Symbiotic Relationship between Microbacterium sp. SK0812 and Candida tropicalis SK090404

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A bacterium growing inside yeast cytoplasm was observed by light microscope without staining. The bacterium was separately stained from yeast cell by a fluorescent dye, 4',6-diamidino-2-phenylindole (DAPI). The bacterium actively moved inside yeast cytoplasm and propagated in company with the yeast growth. The bacterium was separated from the yeast cytoplasm by selective disruption of yeast cells and the yeast without the intracellular bacterium (YWOB) was obtained by selective inactivation of bacterial cells. The yeast and the intracellular bacterium were identified as Candida tropicalis and Microbacterium sp., respectively. The length of Microbacterium sp. and C. tropicalis measured with SEM image was smaller than 0.5 μm and was larger than 5 μm, respectively. The yeast with the intracellular bacterium (YWIB) grew in a starch-based medium but the YWOB was not C. tropicalis has neither extracellular nor intracellular saccharification enzyme. Glucose was produced from starch by the extracellular crude enzyme (culture fluid) of Microbacterium sp.YWIB produced significantly more ethanol from glucose than YWOB but did not from starch. Conclusively, C. tropicalis is thought to catalyze starch dependent upon Microbacterium sp. growing in its cytoplasm and furnish stable habitat for the Microbacterium sp.

Keywords: Microbacterium sp., C. tropicalis, starch-hydrolysis, saccharification enzyme, intracellular bacterium

All eukaryotes interact with microbes in relationships that can be benign, malign, beneficial, or detrimental to one or both organisms (Vivas and Goodrich-Blair, 2001). Especially, intracellular symbiosis which has been studied is found in the relationship of Rhizobium-legume (Valera and Alexander, 1965; Kuydendall and Elkan, 1976; Shantharam and Wong, 1982) and several insect orders (Finlay and Falkow, 1997; Goebel and Gross, 2001; Gross et al., 2003). Fact, facultative or obligate intracellular bacteria can be found throughout the three of life from protists to plants and animals (Houk and Griffiths, 1980; Ishikawa, 1989; Corsaro et al., 1999). Moreover, the first stable intracellular symbiotic association of one prokaryote within another prokaryotic cell was recently described (Von Dohlen et al., 2001). An ectoparasite Bdellovibrio (Guerrero et al., 1986) and the facultative intracellular pathogen Daptobacter were known to be thriving within the cytosol of other bacteria (Martin, 2002; Rendulic et al., 2004). Within an animal host cell, the bacteria can reside in two different compartments. Either they can be localized to a vacuole which may be derived from a phagosome formed during engulfment of the bacteria, or they may colonize the host cell cytosol (Goebel and Gross, 2001; Ochman and Moran, 2001). The mutualistic association between Vibrio fischeri bacteria and Euprymna scolopes squid was reported to be a striking resemblance to the interactions between pathogens and immune systems (McFall-Ngal and Ruby, 1998). This intracellular location may be aimed mainly at the exploitation of host metabolites in order to support bacterial multiplication in a relatively safe host compartment devoid of several potent host defense mechanisms (Lee et al., 1999). Moreover, the intracellular state may contribute to the dissemination of the bacteria within the host and, after evading the host cells, their release into the environment of direct transmission to another host organism (Finlay and Falkow, 1997; Gross et al., 2003).

Some bacterioocyte endosymbionts were reported to be descendents of free-living Enterobacteriaceae; however, the relationship between host bacteria and the intracellular bacteria is still under debate (Canback et al., 2004). Symbiotic associations between single-cell prokaryotes and single-cell protists have been studied based on molecular sequences (Berchtold et al., 1999). A prokaryotic symbiont belonging to the order Bacteroidales was identified as an intracellular endosymbiont of the protist Pseudotrichonympha grussi (Noda et al., 2005). Some gut flagellates are regularly colonized by endosymbionts located in the cytoplasm or in the nucleus (Okhuna, 2003; Stingl et al., 2005). The symbiotic interaction between single cell organisms is not general phenomenon and its physiological mechanism is not definitely examined. We found a moving particle in the yeast cytoplasm and isolated that from the yeast cell, but on the other hand the Microbacterium sp. was cured from the cytoplasm of C. tropicalis.

In this study, we characterized the Microbacterium sp. and C. tropicalis to estimate the possibility that the Microbacterium sp. may be an endosymbiont and C. tropicalis may
be a host. The *C. tropicalis* produced ethanol from glucose like other yeast strain and the *Microbacterium* produced glucose from starch by an extracellular crude enzyme *in vitro* test; however, *C. tropicalis* with the intracellular *Microbacterium* sp. did not produce ethanol from starch. This is the first discover that a bacterium species is growing inside yeast cell.

**Materials and Methods**

**Isolation of yeast**  
A yeast species was isolated from sediment of Jungrangcheon located in Sanggye-dong (Korea). The yeast was cultivated in a medium containing 10 g/L of glucose and 1 g/L of yeast extract. A moving-particle inside yeast cytoplasm was observed under a light microscope, which was separated from yeast cell by selective disruption of yeast.

**Separation of moving particle**  
In order to separate the bacterium growing inside the yeast cytoplasm, yeast cells were disrupted by a mini bead beater (Biospec, USA). The 0.5 mm beads were used for selective disruption of yeast cells. Tubes and beads were autoclaved to protect contamination and all procedures for bacterial separation were performed under aseptic condition. The bead beater was operated at 4°C and 2,500 strokes for 10 min, and then the disrupted cell suspension was diluted and spread on agar plate containing 2 g/L of yeast extract. After incubated at 30°C for 48 h, the morphologically different colonies from yeast cells were transferred to broth medium composed of 2 g/L of yeast extract, which was specified as the intracellular bacterium.

**Curing of intracellular bacterium**  
All procedures to cure the intracellular bacterium from yeast cytoplasm were aseptically performed to protect contamination. 100 ml of yeast culture was centrifuged at 3,000×g and 4°C for 30 min and the precipitant was suspended in 50 ml of saline and then divided into 5 ml in test tubes. The cell suspensions were treated with microwave range and 4°C for 30 min, and then the precipitant was suspended in 5 ml of distilled water by vacuum suction. One hundred microliters of yeast cells were filtered through a black polycarbonate filter (pore size, 0.22 μm, Millipore, USA), which was rinsed twice with 10 ml of double distilled water by vacuum suction. One hundred microliters of DAPI solution (0.5 μg/ml) was dropped on the filter and then incubated at room temperature for 60 min. The filter was rinsed with 50 ml of double distilled water by vacuum suction and immersed with a drop of buffered glycercin, and covered with a cover glass. The oil immersion objective was examined under a fluorescence microscope (Carl Zeiss, Axioskop 50, Germany) with UV light (Saby *et al.*, 1997).

**Preparation of SEM image**  
The yeast and bacterial cells were fixed in 4% glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.4). The procedures for dehydration and sample preparation were performed by the general method (Corsaro *et al.*, 1999). The scanning electron micrograph was prepared in the Korea Basic Science Institute (KBSI) located in Daegu metropolitan city.

**Identification**  
Bacterial 16S-rDNA was amplified via direct PCR using the following universal primers: forward; 5′-GAGTTGGAATCTGGCTCAG-3′ and reverse; 5′-AAGGAGGGATCCAGCGCGC-3′. PCR reaction mixture (50 μl) was consist of 2.5 U Taq polymerase, 250 μM of each dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 100 ng template, 50 pM primer, and 1.5 mM MgCl₂. Amplification was conducted for 30 cycles of 1 min at 95°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C using a PCR machine (T Gradient model, Biometera, Germany). The PCR products were directly sequenced with an ABI Prism 3700 genetic analyzer upon request to a professional company (Macrogen Inc., Korea). The 16S rDNA sequences were analyzed using the GenBank database, and identification was performed on the basis of 16S rDNA sequence homology.

**Growth on carbohydrates**  
The YWOB and intracellular bacterium were cultivated on starch and sugar-base carbohydrates to compare the basic catabolism. Medium composed of 0.1 g/L of yeast extract and 5 g/L of individual carbohydrate was prepared in test tubes with the Durham tube. Pre-cultivated cells in a medium containing 5 g/L of yeast extract were washed with saline by centrifugation at 5,000×g and 4°C for 30 min. Suspended cells in 2 volumes of saline was used as an inoculum and inoculation size was adjusted to 5% (v/v). The growth of bacterium or yeast was determined with gas collected in the Durham tube or based on turbidity increase.

**DAPI staining of yeast and bacterium**  
The culture of YWIB and YWOB were filtered through black polycarbonate filter (pore size, 0.22 μm, Millipore, USA), which was rinsed twice with 10 ml of double distilled water by vacuum suction. One hundred microliters of DAPI solution (0.5 μg/ml) was dropped on the filter and then incubated at room temperature for 60 min. The filter was rinsed with 50 ml of double distilled water by vacuum suction and immersed with a drop of buffered glycercin, and covered with a cover glass. The oil immersion objective was examined under a fluorescence microscope (Carl Zeiss, Axioskop 50, Germany) with UV light (Saby *et al.*, 1997).

**Growth on starch**  
The YWIB, YWOB and the intracellular bacterium were cultivated in a medium containing 0.1 g/L of yeast extract and 10 g/L of starch. During cultivation, 1 ml of culture was sampled from each culture and diluted with saline by 10-folded dilution method. The diluted cultures were spread on agar plate containing 2 g/L of yeast extract. After 3 days of cultivation, the emerged colonies were counted and de-