1), lane 4: molecular weight marker (11–180 kDa), lane 5: yeast Y2 which contained 3 *Staphylococcus*-specific proteins (lane 6, 7, 8 and 9: yeasts Y1, G1, F and G2 which contained 1 *Staphylococcus*-specific proteins.

3.3. Detection of intracellular staphyloccoci by direct immunofluorescence assay

FITC-conjugated antibodies; IgG-H-I and IgG-L-I reacted strongly with *S. hominis*, *S. haemolyticus* and *S. aureus* but not with *E. coli*, *H. pylori*, Y1 and Y2, demonstrating the specific interaction of antibodies with members of the genus *Staphylococcus*. Observations with fluorescence microscopy showed fluorescent green spots inside the vacuole of yeast cells that were stained red with evans blue. These green fluorescent spots were observed inside the vacuole of mother and daughter yeast's cells. These results indicated that FITC-conjugated antibodies reacted with intracellular bacteria, confirming their identity as staphylococci and revealing their localization inside the vacuole of yeast cells (Fig. 3A). Furthermore, occurrence of the intracellular staphylococci in consecutive generations of yeast cells indicated that they were physiologically active and able to multiply in the yeast and be transmitted to next generation (Fig. 3B). Fluorescent microscopy of negative control yeast, showed dark vacuoles without green spots, indicating the specificity of antibodies (Fig. 3C).

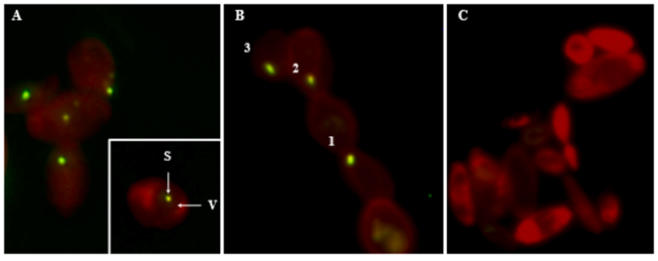

Fig. 3. Interaction of FITC-labeled antibody with intracellular staphylococci in the vacuole of yeast. A) FITC-labeled antibody interacted strongly with *Staphylococcus* cells inside the vacuole of yeast, revealing green fluorescent spots inside the yeast cells that stained red with evans blue. Inset shows intracellular *Staphylococcus* (S) inside the yeast vacuole (V). B) Occurrence of the intracellular staphylococci in consecutive generations (1, 2 and 3) of yeast cells. C) Negative control yeast with dark vacuole, showing lack of non-specific interaction of antibody. Original magnification: X 1000.

3.4. Detection of intracellular staphyloccoci using FISH

Fluorescent microscopy observations on Y1 and Y2 showed yellowish-green fluorescent spots inside two yeasts. The fluorescent signals representing bacterial bodies appeared as shining spots in the yeast's vacuole. Shiny bacterial cells with different numbers were observed in both mother and daughter yeast cells. The lack of non-specific fluorescent signals was *confirmed* by the absence of non-specific interaction of non-target control probe (Fig. 4).

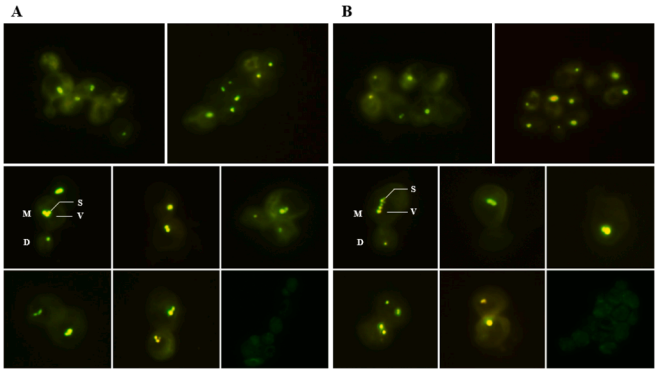

Fig. 4. Detection of intracellular staphylococci within yeast cells using fluorescence *in situ* hybridization. All photographs show intracellular existence of staphylococci (S) as fluorescent spots inside yeast's vacuole (V); Y1 (photographs A) and Y2 (photographs B). Bacterial cells can be observed in mother (M) and daughter (D) yeast cells. Absence of non-specific FISH signal using non-target probe, showed lack of non-specific interaction (NC). Original magnification: X 1000.

4. Discussion

Fluorescent-labeled oligonucleotide probes or antibodies have been used as specific and powerful tools for identification and localization of intracellular bacteria. Reports suggest that active bacteria with enough ribosome can be detected with fluorescent probes. In the other words, dead bacteria, those without rRNA, starved or dormant are not detectable (Amann et al., 1995; Christensen et al., 1999). In this study, WB with anti-staphylococci antibodies raised in rabbit showed occurrence of staphylococcal proteins in the protein pool of *Candida albicans* yeasts. A considerable number of proteins detected in yeasts were similar to those found in staphylococci such as 66 KDa proteins with heparin-sulfate binding activity and 57.5 KDa acting as fibronectin-binding protein A. The information on proteins was extracted from the UniProt database (http://www.uniprot.org). In our previous study, WB with egg yolk antibody against *H. pylori* raised in hen detected six *H. pylori*- specific proteins; thiol peroxidase, peroxiredoxin, urease-A subunit, and small and large subunits of vacuolating cytotoxin A, in the protein pool of gastric and oral yeasts by western blotting (Saniee et al., 2013a). Fluorescent microscopy observations on yeasts that were treated with FITC- conjugated antibodies against staphylococci showed interaction of labeled antibodies with intracellular bacteria. Occurrence of fluorescent spots inside the vacuoles of mother as well as daughter yeasts' cells indicated that the intracellular staphylococci were physiologically active and replicative and transmitted to next generation of yeasts. In our previous study, DIF with FITC-labeled egg yolk antibody against *H. pylori* revealed the identity of intracellular bacteria as *H. pylori* and its localization inside the vacuole of yeasts (Saniee et al., 2013b).

Results of FISH validated the immunodetection of staphylococci inside the vacuole of the two *C. albicans* yeasts. Observation of staphylococcal cells inside the vacuoles of mother as well as daughter yeasts cells indicated that the intracellular staphylococci were physiologically active and heritable. In a study, the bacterial contaminant of *Laccaria bicolor* fungal nurseries was identified by PCR and sequencing as a species of *Paenibacillus.* Because the origin of bacterial contaminant was suspected to be intracellular, *Paenibacillus*-specific FISH probe was used for identification of the bacterium and its localization inside the fungal mycelium. The positive interaction of FISH probe with intracellular bacteria revealed the origin of extracellular bacterial contaminant (Bertaux et al., 2003). FISH was also performed on DNA

extracted from crushed spores of arbuscular mycorrhiza fungus *Gigaspora margarita*, using two different probes for identification of two unrelated intracellular bacteria. The two types of endosymbiotic bactreia were identified as a rod-shaped, Gram-negative *Candidatus* Glomeribacter gigasporarum (Bianciotto et al., 2003) and a Gram-positive coccoid bacterium belonging to Mollicutes-related endobacteria (Mre) taxon (Naumann et al., 2010). Glomeribacter endobacterium showed stable association with its fungal host and was heritable (Bianciotto et al., 2004) while Mre association remained uncertain. These results showed that AMF could host different endobacteria (Desiro et al., 2014).

Detection of staphylococcal proteins and genes inside *Candida albicans* yeast indicates the existence of intracellular bacteria inside the vacuole of yeast. These results suggest yeast as the potential reservoir of medically important bacteria that might release them under certain conditions such as exposure to drugs like amphotericin B (Tavakolian et al., 2018). Reports indicate that *S. aureus* once considered an extracellular pathogen, is capable of surviving inside the vacuole of phagocytic and non-phagocytic cells (Strobel et al., 2016). Survival of *S. aureus* in macrophages has been shown to be promoted by up-regulating of anti-opoptotic factors (Koziel et al., 2009), tolerating acidic conditions (Weinrick et al., 2004), reducing acidification of the phagosome and preventing phagolysosomal maturation (Jubrail et al., 2016; Kahl et al., 2000), and resisting or degrading reactive oxygen (Liu et al., 2005), and nitrogen (Richardson et al., 2008) species. However, like other intracellular pathogens such as *Shigella*, *Francisella*, *Listeria* and *Rickettsia* (Hybiske and Stephens, 2008), *S. aureus* can escape from phagosomes into the host cell cytosol, ultimately causing death to the host cell (Gresham et al., 2000; Lâm et al., 2010). These results suggest that a continuous cycle of lysis and uptake turns macrophages into persistent reservoirs that can disseminate intracellular *S. aureus* throughout the body (Jubrail et al., 2016; Kubica et al., 2008). It has been proposed that bacterial resistance strategies against phagosome-mediated destruction have been learned in free-living amoebae as a pre-adaptation to survive within phagocytic cells (Bozue and Johnson, 1996). This indicates the long co-evolution of bacteria with eukaryotic cells that may have begun more than one billion years ago and led to bacterial endosymbiosis with eukaryotes (Greub et al., 2003).

The entire bacterial genomes have been assembled from the fungal-bacterial DNA preparations by the advanced genome-sequencing technology (Uehling et al., 2017). Moreover, BLAST analysis for 1980 yeast genes showed strongest hit to a eubacterial gene (Cotton and McInerney, 2010). Frequent detection of intracellular bacterial genes in the genomic map of fungi should be regarded as the indication of an evolutionary association between two partners that benefits both. Endosymbiotic bacteria that establish inside fungal vacuole as permanent or transient residents could serve as genetic depositories whose products drive the vital activities of themselves and their fungal host. It has been suggested that endosymbiotic association of bacteria with fungi may be important in fungal adaptation to a certain habitat, while fungal host could serve as an environmental reservoir for endosymbiotic bacterial partner (Deveau et al., 2018).

Details of endosymbiotic association of bacteria with yeast may be elucidated by characterization of the intracellular bacteria. In this study, detection of Staphylococcal proteins in the protein pool of *C. albicans* by WB and bacterial localization inside the yeast's vacuole by DIF indicated the likelihood of intracellular occurrence of staphylococci inside the yeast's vacuole. Furthermore, results of FISH confirmed the identity and localization of intracellular bacteria, also indicating that they were physiologically active and heritable.

Declaration of Competing Interest

None.

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